**ABSTRACT**

Insect chitin deacetylases (CDAs) catalyze the removal of acetyl groups from chitin and modify this polymer during its synthesis and reorganization. CDAs are essential for insect survival and therefore represent promising targets for insecticide development. However, the structural and biochemical characteristics of insect CDAs have remained elusive. Here, we report the crystal structures of two insect CDAs from the silkmoth *Bombyx mori*: *BmCDA1*, which may function in cuticle modification, and *BmCDA8*, which may act in modifying peritrophic membranes in the midgut. Both enzymes belong to the carbohydrate esterase 4 (CE4) family. Comparing their overall structures at 1.98–2.4 Å resolution with those from well-studied microbial CDAs, we found that two unique loop regions in *BmCDA1* and *BmCDA8* contribute to the distinct architecture of their substrate-binding clefts. These comparisons revealed that both *BmCDA1* and *BmCDA8* possess a much longer and wider substrate-binding cleft with a very open active site in the center than the microbial CDAs including *VcCDA* from *Vibrio cholerae* and *ArCE4A* from *Arthrobacter* species AW19M34-1. Biochemical analyses indicated that *BmCDA8* is an active enzyme that requires its substrates to occupy subites 0, +1, and +2 for catalysis. In contrast, *BmCDA1* also required accessory proteins for catalysis. To the best of our knowledge, our work is the first to unveil the structural and biochemical features of insect proteins belonging to the CE4 family.
Structural activity of two insect chitin deacetylases

removal of acetyl groups from chitin to form chitosan (1), a polymer of $\beta$-(1,4)-linked D-glucosamine residues. CDAs are widely distributed in protists, diatoms, bacteria, fungi, nematodes and insects (2-8), playing vital roles in chitinous matrix formation and modification (9) as well as in biological attack of fungal pathogens (5,10). CDAs have been considered promising targets for the design of antifungal, antibacterial and pest control reagents (2,11-13).

To date, six crystal structures of CDAs from fungi and bacteria have been determined, including ClCDA from Colletotrichum lindemuthianum (14), AnCDA from Aspergillus nidulans (15), ArCE4A from Arthrobacter species AW19M34-1 (16), two chitooligosaccharide deacetylases VcCDA from Vibrio cholerae (17), VpCDA from Vibrio parahaemolyticus (18) and one putative CDA (EcCDA) from Encephalitozoon cuniculi (19). Except the structure of EcCDA which is not capable of deacetylating chitin, the other structures exhibit conserved critical residues in the active site, indicating that all CDAs use the same metal-assisted general acid/base catalytic mechanism conserved across CE4 enzymes (14-18,20-28). However, the large discrepancies in the shape of the substrate-binding site among these enzymes lead to varied substrate preferences and deacetylation modes. The crystal structures of VcCDA and ArCE4A represent the only two known CDA structures resolved in the presence of their oligosaccharide substrates (16,17). The VcCDA-substrate complex structures provide evidence that six critical loops adopt conformational changes to effectively trap chitooligosaccharides in the substrate-binding pocket. VpCDA has a substrate-binding pocket nearly identical to that of VcCDA and is highly active toward (GlcNAc)$_2$ (18,29). In contrast, there are marked differences in the loops surrounding the substrate-binding site in ArCE4A, ClCDA and AnCDA (16,17). All of them adopt shorter loops to form a relatively open substrate-binding cleft and are active toward both chitooligosaccharide and chito-polysaccharide substrates. Sequence alignment indicates that insect CDAs do not share the specific loops observed in other CDAs (30,31). Unfortunately, the structures of insect CDAs have long been pursued without success.

Insects possess a greater number of CDAs than any other organism. An insect may possess as many as five groups of genes encoding CDAs (32), which may function in the epidermis, tracheal tubes and midgut (33-36). Previous research suggested that chitosan was present in the precise locations where the flexibility of chitin fibers was required (37). Two cda gene mutants of Drosophila melanogaster embryos resulted in elongated and tortuous tracheal tubes (34,35). RNA interference (RNAi) of all nine cda genes from Tribolium castaneum resulted in abnormal phenotypes in the tracheal tubes and cuticle and in joint defects, followed by molting failure or even death (33). RNAi of LmCDA2 from Locusta migratoria changed the chitin organization in the procuticle from a helicoidal to a unidirectional orientation (38). However, many efforts have failed to demonstrate the deacetylation activities of insect CDAs toward chitinous substrates in vitro (30,39). Data about the biochemical characteristics and structure-function relationship of insect CDAs remain scarce.

In this study, two CDAs from Bombyx mori, BmCDA1 and BmCDA8, were crystallized and resolved, providing the first insight into the structural characteristics, activity profiles and deacetylation mode of insect CDAs. The structures revealed that insect CDAs possess several unique structural features that distinguish them from other known CE4 enzymes. This work will also assist the development of specific agrochemicals for pest control.
RESULTS

Overall structure of BmCDA1-CAD—BmCDA1 is composed of an N-terminal signal region (residues 1–23), a chitin-binding domain (CBD, residues 24–122), a low-density lipoprotein receptor domain (LDLa, residues 123–161) and a catalytic domain (CAD, residues 162–539) (Fig. 1A). Our experiments showed that full-length recombinant BmCDA1 was not stable and underwent autocleavage when incubated with the crystallization reagent. Thus, the truncated form of BmCDA1 (BmCDA1-CAD, residues 162–539) was cloned, expressed and purified for crystallization. Diffraction data were collected to 1.98 Å and 2.4 Å on native and SeMet protein crystals, respectively, and the structure was solved using the SeMet single-wavelength anomalous diffraction technique.

BmCDA1-CAD was crystallized in the trigonal space group I4 with one molecule in the asymmetric unit (Table 1). The overall structure of BmCDA1-CAD consists of two regions: a CE4-conserved NodB homology subdomain and C-terminal loops (Fig. 1A). The NodB homology domain is a (β/α)7 barrel (residues 162–471) composed of seven parallel β-strands arranged in a barrel that is surrounded by six α-helices. Notably, the (β/α)7 barrel contains one loop insertion (residues 331–402) between β5 and α5. The C-terminal loops (residues 472–539) consists of α8, a pair of antiparallel β-strands (β9 and β10) and several loops. These structural elements, the loop insertion of the (β/α)7 barrel and the C-terminal loops, appear to be unique because they are not present in any of the other CE4 structures determined to date.

Active site and substrate-binding cleft of BmCDA1-CAD—The active site of BmCDA1-CAD is located at the top center of the (β/α)7 barrel and contains a metal-binding triad conserved across the CE4 family, namely, a zinc ion coordinated by Asp206, His261 and His265 (Fig. 1B & Fig. 2A). Like most CE4 family members, BmCDA1-CAD contains an active site shaped by five motifs, Motifs 1-5 (Fig. 1B). Motif 1 (TFDD) contains the catalytic base Asp205 and the zinc-binding residue Asp206. Motif 2 (HSITH) contains two zinc-binding residues, His261 and His265. Motif 3 (RAPYL) contains the canonical Arg306 responsible for stabilizing the catalytic base Asp205. Structure-based sequence alignment indicates that Motif 4 (AMVDS) is less conserved because most CE4 enzymes possess a Motif 4 with the sequence DxxD[W/Y] (Fig. 1B). As the residue W/Y forms one wall of the active pocket, the replacement of W/Y by S results in an open active site for BmCDA1. Motif 5 (YFH) is also less conserved because most CDAs’ Motif 5 contain LxH. The canonical L contributes to form a hydrophobic patch (21). Taken together, the differences in Motif 4 and Motif 5 confer BmCDA1 a more open and wider active pocket.

A long substrate-binding cleft was observed on the surface of BmCDA1-CAD (Fig. 2C), with only one solvent-exposed hydrophobic residue (Tyr242) that might aid in substrate binding. The substrate-binding cleft is shallow and open when compared with the other microbial CDAs due to the lack of several loops that give microbial CDAs their characteristically deep and narrow substrate-binding cleft (Fig. S1).

Crystal structure of BmCDA8—BmCDA8 (residues 19–381) lacking the N-terminal signal peptide was expressed, purified and crystallized. Diffraction data were collected to 2.4 Å. The structure was resolved by molecular replacement with BmCDA1-CAD as the search model. BmCDA8 was crystallized in the trigonal space group P321 with one molecule in the asymmetric unit (Table 1). Residues 19–22 were not included in the final structure due to a lack of interpretable electron density. BmCDA8 showed 37% sequence
identity with BmCDA1-CAD. The overall architecture of BmCDA8 was similar to that of BmCDA1-CAD (Fig. 2A), corresponding to an r.m.s. deviation of 1.31 Å for 345 equivalent Cα atoms.

Similar to BmCDA1, BmCDA8 (residues 23–381) consists of two regions, a CE4-conserved NodB homology subdomain and C-terminal loops. The active site of BmCDA8 is located at the top center of the (β/α)7-barrel (Fig. 2A). The conserved metal-binding triad is Asp63-His117-His121 coordinated with a zinc ion, and the active site is shaped by Motifs 1–5 (Fig. 1B & Fig. 2A).

However, two differences between BmCDA8 and BmCDA1-CAD were observed. One obvious difference is the replacement of Ala392 (BmCDA1) by Ser241 (BmCDA8) in Motif 4. The other difference is the substrate-binding cleft. Unlike BmCDA1-CAD, BmCDA8 contains a narrower and deeper substrate-binding cleft that passes through the active site where the catalytic reaction occurs (Fig. 2B, C). As shown in Fig. 2B, this cleft has an extended structure with two open ends. One end is shaped by the loop insertion. In detail, surface-exposed Phe238, Phe247 and Phe248 form a hydrophobic “claw” at the top. The other end is shaped by the C-terminal domain that provides two aromatic residues Phe347 and Tyr361.

**Enzymatic activity and the deacetylation mode of BmCDA8**—The activity assay indicated that BmCDA8 instead of BmCDA1 is active. To investigate the catalytic characteristics of BmCDA8, the enzymatic activity was determined using various kinds of chitinous substrates, including (GlcNAc)1–6, and the polymeric substrates ethylene glycol chitin and colloidal chitin. Among oligomeric chitinous substrates, BmCDA8 showed no activity toward (GlcNAc)1–2 but did exhibit activities toward (GlcNAc)3–6. As for (GlcNAc)3–6, the kcat/Km values of BmCDA8 increased as the degree of polymerization increased. In contrast to its relatively low affinities toward chitooligosaccharides, BmCDA8 showed higher affinities toward the polymeric substrates, ethylene glycol chitin and colloidal chitin (Table 2).

The deacetylation mode of BmCDA8 was investigated by a two-step analysis of the deacetylated products of (GlcNAc)3 (Fig. 3A). In the first step, electrospray ionization mass spectrometry (ESI-MS) analysis was performed to determine the number of deacetylated GlcNAc residues. In the second step, ESI-MS analysis was performed again to determine the deacetylation sites. Before the ESI-MS analysis, the deacetylated products were pretreated with the enzyme OfHex1, which specifically cleaves β-1,4 linked GlcNAcs instead of GlcNs from the non-reducing ends of the chitooligosaccharides. Thus, only chitooligosaccharides with a GlcN at the non-reducing end remained for the second ESI-MS analysis. For the substrate (GlcNAc)3, the first-step ESI-MS analysis showed that the products included (GlcNAc)3 and a mono-deacetylated product with a mass loss of 42.0, the mass of a CH3CO−H group. The mono-deacetylated product was not further hydrolyzed by OfHex1 in the second step, indicating that the product was GlcN-GlcNAc-GlcNAc (Fig. 3B). Notably, neither GlcN nor mono-deacetylated products of (GlcNAc)2 were present, indicating that deacetylation occurred at the first GlcNAc at the non-reducing end. Since the biochemical data indicated that BmCDA8 was not able to deacetylate GlcNAc or (GlcNAc)2 (Table 2), we deduced that BmCDA8 activity requires substrates to occupy subsites 0, +1 and +2 (Fig. 3C), where 0 is the catalytic site and “+” refers to the reducing ends, according to the nomenclature commonly used for CE4 enzymes (40-42).

To further confirm the requirement of the subsites for catalysis, mutagenesis of Gln125 at +1 subsite and Ser241 at +2 subsite was performed. The two substrate-binding sites were predicted by
molecular dynamics stimulations (Supplementary Information). The activity of the mutants toward (GlcNAc)$_3$ was determined. As shown in Fig. 3D, both Q125A and S241A showed markedly lower specific activity than the wild-type protein. The mutation of Ser$_{241}$ caused much more serious impairment of activity than the mutation of Gln$_{125}$, even though Gln$_{125}$ interacts with both subsites +1 and +2. This difference suggests that subsite +2 might be more crucial for (GlcNAc)$_3$ binding.

Enzymatic activity of BmCDA1 requires accessory proteins—Our study showed that the deacetylation activity of BmCDA1 was undetectable towards various chitinous substrates even with prolonged incubation time to 120 h or with higher enzyme concentrations at 200 μM. To understand the activation mechanism of BmCDA1, we added molting fluid (MF), which is a protein cocktail secreted by insect epidermal cells that facilitates old cuticle shedding, into the reaction mixture. Strikingly, the activity of BmCDA1 toward ethylene glycol chitin and colloidal chitin was boosted in the presence of MF when compared with that of BmCDA1 alone and the catalytic residue-mutated form D205S (Fig. 4A). To further understand what proteins are involved in the activation of BmCDA1, a cuticular chitin-binding protein CPAP-3A1, it is a homolog of Obstructor A in Drosophila that physically interacts with Serpentine (the CDA1 homolog in Drosophila) (43), was mixed with an equimolar amount of BmCDA1. The enzymatic activity assay showed a significant increase in the deacetylation activity of BmCDA1 in the presence of CPAP3-A1 (Fig. 4B). The in vitro pull-down assay illustrated that CPAP3-A1 can pull down BmCDA1 (Fig. S2). However, CPAP3-D, which belongs to the same CPAP family as CPAP3-A1 and shows the highest binding affinity to deacetylated chitin among CPAP family members (44), could not activate nor pull down BmCDA1 (data not shown). Taken together, these data indicated that BmCDA1 requires specific accessory proteins to achieve activity.

DISCUSSION

This study on BmCDA1 and BmCDA8 provides the first structural and biochemical comparisons of insect CDAs. As revealed by chemical and spectroscopic analyses, chitin in insect is deacetylated at a degree of 5–25% (45). The deacetylation activity of insect CDAs appears to be necessary because abrogation of its activity by gene knockdown results in chitinous laminar organization disorder or even lethality (38,46). Surprisingly, most studies indicate that insect CDAs are ineffective enzymes in in vitro testing assays.

Insect CDAs seem to be designed less active. The deacetylated degree of the insect chitin matrix (5–25% chitosan) was relatively low when compared with that of the fungal cell wall (~75% chitosan in Mucor rouxii) (47). MrCDA from M. rouxii was highly active toward chitinous substrates (4). The specific activity of MrCDA toward (GlcNAc)$_3$ was 467 μM/min/μM (48) which was 65.7-fold higher than that of BmCDA8. The weak activity of insect CDAs might help to maintain the low degrees of chitin deacetylation observed in vivo. Structural alignment of BmCDA1/BmCDA8 with other CE4 enzymes including peptidoglycan deacetylases, poly-β-1,6-N-acety-D-glucosamine deacetylases, acetylxylan de-O-acetylases, chitin deacetylases and chitin oligosaccharide deacetylases shows strong conservation of the active site (Fig. S3). The similarity of the active sites suggests that insect CDAs use the same catalytic mechanism. However, the different architectures of the substrate-binding clefts of the CE4 CDAs confer different catalytic properties to these enzymes. The narrow entrance of the substrate-binding pocket of VcCDA is composed of six loops close to each other, contributing a specific and high catalytic efficiency.
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The openness and shortness of the substrate-binding cleft of \( \text{ArCE4A} \) represents a common feature of other CE4 enzymes, conferring them with higher rates for various substrates (16). Unlike \( \text{VcCDA} \) and \( \text{ArCE4A} \), the only two known structures complexed with chitooligosaccharide substrates, both \( \text{BmCDA1} \) and \( \text{BmCDA8} \) possess a much longer, wider and more open substrate-binding cleft (Fig. 2 B, C). The lack of steric constraints within the substrate-binding clefts may reduce the effectiveness of trapping substrates once bound, perhaps explaining the weak activity observed for \( \text{BmCDA1} \) and \( \text{BmCDA8} \). The longer substrate-binding cleft formed by unique loops appears to be an intentional feature of insect CDAs to fit chitin fibers \textit{in vivo}. To illuminate the functions of the unique loops in insect CDAs, we have constructed nine truncates of \( \text{BmCDA8} \), each of which lacks partial or whole loops (Supporting Information). Unfortunately, we failed to obtain any recombinant proteins. The function of loops unique in insect CDAs requires further investigation.

The activation of \( \text{BmCDA1} \) in the presence of molting fluid suggests that the activity of insect CDAs could be regulated by an unknown mechanism. As a component of molting fluid, the chitin binding protein \( \text{CPAP3-A1} \) instead of \( \text{CPAP3-D1} \) was capable of activating \( \text{BmCDA1} \), suggesting that the activation mechanism is complex. A possibly similar case has been shown for \( \text{PgaB} \), a poly-\( \beta \)-1,6-N-acety-D-glucosamine deacetylase from \( \text{Escherichia coli} \). \( \text{PgaB} \) is composed of two domains, an N-terminal deacetylase domain and a C-terminal GH18/GH20-like domain, the association of which is proposed to create a substrate-binding cleft during de-N-acetylation (49). In \( \text{Drosophila} \), the \( \text{CPAP3-A1} \) homolog \( \text{Obstructor-A} \) chitin-binding protein interacts with the deactylation domain protein \( \text{Serp (CDA1)} \) (43). We further verified the interaction of \( \text{BmCDA1} \) and \( \text{CPAP3-A1} \) using an \textit{in vitro} pull-down assay. One may deduce the activation of \( \text{BmCDA1} \) requires an accessory factor, e.g., \( \text{CPAP3-A1} \). Future structural studies of the complex will provide information about the activation mechanism.

Taken together, the structural and biochemical data provide insights into the novel characteristics of insect CDAs. The lack of available and clear information regarding insect CE4 enzymes highlights the importance of the addition of the \( \text{BmCDA1-CAD} \) and \( \text{BmCDA8 structure} \) to the CE4 structural database, adding new knowledge about the CE4 family.

**EXPERIMENTAL PROCEDURES**

**Gene Cloning and Expression Plasmid Construction**—Total RNA was extracted from five \( \text{B. mori} \) specimens at the 5th instar (day 5) using \text{RNAiso}™ Plus (TaKaRa, Japan) according to the manufacturer's protocol. The cDNA was synthesized using the \text{PrimeScript}™ RT Reagent Kit (TaKaRa, Japan). The gene encoding \( \text{BmCDA1-CAD} \) and \( \text{BmCDA8} \) was amplified from the cDNA with the primers listed in Table S1 A 6×His affinity tag was introduced at the C-terminal. The product was ligated into the EcoRI and XhoI restriction sites of the pPIC9 expression vector using the In-Fusion Kit (Clontech, Palo Alto, California). The resulting expression plasmids were subsequently linearized with the restriction enzyme SalI to allow integration into the chromosomal DNA of \( \text{Pichia pastoris GS115} \) (Invitrogen, Carlsbad, California, USA).

**Expression and Purification**—Recombinant \( \text{P. pastoris} \) was first grown in buffered complex medium containing glycerol (BMGY, Invitrogen) at 301 K to an optical cell density of 4.0 at 600 nm. The cells were collected by centrifugation, resuspended in buffered methanol complex medium (BMMY, Invitrogen) and transferred into a 5 L fermentation tank. The volume of cultures for
production of recombinant proteins is 3 L. The pH was controlled with a sterilized base solution of 1 M KOH. Protein production was induced by delivering methanol to the vessel at a constant feed rate. The fermentation proceeded for 92 h at 301 K. The culture supernatant was obtained by centrifugation. The supernatant was subjected to ammonium sulfate precipitation with 75% saturation at 277 K for 12 h. After centrifugation, the supernatant was removed, and the precipitate was resuspended in distilled water and then desalted in buffer A (20 mM sodium phosphate, 0.5 M sodium chloride, pH 7.4) using a HiTrap Desalting column (5 ml; GE Healthcare, USA). The resulting sample was then loaded into a HisTrap HP affinity column (5 ml; GE Healthcare, USA) equilibrated in Buffer A. The target protein was eluted with 20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole pH 7.4. The eluted protein was >95% pure, as analyzed by SDS-PAGE. The purified protein was then desalted in 20 mM Tris (pH 7.4) and 20 mM NaCl and concentrated to an appropriate concentration for the crystallization experiments. The yields for the recombinant proteins are 0.2 mg/L (BmCDA8), 1 mg/L (BmCDA1) and 5 mg/L (BmCDA1-CAD). Mutations were introduced by PCR-based site-directed mutagenesis, and the mutated proteins were purified using the same protocol described above.

Expression of Selenomethionine-containing BmCDA1-CAD—The same strain was used for the expression of SeMet-incorporated BmCDA1-CAD. The growth conditions before induction were the same as those described above. The cells were then washed three times with PBS and resuspended in buffered methanol medium with the following modification. The medium contained 100 mM potassium phosphate at pH 6.0, 1.34% (w/v) yeast nitrogen base without amino acids (Invitrogen), 0.09 mg/mL adenine sulfate, 0.09 mg/mL uracil, 0.34 mg/mL thiamine, 0.3 mg/mL succinic acid, 0.01 mg/mL inositol, 0.09 mg/mL L-tryptophan, 0.09 mg/mL L-histidine, 0.09 mg/mL L-arginine, 0.09 mg/mL L-tyrosine, 0.09 mg/mL L-leucine, 0.09 mg/mL L-soleucine, 0.09 mg/mL L-lysine, 0.15 mg/mL L-phenylalanine, 0.3 mg/mL L-glutamic acid, 0.3 mg/mL L-aspartic acid, 0.45 mg/mL L-valine, 0.6 mg/mL L-threonine, 1.2 mg/mL L-serine, 0.12 mg/mL L-cysteine, 0.3 mg/mL L-glutamine, 0.2 mg/mL L-proline, 0.2 mg/mL L-alanine and 0.1 mg/mL selenomethionine. The induction of the expression and purification procedures were the same as those described above.

Enzymatic Activity Assays—The enzyme activity was determined based on the detection of acetate released by the action of BmCDA8. The assays were performed using the K-ACETRM Kit (Megazyme© International, Wicklow, Ireland) according to the manufacturer’s instructions. A deacetylation activity assay was performed using different chitinous substrates, including both polymeric substrates [ethylene glycol chitin (EGC, Wako Pure Chemicals, Japan), colloidal chitin, and α-chitin (Sigma-Aldrich, USA)] and oligomeric substrates (monomer to hexamer, Qingdao BZ Oligo Biotech Co., Ltd., China). The conditions of the enzymatic assay on chitin were as follows: 100 μL of a mixture containing 6 μM enzyme and 0.5 mg of the polymeric substrate in 20 mM Tris and 20 mM NaCl (pH 7.4) was incubated at 30 °C for 30 min. The reaction was terminated by boiling at 100 °C for 1 min. The amount of acetic acid was determined using the K-ACETRM Kit. For oligomeric substrates, Michaelis-Menten parameters were determined. Reaction components were incubated in a final volume of 100 μL at 30 °C for 2 hours in the presence of 20 mM Tris (pH 7.4) and 20 mM NaCl, 6 μM enzyme and 1-10 mM (GlcNAc)1-6. Then enzyme reaction was stopped by boiling at 100 °C for 1 min and the concentration of released acetic acid was then measured by the K-
ACETRM Kit according to the manufacturer’s protocol. Data analysis was performed with OriginPro 8.5 (OriginLab, USA).

*BmCDA1 Activation Reactions*—To test the activation of *BmCDA1* by molting fluid and CPAP3-A1, the recombinant *BmCDA1* protein (100 μg) was incubated with 100 μg of molting fluid and CPAP3-A1 respectively in a total volume of 50 μL in buffer containing 20 mM Tris and 20 mM NaCl (pH 7.4) at 30 °C for multiple time intervals as followed: 0 h, 2 h, 5 h, 10 h and 24 h. Ethylene glycol chitin and colloidal chitin were used as substrates to assess the activation of *BmCDA1* by molting fluid and CPAP3-A1. The molting fluid was extracted as mentioned by Qu et al. (50). CPAP3-A1 was recombinantly expressed and purified as previously described (44).

*ESI-MS Analysis*—For this experiment, 10 mM (GlcNAc)_3 was treated with 10 μM *BmCDA8* in 20 mM Tris (pH 7.4) and 20 mM NaCl at 30 °C for 48 h. The samples were boiled at 100 °C for 2 min and then centrifuged at 17,000×g for 10 min. Purified *Ostrinia furnacalis* hexosaminidase1 (O*H*ex1) (51) was added to the samples to a final concentration of 10 μM. The resulting solution was incubated at 30 °C for 48 h. NaNO₃ was added to all the samples at a final concentration of 0.03% to prevent bacteria growth. The reactions were terminated by boiling for 2 min. The samples treated with and without O*H*ex1 were subjected to ESI-MS and recorded in both positive and negative mode using an Agilent 6224 TOF LC/MS system with a dual-nebulizer ESI source (Agilent Technology, USA).

*In vitro GST Pull-down Assays*—GST and GST-CPAP3-A1 were expressed in *Escherichia coli* strain BL21 (DE3). Pull-down assay was performed using BeyoGold™ GST-tag Purification Resin (Beyotime) according to the manufacturer’s instructions. The GST proteins were incubated with 50 μL resins in 20mM Tris (pH 8.0), 200mM NaCl, and 0.5% (v/v) NP40 for 2 hours at 4°C. The resins were washed five times and then incubated with equal amount of His- *BmCDA1* over night at 4°C. The beads were washed five times again, and the presence of His- *BmCDA1* was detected by western blot using anti-*BmCDA1* antibody.

*Crystallization, Data collection and Structure determination of *BmCDA1-CAD*—Crystals of native *BmCDA1-CAD* were grown by vapor-phase diffusion using the hanging drop method with an equal volume (1 μL) of protein (12 mg/mL in 20 mM Tris and 20 mM NaCl, pH 7.4) and reservoir solution (0.2 M Sodium malonate and 20% polyethylene glycol 3350 with a final pH 7.0) at 277 K. Crystals were harvested in rayon fiber loops, bathed in a solution containing reservoir solution and 25% (v/v) glycerol as a cryoprotectant prior to flash freezing in liquid nitrogen. Diffraction data were collected using BL18U of the National Facility for Protein Science Shanghai (NFPS) at the Shanghai Synchrotron Radiation Facility in China. The diffraction data were processed and integrated using the HKL-3000 package (52). Data processing statistics are given in Table 1.

The structure of *BmCDA1-CAD* could not be solved using any of the deposited CE4 structures as a search model in molecular replacement calculations. Phasing information was thus obtained using single wavelength anomalous dispersion with selenium derivative data. The crystals of selenium-containing *BmCDA1-CAD* protein were harvested using the same procedure under the same conditions. Diffraction data were collected using BL17U at the Shanghai Synchrotron Radiation Facility in China (53). Detection of heavy atom sites, phasing, and density modification was performed with AutoSol (54) in Phenix (55). The initial structure was built using Autobuild (56) in Phenix. COOT (57) was used to make manual corrections to the model between...
further cycles of refinement using Phaser (58). The selenium-containing BmCDA1-CAD structure was used as the starting model for refinement of the native BmCDA1-CAD data. The native BmCDA1-CAD data were handled in the same way as the selenium-containing data. Final data and refinement statistics for the two structures are shown in Table 1.

**Crystallization, Data collection and Structure determination of BmCDA8**—The crystallization conditions of BmCDA8 were screened by means of hanging drop vapor diffusion in 96-well VDX plates at 277 K. The drop consisted of equal volumes (1 μL) of protein (10 mg/mL) and reservoir solution. The crystallization condition (0.02 M calcium chloride dihydrate, 0.1 M sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-methyl-2,4-pentanediol) was obtained from Crystal Screen 2 (Hampton Research). Crystals of BmCDA8 were harvested in rayon fiber loops and flash frozen in liquid nitrogen. The diffraction data were collected on Beamline BL18U1 of the National Facility for Protein Science Shanghai (NFPS) at the Shanghai Synchrotron Radiation Facility in China. The data were processed and scaled using HKL3000 (52). Data analysis was performed using Phenix (55).

The structure of BmCDA8 was resolved by molecular replacement with Phaser (58) using native BmCDA1-CAD structure as the model. The structure figures were created using the molecular visualization software PyMol. Secondary structure topology cartoons were analyzed by Pro-origami (59), which is available at http://munk.csse.unimelb.edu.au/pro-origami/. The structures were validated using the website https://validate-rcsb-1.wwpdb.org/.

**MD Simulation**—Three independent MD simulations were performed using NAMD 2.10b2 with the force field charmm36. The systems were solvated with TIP3P water molecules, sodium ions were added to 0.15 M in water, and chloride ions were added to neutralize the system. An isothermal-isobaric (NPT) ensemble was employed at 310 K and 1 atm with a Langevin thermostat. Each system was equilibrated for 10 ns (the total energy was stable) with position restraints. Then, 50 ns MD simulations without restraints were performed for each system. The root-mean-square deviation (RMSD) value was calculated from protein Cα atoms superimposed on the starting structure.

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

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Footnotes
The abbreviations used are: AnCDA, chitin deacetylase from *Aspergillus nidulans*; ArCE4A, chitin deacetylase from a marine *Arthrobacter* species; BmCDA1, chitin deacetylase 1 from *Bombyx mori*; BmCDA1-CAD, the catalytic domain of chitin deacetylase 1 from *Bombyx mori*; BmCDA8, chitin deacetylase 8 from *Bombyx mori*; CDA, chitin deacetylase; CE4, carbohydrate esterase 4 family; CICDA, chitin deacetylase from *Colletotrichum lindemuthianum*; CPAP3, cuticular proteins analogous to peritrophin 3; EcCDA, chitin deacetylase from *Encephalitozoon cuniculi*; EGC, ethylene glycol chitin; ESIMS, electrospray ionization mass spectrometry; GlcN, glucosamine; GlcNAc, N-acetyl-D-glucosamine; MD, Molecular Dynamics; MF, molting fluid; OfHex1, hexosaminidase 1 from *Ostrinia furnacalis*; RMSD, root-mean-square deviation; RNAi, RNA interference; VcCDA, chitin deacetylase from *Vibrio cholerae*; VpCDA, chitin deacetylase from *Vibrio parahaemolyticus*. 
Table 1. Data Collection and Structural Refinement Statistics

|                          | Se-BmCDA1-CAD | Native-BmCDA1-CAD | BmCDA8       |
|--------------------------|--------------|------------------|--------------|
| **Data Collection**      |              |                  |              |
| Wavelength (Å)           | 0.979452     | 0.97776          | 0.97775      |
| Resolution (Å)           | 2.33-50 (2.33-2.41) | 1.98-50 (1.98-2.01) | 2.30-50 (2.30-2.34) |
| Temperature (K)          | 100          | 100              | 100          |
| Space group              | I4           | I4               | P3 21        |
| a, b, c (Å)              | 134.941,134.941,77.120 | 136.006,136.006,77.209 | 115.017,115.017,106.510 |
| a, β, γ (°)              | 90,90,90     | 90,90,90         | 90,90,120    |
| Unique reflections       | 29817 (2917) | 49155 (2407)     | 36623 (1815) |
| Completess (%)           | 99.9 (98.8)  | 100 (100)        | 100 (100)    |
| R_ave %*                | 7.7 (20)     | 15.2 (83.5)      | 15.4 (75.1)  |
| Redundancy               | 15.0 (14.6)  | 13.5 (12.7)      | 11.2 (10.5)  |
| I/σ(I)                  | 15.9 (14.2)  | 4.3 (2.5)        | 3.0 (2.1)    |
| CC1/2                    | 0.991        | 0.826            | 0.834        |
| Wilson B factor (Å^2)    | 26.86        | 23.59            | 37.59        |

**Statistics for Refinement**

|                          |              |                  |              |
| Resolution (Å)           | 2.396-42.67 (2.396-2.482) | 1.98-47.78 (1.98-2.05) | 2.399-39.07 (2.399-2.485) |
| No. of reflections       | 27317 (2673) | 49077 (4834)     | 28971 (2523) |
| Completess (%)           | 99.8 (99.14) | 99.81 (99.42)    | 89.7 (79.67) |
| R_ave/R_free (%)         | 16.27 (18.17)/19.09 | 16.66 (23.89/18.96) | 16.97 (21.18/18.88) |
| Average B factor (Å^2)   | 36.2         | 30.55            | 43.67        |
| Protein atoms            | 3095 (35.64) | 3075 (29.33)     | 2907 (43.31) |
| Ligand                   | 43 (54.45)   | 43 (50.66)       | 29 (62.94)   |
| Water molecules          | 236 (40.22)  | 399 (37.84)      | 170 (46.43)  |
| Other atoms              | 0            | 0                | 0            |
| **Rmsd**                 |              |                  |              |
| Bond angles (°)          | 1.03         | 0.99             | 1.02         |
| Bond length (Å)          | 0.01         | 0.01             | 0.013        |
| **Ramachandran plot (%)**|              |                  |              |
| Favored region           | 96.3         | 96.8             | 97.8         |
| Allowed region           | 3.7          | 3.2              | 2.2          |
| Outliers                 | 0            | 0                | 0            |
| **PDB ID**               | 5ZNS         | 5ZNT             | 5Z34         |

*Values in parentheses are for highest-resolution shell.
Table 2. Kinetic parameters of BmCDA8.

| Substrate | $K_m$ (mM) | $K_m$ (mg mL$^{-1}$) | $V_{max}$ (mM min$^{-1}$) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$mM$^{-1}$) |
|-----------|------------|----------------------|--------------------------|----------------------|-----------------------------------|
| GlcNAc   | -          | -                    | -                        | -                    | -                                 |
| (GlcNAc)$_2$ | -          | -                    | -                        | -                    | -                                 |
| (GlcNAc)$_3$ | 76.7      | 48.1                 | 0.0162                   | 7.62                 | 0.099                             |
| (GlcNAc)$_4$ | 12.3       | 10.2                 | 0.0119                   | 5.59                 | 0.45                              |
| (GlcNAc)$_5$ | 15.7       | 16.2                 | 0.0160                   | 7.52                 | 0.48                              |
| (GlcNAc)$_6$ | 9.2        | 11.4                 | 0.0193                   | 9.07                 | 0.98                              |

$K_m$ (mg mL$^{-1}$) | $V_{max}$ (mg mL min$^{-1}$) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$mL mg$^{-1}$)

| EGC* | 1.926 | 0.085 | 0.097 | 0.050 |
| Colloidal chitin | 1.599 | 0.011 | 0.012 | 0.0077 |

*Ethylene glycol chitin
Figure 1. Domain architecture, overall structure and active site of BmCDA1. (A) Color-coded domain organization of full-length BmCDA1 (upper panel) and cartoon representations of the architecture of the NodB homology subdomain (slate, barrel; white, α-helices), C-terminal loops (blue) and loop insertion (orange) of BmCDA1-CAD (lower panel). SP, signal peptide. The zinc iron is represented as a deep orange sphere. (B) Surface representations of the active site (left panel). Motifs 1–5 of the active site are colored cyan (Motif 1), lime green (Motif 2), magenta (Motif 3), yellow (Motif 4) and blue (Motif 5). The key residues in the five motifs are shown in stick representation using the same color scheme. Structure-based sequence alignment of five CDAs showing the conserved Motifs 1–5 (right panel). The highly conserved residues are indicated.
Figure 2. Structural comparison of BmCDA1 and BmCDA8. (A) Structural alignment of the overall structure (left panel) and active site (right panel) of BmCDA1 and BmCDA8. Substrate-binding clefts of BmCDA8 (B) and BmCDA1 (C). The substrate-binding clefts are shown in surface representation, while the remaining regions are shown in cartoon representation. In BmCDA8, the surfaces are colored slate (active site), deep blue (residues from the C-terminal loops), hot pink (residues from the loop insertion) and white (other regions). The surface-exposed aromatic residues that line in BmCDA8 and BmCDA1 are shown in stick representation in black. The structures of the substrate-binding clefts are each viewed from two angles rotated 90° along the vertical axes.
Figure 3. Structural analysis of deacetylated products of (GlcNAc)₃. (A) Scheme of the two-step analysis method. (B) ESI-TOF MS spectra of the deacetylated products of (GlcNAc)₃. (C) Schematic representations of the binding mode of (GlcNAc)₃ to BmCDA8. Symbols: (○) GlcNAc residue; (●) GlcN residue. (D) Specific activities of the wild-type protein and mutants of BmCDA8 toward (GlcNAc)₃.
Figure 4. The requirement of accessory proteins for BmCDA1 to achieve activity. The addition of molting fluid (MF) (A) and CPAP3-A1 (B) substantially stimulated BmCDA1 activity toward ethylene glycol chitin (EGC) (left panel) and colloidal chitin (right panel).
Structural and biochemical insights into the catalytic mechanisms of two insect chitin deacetylases of the carbohydrate esterase 4 family
Lin Liu, Yong Zhou, Mingbo Qu, Yu Qiu, Xingming Guo, Yuebin Zhang, Tian Liu, Jun Yang and Qing Yang

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