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# Cloning, Expression, and Localization of a Molt-Related β-*N*-Acetylglucosaminidase in the Spruce Budworm, *Choristoneura fumiferana*

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A β-*N*-acetylglucosaminidase cDNA (*Cf*GlcNAcase) was cloned from the spruce budworm, *Choristoneura fumiferana*. Western blotting analysis of developmental *Cf*GlcNAcase expression revealed high levels of expression of the gene on the last day of the 5th instar larvae and the first day in the 6th instar larvae, followed by a decrease to background levels during the intermolt of the 6th instar. *Cf*GlcNAcase was detected again from the last day of the 6th instar to day 2 of pupal stage. *Cf*GlcNAcase expression was induced by tebufenozide at 24 h post treatment and remained at high levels until 72 h. Immunohistochemical localization analysis of *Cf*GlcNAcase indicated that *Cf*GlcNAcase was present in the molting fluid, epidermis, trachea, and hemolymph in prepupae during the transformation from larva to pupa. *Cf*GlcNAcase cDNA was expressed into a recombinant protein in bacterial and baculovirus systems and the protein expressed in the baculovirus system had a higher chitinolytic activity than in the bacterial system and appeared to be secreted. Arch. Insect Biochem. Physiol. 68:49–59, 2008. © 2008 Wiley-Liss, Inc.

KEYWORDS: β-*N*-acetylglucosaminidase; endochitinase; chitin; cuticle; 20-hydroxyecdysone; molt

## INTRODUCTION

During molting and metamorphosis, insects shed their old cuticle to allow for growth and morphological changes. At least two major types of enzymes are involved in the degradation of proteins and chitin in the cuticle, proteases and chitinolytic enzymes, which degrade the cuticle proteins and chitin in the cuticle, respectively. Endochitinase, β-*N*-acetylglucosamidase (or exochitinase) and chitin deacetylase are the major chitinolytic enzymes that degrade chitin and chitin-oligosaccharides during insect molt (Fukamizo and Kramer, 1985a,b; Baker and Kramer 1996). While endochitinase is an endo-splitting chitinase cleaving internally within chitooligosaccharide chains, β-*N*-acetylglucosamidase (GlcNAcase) is an exo-splitting enzyme cleaving off the GlcNAc unit sequentially from the non-reducing end of *N*-acetylated chitooligosaccharides (Fukamizo and Kramer, 1985a,b).

A lepidopteran GlcNAcase was first isolated from the molting fluid, integument, and pupal hemolymph of *Manduca sexta* (Koga et al., 1982). Fukamizo and Kramer (1985a,b) studied the hydrolysis of insect chitin to *N*-acetylglucosamine by endo-splitting chitinase and exo-splitting GlcNAcase and found that a combination of these two enzymes resulted in a synergistic effect on chitin catabolism, such that the combined enzyme activity is much more effective than each of the individual enzymes. They suggested that the chitin degradation is dominated by the endo-splitting chitinase,

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which initiated hydrolysis by attacking the polymeric substrate in a random fashion. The resultant small oligosaccharide intermediates are then further digested by exo-splitting GlcNAcase into *N*-acetylglucosamine (Fukamizo and Kramer, 1985a,b).

Koga et al. (1989) studied the timing of the appearance of chitinase and GlcNAcase in integuments of *Bombyx mori* during the larval-pupal transformation and found that GlcNAcase activity appeared earlier than endochitinase, the latter being present one day before ecdysis. Koga et al. (1992) used polyclonal antibodies against *B. mori* chitinase and GlcNAcase to study chitinolytic enzymes in the integument of *B. mori* and *M. sexta*. They identified several endochitinase-like proteins in the integument and molting fluid, but only one GlcNAcase-like protein was detected (Koga et al., 1992). Nagamatsu et al. (1995) purified three GlcNAcase isoforms from the integument tissues of *B. mori*. Koga et al. (1989, 1992) also suggested that endochitinases in *B. mori* and *M. sexta* are synthesized in the integuments as zymogens, which are activated by limited proteolysis.

Insect hormones regulate the activity of GlcNAcase during development. The activity of *M. sexta* GlcNAcase was enhanced by treating 5th instar larvae with low levels of 20-hydroxyecdysone (20E) (1 µg/g; Fukamizo and Kramer, 1987). High levels of 20E (15 µg/g) had no effect on GlcNAcase activity, but significantly increased endochitinase activity (Fukamizo and Kramer, 1987). Zen et al. (1996) found that the expression of *M. sexta* GlcNAcase gene was induced by the juvenile hormone (JH) analog fenoxycarb.

Because endochitinase and GlcNAcase are capable of degrading chitin, one of the important components of the integument and the midgut of insects, these enzymes have long been suggested to be potential targets for biological control agents (Kramer et al., 1997). We reported the cloning and characterization of a molting-associated endochitinase from *C. fumiferana* (Zheng et al., 2002, 2003). In this study, we report on the cloning and characterization of a molting-associated *C. fumiferana* GlcNAcase from the same species. We have investigated developmental expression, tebufenozide-induced expression, protein localization, and enzymic activity of *Cf*GlcNAcase.

## MATERIALS AND METHODS

### Insect Preparation, Treatment With Tebufenozide (RH5992), and Collection of Molting Fluids

Third instar larvae of the spruce budworm (*Choristoneura fumiferana* Clen Lepidoptera:Tortricidae) were reared on a synthetic diet (McMorran, 1965) at 22°C, 70% relative humidity, and a photoperiod of 12-h light and 12-h dark cycle until they reached pupae or adult moths. Fifth instar larvae were selected after the insects underwent head capsule slippage in order to synchronize their next developmental stages.

For ecdysone hormone analog treatment, tebufenozide (RH5992; Rohm & Hass, Co.) was dissolved in 95% ethanol and then diluted to a final concentration of 20 ng/µl in an aqueous imbibing solution containing 1% sucrose. Two microliters of the hormone solution (40 ng) were injected into one-day-old 6th instar larvae on the side of the abdomen using a microinjector.

To collect the molting fluid samples, a small hole was made with a needle at the bottom of a 0.5-ml PCR tube, which was then put in a 1.5-ml tube containing 1.5–2.0 µl cold phosphate buffered saline buffer. Old skin that had just separated from prepupae was collected and put in the 0.5-ml PCR tube, which was then centrifuged (10,000 rpm) for a few seconds at 4°C. The buffered molting fluid was collected in the 1.5-ml tube and kept at –20°C until ready for SDS-PAGE and Western blotting analyses.

### Cloning of *Cf*GlcNAcase cDNA

An expressed sequence tag (EST) fragment encoding a GlcNAcase was identified from a spruce budworm epidermis EST project. DNA from the corresponding EST plasmid clone was purified and labeled with $^{32}$P-dATP according to standard procedures, and used as a probe to screen an epidermis cDNA library constructed in the Uni-ZAP XR

vector (Stratagene, La Jolla, CA). Eight positive clones were isolated and sequenced from both directions. Because all clones appeared to encode identical sequences, only the longest one was completely sequenced in both directions and used for the rest of the experiments.

## In Vitro Expression of *Cf*GlcNAcase cDNA

Two different protein expression systems, *E. coli* and baculovirus, were used to produce recombinant GlcNAcase. In the bacterial system, the open reading frame of *Cf*GlcNAcase was amplified by PCR and inserted into the pPROEX™ HT expression vector (Invitrogen) between the *Spe* I and *Xho* I restriction sites. The recombinant pPROEX™ HT-*Cf*GlcNAcase plasmid was used to transform DH10B™ *E. coli* cells for protein expression.

The Bac-To-Bac system (Invitrogen) was also used for *Cf*GlcNAcase expression. The open reading frame of *Cf*GlcNAcase was inserted into the donor plasmid pFastBac™ between the *Spe* I and *Xho* I and the recombinant donor plasmid DNA was used to transform DH10Bac cells for transposition, generating the recombinant bacmid AcMNPV-*Cf*GlcNAcase. The construction was confirmed by using PCR and sequencing. Sf21 cells were transfected with the recombinant AcMNPV-*Cf*GlcNAcase for protein expression according to the manufacturer's instruction.

## Antibody Production and Purification

The recombinant *Cf*GlcNAcase protein from the bacterial system was separated in 7.5 % SDS-PAGE gels and the target band was cut out of the gels. A total of 200 μg protein in the SDS gel slices was used for polyclonal antibodies production (Cocalico Biologicals, Inc., Reamstown, PA). *Cf*GlcNAcase-specific antibodies were purified from the antiserum according to Rybicki's method (Rybicki, 1986). The recombinant *Cf*GlcNAcase protein was transferred from gels to membranes, which were stained with Ponceau S solution for 5 min, followed by washing with 1× PBS buffer. The target bands were cut out and washed in 1× PBS buffer to de-stain. The membrane slices were incubated with the antiserum at 4°C overnight with slow shaking, after which they were successively washed in 0.15 mol/L NaCl and 1× PBS, for 20 min each, at room temperature. Antibodies that bound to the *Cf*GlcNAcase protein on the membrane slices were eluted off with glycin buffer (0.2 mol/L glycin, pH 2.9, 1 mmol/L Ethyleneglycol-bis [β-amino-ethylether]-*N*, *N*, *N*′, *N*′-tetraacetic acid).

## SDS-PAGE and Western Blotting

Protein samples were boiled for 3 min after adding an equivalent volume of SDS sample buffer. SDS-PAGE was performed in 7.5% polyacrylamide gels, which were stained with Coomassie Brilliant Blue R-250 solution. For Western blot analysis, proteins were transferred from the acrylamide gel to a Hybond-C Extra nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was blocked with 3% BSA in 1× PBS buffer for 30 min at room temperature, and then incubated with the purified *Cf*GlcNAcase antibodies (1:100) at room temperature for 1 h. Goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody at a dilution of 1:1,000. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for color development.

## Immunohistochemistry

Whole larvae at the selected stages were fixed with 4% formaldehyde in 1× PBS buffer for 24 h at 4°C and embedded in paraffin. Five-micrometer-thick series cross-sections were prepared for immunostaining. Deparaffined section samples were blocked by incubating for 1 h in 1× PBS buffer containing 3% BSA and 1.5% sheep serum. The sections were then incubated overnight with purified anti-*Cf*GlcNAcase antibodies at 4°C. After washing three times in 1× PBS buffer, the sections were incubated with fluorescein-labeled sheep anti-rabbit IgG fragment (Boehringer Mannheim, Montreal, Canada) at a dilution of 1:200 for 1 h. After washing three times in 1× PBS buffer, the samples were observed and photographed under a fluorescence microscope.

### Assay of *Cf*GlcNAcase Activity

Chitinolytic activity of the recombinant *Cf*GlcNAcase was tested according to the method of Nagamatsu et al. (1995). AcMNPV-*Cf*GlcNacase-infected Sf21 cells and media were collected, respectively, at day 3 post-infection. Twenty micrograms of proteins from the medium or 80 μg of proteins from the infected cells were used for each assay. The assays were conducted with 1 mM p-nitrophenyl-β-D-GlcNAc (Sigma) as a substrate in 40 mM sodium phosphate buffer containing 0.8 mg/ml bovine serum albumin at pH 6.8 and 25°C. The reactions were stopped after 1 h by adjusting pH value to 8.0 with an equal volume of 0.5 M sodium carbonate. Released p-nitrophenol was measured at $A_{405}$. Protein extracts from Sf21 cells (or from the media) infected with empty bacmid DNA were used as controls.

## RESULTS

### Cloning and Sequence Analysis

A partial sequence encoding a β-GlcNAcase was identified in a *C. fumiferana* epidermis EST dataset by Blast search algorithm. A 1.5-kb cDNA insert was isolated from the corresponding bacterial clone and labeled with $^{32}$P-dATP and used as a probe to screen an epidermis cDNA library to obtain the full-length sequence. Eight positive clones were isolated from $4 \times 10^4$ plaques. These clones were identical in nucleotide sequence as indicated by sequencing. The longest insert (2.3 kb) was then completely sequenced from both of the 5′ and 3′ ends. A BLAST search in GenBank revealed that this cDNA clone was highly similar to GlcNAcases from other Lepidopteran insects. The cDNA was 2,311 nucleotides in length and coded for a 593-amino acid protein with a predicted molecular mass of 67.8 kDa and a pI of 5.15 (Fig. 1). Two putative N-glycosylation sites (NYT and NAT) were present at amino acid positions 151–153 and 164–166, respectively. A putative signal peptide consisting of 23 amino acid residues at the *N*-terminal end was found based on the sequence alignment with *M. sexta* (Zen et al., 1996) and *B. mori* (Nagamatsu et al., 1995) GlcNacases (Fig. 2A).

Sequence alignment of nine GlcNAcases from different insect species revealed that *Cf*GlcNAcase was very similar to other lepidopteran homologues, such as those from *B. mori*, *M. sexta*, and *Trichoplusia ni* (Fig. 2A). Two GlcNAcase regions believed to be involved in catalysis and/or substrate binding (region I: aa 202–252; region II: aa 358–373) are highly conserved across all species. Several other regions of *Cf*GlcNAcase also appeared to be well conserved, such as the amino acid residues of 73–93, 289–310, and 321–338. Phylogenetic analysis of insect GlcNAcases showed two major groups: lepidopteran and dipteran groups (Fig. 2B). In addition to *Cf*GlcNAcase, the lepidopteran group consisted of *M. sexta* GlcNAcase (Zen et al., 1996; AAQ97603), *B. mori* GlcNAcase (Nagamatsu et al., 1995; AAC60521), *B. mandarina* GlcNAcase (Goo et al., 1999; AAG48701), and *T. ni* GlcNAcase (AAL82580). Identities of these GlcNAcases with *Cf*GlcNAcase ranged between 72 to 97%. The dipteran group includes *Drosophila melanogaster* GlcNAcases B (AAG22248) and D (AAN11596), and *Anopheles gambiae* GlcNAcase (EAA10994). Lepidopteran and Dipteran GlcNAcase formed a cluster that appeared distinct from the single Hymenopteran representative, the honeybee (*Ap*GlcNAcase).

### In Vitro Expression of *Cf*GlcNAcase

The *Cf*GlcNAcase cDNA was expressed into a recombinant protein in both *E. coli* and baculovirus expression systems (Fig. 3). *Cf*GlcNAcase protein expressed in the bacterial system had an apparent molecular mass of 68 kDa in SDS gels (Fig. 3A, lane 4), close to the predicted size based on the deduced amino acid sequence. However, the protein expressed in the baculovirus system had a molecular mass of 71 kDa (Fig. 3A, lane 2). The difference in size may be due to post-translation modifications of the protein, for instance by the addition of sugar moieties at one or both of the NYT and NAT glucosylation sites (Fig. 2A). Anti-

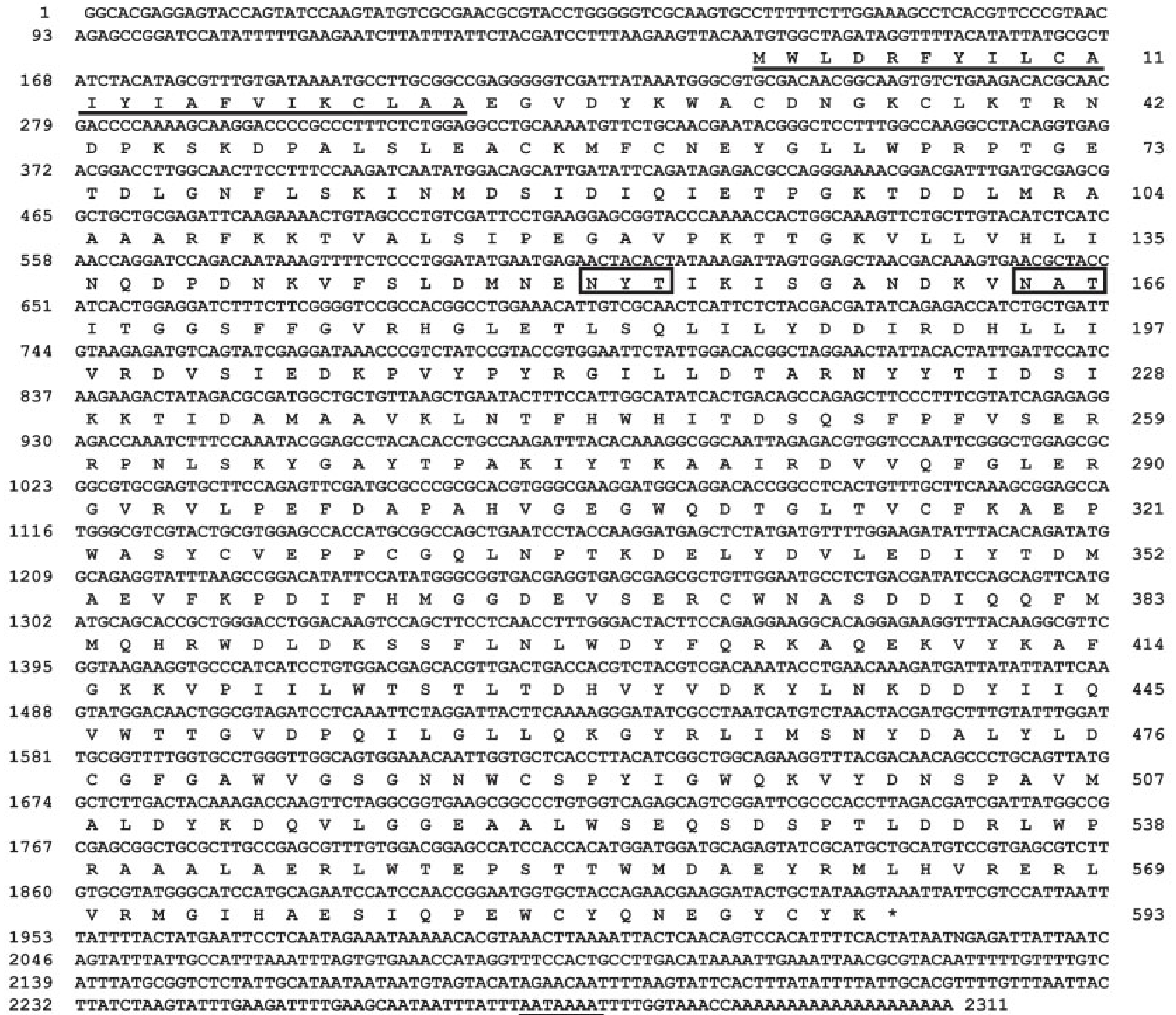

Fig. 1. Nucleotide and deduced amino acid sequences of *C. fumiferana* β-*N*-acetylglucosaminidase cDNA (*Cf*GlcNAcase). A putative signal peptide is underlined. A putative polyadenylation site (AATAAA) is double underlined. Two putative *N*-glycosylation sites are boxed. The numbers on the left refer to the nucleotide positions while the numbers on the right refer to the amino acid positions. The GenBank accession numbers for the nucleotide and deduced amino acid sequences are DQ005717 and AAX94571, respectively.

bodies generated against the recombinant protein from the bacterial system could immunologically react with the proteins expressed in the baculovirus system (Fig. 3B). This result of in vitro expression of *Cf*GlcNAcase cDNA confirmed that the open reading frame of the *Cf*GlcNAcase cDNA did code for a protein with the same size as predicted based on the deduced sequence.

### Activity Assays of Recombinant *Cf*GlcNAcase

To test whether or not the recombinant protein produced through the AcMNPV-*Cf*GlcNAcase expression system had enzymatic activity, infected Sf21 cells and culture medium were collected at day 3 post-infection and used in activity assays according to Nagamatsu et al. (1995). The rationale

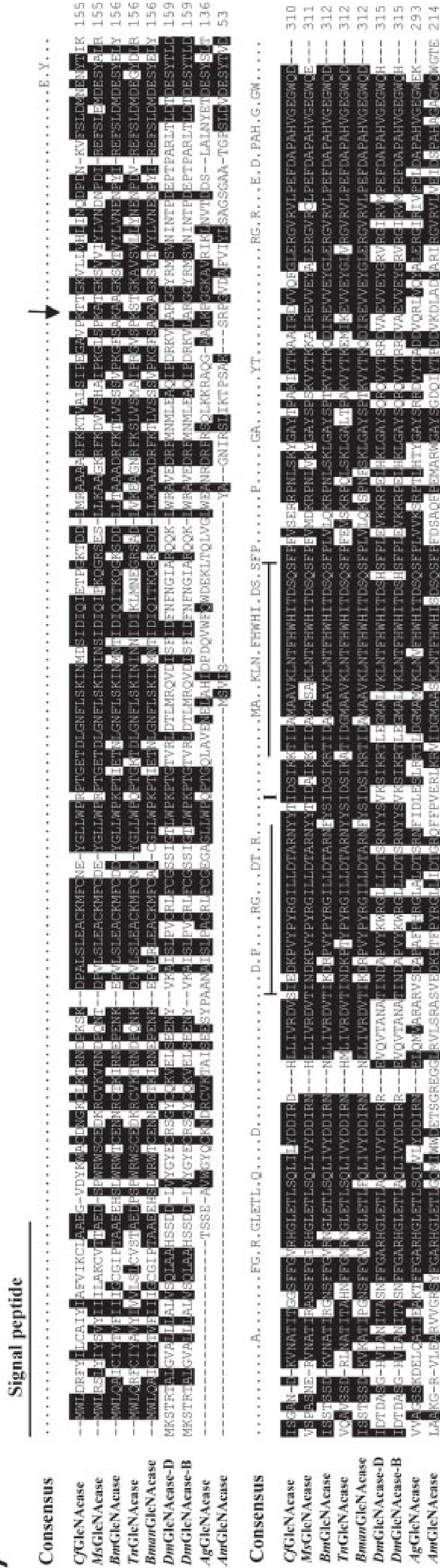


Figure 2.

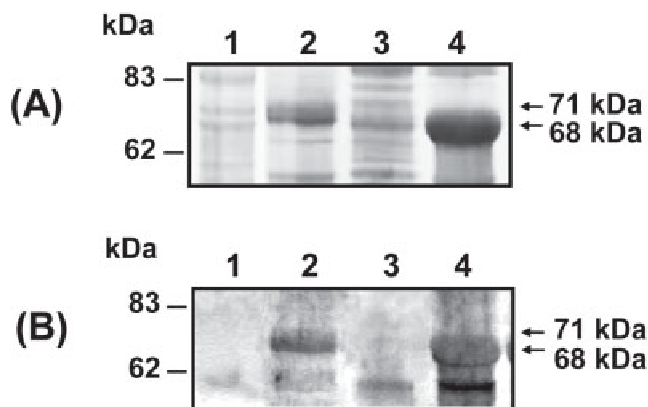


Fig. 3. SDS-PAGE (**A**) and Western blot (**B**) analysis of *Cf*GlcNAcase proteins expressed in vitro in baculovirus and *E. coli*. The *Cf*GlcNAcase protein expressed from the baculovirus system (Lane 2) was detected in the medium and has a size of 71 kDa and about 3 kDa larger than the *Cf*GlcNAcase protein from the bacterial system (Lane 4), in which the protein was detected in the *E. coli* cells. Lane 1 shows the control proteins extracted from DH10B™ *E. coli* cells containing pPROEX™ HT vector alone. Lane 3 shows the proteins from Sf21 cells infected with AcMNPV bacmid DNA. The antibody was made against the recombinant protein produced in the bacterial system.

Fig. 2. **A:** Alignment of the amino acid sequences of insect GlcNAcases. Consensus residues that are identical among all sequences are shown at the top of the alignment. Numbers on the left indicate the positions of amino acid residues. The arrow indicates the cleave site by limited proteolysis with trypsin in *B. mori* GlcNAcase (Nagamatsu et al., 1995). Regions I and II refer to *M. sexta* GlcNAcase regions that are believed to be critical for catalysis and/or substrate binding (Zen et al., 1996). **B:** Phylogenetic analysis of GlcNAcase amino acid sequences. *Cf*GlcNAcase (this study, AAX94571); *Bm*GlcNAcase: *B. mori* GlcNAcase (Nagamatsu et al., 1995; AAC60521); *Bman*GlcNAcase: *B. mandarina* GlcNAcase (Goo et al., 1999; AAG48701); *Ms*GlcNAcase: *M. sexta* GlcNAcase (Zen et al., 1996; AAQ97603); *Tn*GlcNAcase: *T. ni* GlcNAcase (AAL82580); *Dm*GlcNAcase-D: *D. melanogaster* GlcNAcase isoform D (AAN11596); *Dm*GlcNAcase-B: *D. melanogaster* GlcNAcase isoform B (AAG22248); *Ag*GlcNAcase: *A. gambiae* GlcNAcase (EAA10994); *Am*GlcNAcase: *Apis mellifera* GlcNAcase (XP_394963).

for using culture media to perform the activity assay is that *Cf*GlcNAcase was predicted to be a secreted protein. The results revealed that the medium of AcMNPV-*Cf*GlcNAcase-infected Sf21 cells had more than 10-fold higher chitinolytic activity than that of AcMNPV-infected cells (Fig. 4). In contrast, the AcMNPV-*Cf*GlcNAcase-infected Sf21 cells fraction had about 3-fold higher activity than the control AcMNPV-infected cell fraction. These results indicated that the recombinant *Cf*GlcNAcase possessed chitinolytic enzymatic activity and it was secreted into the medium after synthesis. A very low level of enzymatic activity was detected for the recombinant *Cf*GlcNAcase produced in the bacterial system (data not shown).

## Developmental Expression of *Cf*GlcNAcase

Expression of *Cf*GlcNAcase in late 5th instar larvae, 6th instar larvae, and pupae at early stages was examined by Western blotting analysis (Fig. 5A). A high level of *Cf*GlcNAcase expression was detected on the last day (D3) of 5th instar larvae that

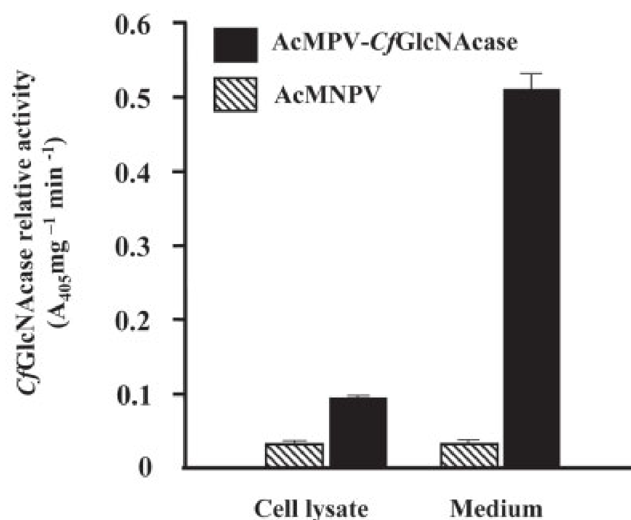


Fig. 4. Chitinolytic activity of *Cf*GlcNAcase expressed by AcMNPV-*Cf*GlcNAcase in the medium and the cell fraction. The activity was measured using p-nitrophenyl-β-GlcNAc as a substrate at $A_{405}$ according to the method of Nagamatsu et al. (1995). The proteins from Sf21 cells infected with wild type AcMNPV and the medium were used as controls.

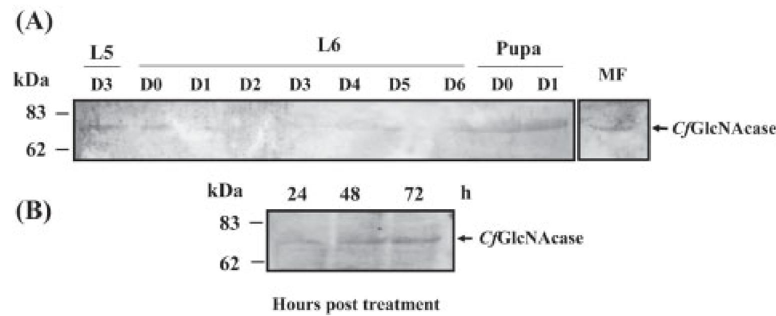


Fig. 5. **A:** Developmental expression of *Cf*GlcNAcase in the spruce budworm larvae from 5th instar to pupal stage. Proteins collected from whole larvae from the last day (D3) of 5th instar to one-day-old pupal stage (at 24 h intervals) were analyzed by Western blotting using purified antibodies against *Cf*GlcNAcase. Proteins collected from the molting fluid (MF) of the prepupae were similarly analyzed. **B:** Western blotting of the induced-expression of *Cf*GlcNAcase by tebufenozide (RH5992). One-day-old 6th instar larvae were injected with 40 ng/larva of tebufenozide. Proteins were isolated from whole larvae and analyzed by Western blotting as above.

were about to molt into 6th instar. *Cf*GlcNAcase protein was also detected on the day (D0) of molting into 6th instar, but the expression decreased to background levels during the intermolt stage (D1–D5) of the 6th instar. High levels of *Cf*GlcNAcase were detected again on the last day (D6) of 6th instar larvae, in the prepupae (D0) and pupae (D1) (Fig. 5A). *Cf*GlcNAcase was also detected in the molting fluid of prepupae. This result indicated that the expression of *Cf*GlcNAcase was correlated with the ecdysis process: immediately prior to molting and during the molt itself, expression of the gene was increased, while during the intermolt periods, the expression was low.

## Effect of RH5992 on *Cf*GlcNAcase Expression

Because the developmental expression of *Cf*GlcNAcase appeared to be ecdysis associated, we tested whether or not the application of exogenous nonsteroid 20E agonist tebufenozide (RH5992) could induce *Cf*GlcNAcase gene expression. When D1 6th instar larvae (which otherwise had only trace levels of *Cf*GlcNAcase at this stage, Fig. 5A), were treated with 40 ng of RH5992, increased levels of *Cf*GlcNAcase protein were detected 24 h later and in the next two days post-treatment (Fig. 5B). Furthermore, treated insects shed old cuticles and molted into an incomplete extra larval stage (7th instar) with a small body size and died after three days post-treatment.

## Immunohistochemical Localization of *Cf*GlcNAcase

Localization of *Cf*GlcNAcase in prepupae was examined using immunohistochemical staining (Fig. 6). At the early stage of molting from 6th instar larvae to prepupae, *Cf*GlcNAcase protein was detected dominantly in the hemolymph (Fig. 6A and C) and epidermis, with very low levels of the *Cf*GlcNAcase proteins on the inner surface of the detached old cuticle (Fig. 6A). At 7 h after apolysis, the *Cf*GlcNAcase proteins were detected in the degrading exocuticle and molting fluid (Fig. 6B). The *Cf*GlcNAcase protein was also detected in the molting fluid between the old and new tracheae (Fig. 6D). No *Cf*GlcNAcase protein was detected in the fat body (Fig. 6A–C) and the midgut (Fig. 6C). This observation indicates that during the early phase of ecdysis, GlcNAcase might not be necessary for the digestion of the endocuticle, but during the late phase of ecdysis, it may play an important role for the digestion of the exocuticle.

## DISCUSSION

Insects periodically shed their old cuticular exuviae and tracheas during molting and metamorphosis. To do so, cuticular chitin in the integument and tracheae has to be degraded. Endochitinase, GlcNAcase and chitin deacetylase are the major enzymes that hydrolyze chitin and chitin-oligosaccha-

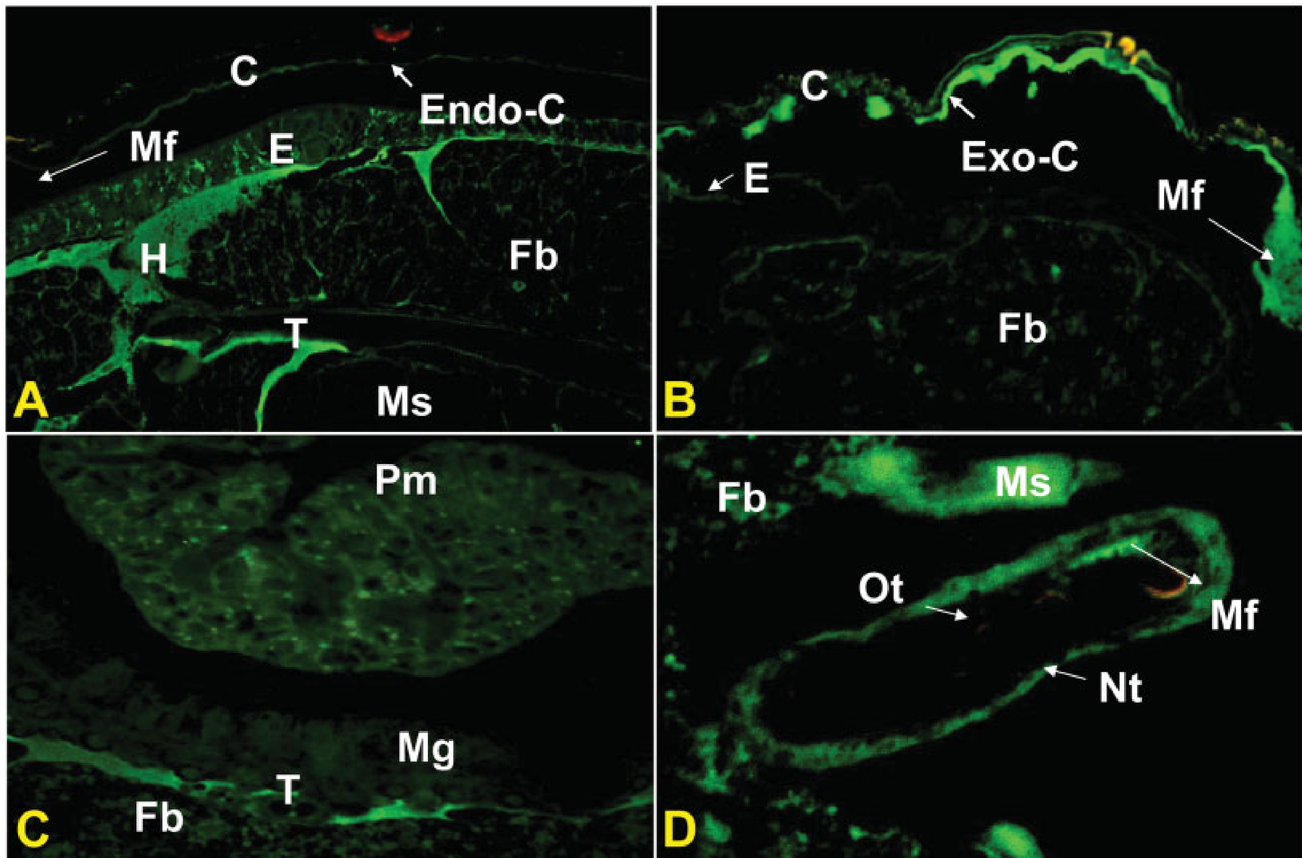


Fig. 6. Immunohistochemical staining of cross-sections of *C. fumiferana* larvae sampled at various times during the larval-pupal molt. The bright green fluorescence indicates the presence of *Cf*GlcNAcase. **A:** Cross-section of an early stage prepupae at the time of endocuticle (Endo-C) digestion and its separation from the epidermis. **B:** Cross-section of a late stage prepupae, sacrificed approximately 7 h later than the one in A, showing exocuticle (Exo-C) digestion and the co-localization of *Cf*GlcNAcase. **C:** Cross-section of the prepupae midgut. **D:** Prepupal trachea showing separated old (Ot) and new (Nt) cuticle layers. C: cuticle; E: epidermis; H: hemolymph; T: trachea; Fb: fat body; Ms: muscle; Mf: molting fluid; MG: midgut; Pm: peritrophic membrane.

rides in the old cuticle into GlcNAc residues (Fukamizo and Kramer, 1985a,b). The resulting GlcNAcs may be recycled and used for the synthesis of the new chitin, which forms new cuticle layers on the surface of the insect body (Gwinn and Stevenson, 1973; Surholt, 1975). On the other hand, the GlcNAcase may also be involved in metabolism of carbohydrates other than chitin (Koga et al., 1982, Altmann et al., 1995).

We cloned an endochitinase cDNA from *C. fumiferana* (Zheng et al., 2002, 2003). For a more detailed understanding of the molting process, we report here on the cloning and characterization of a *Cf*GlcNAcase cDNA from the same insect. Several findings in this study are noteworthy. First, at the time of apolysis of the larval-pupal molt, *Cf*GlcNAcase was present in the epidermis and hemolymph, but not in the molting fluid. However, at 7 h post-apolysis, *Cf*GlcNAcase appeared in the molting fluid after the endocuticle had been digested or when the exocuticle started to be digested. This appearance pattern is different from that of endochitinase (Zheng et al., 2003). *C. fumiferana* endochitinase was detected at high levels in the epidermis when both the endocuticle and exocuticle were digested during molting (Zheng et

al., 2003). Thus, GlcNAcase may not be the most important enzyme involved in the digestion of the endocuticle and its detachment from the epidermis. However, high expression levels of *Cf*GlcNAcase after endocuticle digestion suggest that it is required for endocuticle recycling or exocuticle digestion in the late phase of the molting process. This finding is consistent with the observation in *M. sexta* (Kramer et al., 1993; Zen et al., 1996) and the hypothesis by Fukamizo and Kramer (1987) that chitinase and GlcNAcase act in tandem to catabolize cuticular chitin. In *M. sexta*, endochitinase mRNA is expressed and disappears earlier than GlcNAcase, which remains at high levels until the later stages of the 5th instar molt (Kramer et al., 1993; Zen et al., 1996). Chitin degradation is initiated by endochitinase, which hydrolyses by the polymeric substrate in a random fashion. The resultant small oligosaccharide intermediates are then digested by the exo-splitting GlcNAcase into *N*-acetylglucosamine (Fukamizo and Kramer, 1985a,b). Our data further indicate that endocuticle digestion may require high levels of endo-splitting chitinase activity during the early stage of a molt, whereas exocuticle digestion likely needs both endo-splitting and exo-splitting enzyme activities during the late cuticle recycling stage.

A second significant finding is the presence of *Cf*GlcNAcase in the hemolymph at the early stage of the molt from 6th instar larvae to prepupae. At this stage of the molting cycle, *Cf*chitinase was not detected in the hemolymph and was mainly present in the epidermis and molting fluid (Zheng et al., 2003). *Cf*chitinase was secreted into the endocuticle after it was synthesized in the epidermis. However, *Cf*GlcNAcase may be secreted into the hemolymph after it is synthesized in the epidermis at the early stage of prepupae, although it was eventually present in the molting fluid. The different spatial distribution patterns of these two enzymes may reflect that expression of *Cf*chitinase and *Cf*GlcNAcase is temporally regulated and act sequentially on different target cuticles.

Third, like *Cf*chitinase (Zheng et al., 2002), expression of *Cf*GlcNAcase could be induced by RH5992. *Cf*GlcNAcase was detected at 24 h post treatment and the expression remained at high levels until 72 h before the larvae died. Similar results have been observed in *M. sexta*, in which 20E-induced expression of GlcNAcase mRNA was observed in two days post-treatment (Zen et al., 1996). However, *Cf*chitinase was detected as early as 12 h post-treatment and levels peaked at 36 h and then decreased (Zheng et al., 2002). This difference between endochitinase and GlcNAcase in the timing of protein expression in response to the ecdysone agonist indicates that the mechanisms for the ecdysone-induced expression of these two genes may be different. Therefore, although both enzymes are involved in the ecdysone-initiated molting process, endochitinase and GlcNAcasse may play distinct roles in cuticle degradation.

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