A gut-specific chitinase from the mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae): cDNA cloning, gene structure, expression and enzymatic activity

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Key words. Cerambycidae, *Apriona germari*, baculovirus expression vector, cDNA cloning, chitinase, enzyme, gene structure, mulberry longicorn beetle

Abstract. A gut-specific chitinase gene was cloned from the mulberry longicorn beetle, *Apriona germari*. The *A. germari* chitinase (*AgChi*) gene spans 2894 bp and consists of five introns and six exons coding for 390 amino acid residues. AgChi possesses the chitinase family 18 active site signature and three N-glycosylation sites. Southern blot analysis of genomic DNA suggests that *AgChi* is a single copy gene. The *AgChi* cDNA was expressed as a 46-kDa polypeptide in baculovirus-infected insect Sf9 cells and the recombinant AgChi showed activity in a chitinase enzyme assay. Treatment of recombinant virus-infected Sf9 cells with tunicamycin, a specific inhibitor of N-linked glycosylation, revealed that AgChi is N-glycosylated, but the carbohydrate moieties are not essential for chitinolytic activity. Northern and Western blot analyses showed that AgChi was specifically expressed in the gut; AgChi was expressed in three gut regions, indicating that the gut is the prime site for AgChi synthesis in *A. germari* larvae.

INTRODUCTION

Chitin is the second most abundant polysaccharide in nature and is a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues. It is one of the most unique biochemical constituents found in the cell walls of fungi, the cuticle and peritrophic membrane of insects, the eggshells of nematodes, and the integument of arthropods. Chitinolytic enzymes that catalyze the hydrolysis of chitin have been found in chitin-containing organisms as well as in microorganisms that do not have chitin. The enzymes, chitinases (EC 3.2.1.14), from various organisms have various biological functions. In insects, chitin is the major component of the cuticle and peritrophic membrane, where it functions as a protective structural polysaccharide. During molting and metamorphosis, chitin degradation is achieved through tissue-specific expression of chitinases (reviewed by Merzendorfer & Zimoch, 2003).

Chitinase genes have been cloned from several insect species. Insect chitinases belong to family 18 of the glycohydrolase superfamily and are characterized by a multiple domain structure with molecular masses of 40–85 kDa (Kramer & Muthukrishnan, 1997; Merzendorfer & Zimoch, 2003). Chitinase genes offer several opportunities for gene manipulation for a variety of purposes, i.e., the enhancement of host plant resistance and pathogenecity in transgenic plants and biological control agents (Bonning & Hammock, 1996; Wang et al., 1996; Estruch et al., 1997; Kramer & Muthukrishnan, 1997; Ding et al., 1998; You et al., 2003; Fitches et al., 2004; Kabir et al.,

2006). Furthermore, a recent study showed the wide-ranging applications of biotechnological aspects of chitinolytic enzymes (Dahiya et al., 2006).

This paper describes the cloning of a novel gene encoding chitinase from the larvae of the mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae), that feed on the mulberry tree, tunneling inside the stem and ingesting the living wood. We report here the gene structure, expression and enzymatic activity of a gut-specific chitinase of *A. germari* (AgChi). The distribution of AgChi was determined by transcriptional and translational levels in *A. germari* larvae. The *AgChi* cDNA was expressed functionally in baculovirus-infected insect cells and the recombinant AgChi was assayed for enzymatic activity.

MATERIAL AND METHODS

Animals

Larvae of the mulberry longicorn beetle, *Apriona germari* Hope (Coleoptera: Cerambycidae), were reared on an artificial diet as previously described (Yoon & Mah, 1999). Larvae of *A. germari* were maintained at 25°C, 60% humidity with a 14L : 10D photoperiod.

cDNA library screening, nucleotide sequencing and data analysis

The clone harboring the cDNA insert was selected from the expressed sequence tags (ESTs) that were generated from a cDNA library using whole bodies of *A. germari* beetle larvae (Lee et al., 2004). The plasmid DNA was extracted using a Wizard's mini-preparation kit (Promega, Madison, WI, USA) and sequenced using an ABI 310 automated DNA sequencer

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(Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST programs provided by NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (Version 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the amino acid sequences of chitinase.

Genomic DNA isolation, PCR of the *AgChi* gene and Southern blot analysis

Genomic DNA, extracted from the gut tissues of a single *A. germari* larva using a Wizard Genomic DNA Purification Kit (Promega), was used for PCR amplification with oligonucleotide primers designed from *AgChi* cDNA sequences. The primers used for amplification of the *AgChi* genomic DNA were 5'-ATGAAGGTTACTACACTGTTT-3' for the *AgChi* F-primer and 5'-TTAGTTATCTAGTGTTTTCTT-3' for the *AgChi* R-primer. After a 35-cycle amplification (94°C for 30 s; 48°C for 40 s; 72°C for 2 min), the PCR products for sequencing were cloned into the pGem-T vector (Promega). The construct was transformed into *Escherichia coli* TOP10F' cells (Invitrogen, Carlsbad, CA, USA). The nucleotide sequence was determined using a BigDyeTerminator cycle sequencing kit and an automated DNA sequencer.

Genomic DNA extracted from gut tissue of *A. germari* was digested with *Apa*I, *Bam*HI or *Hind*III, and electrophoresed on a 1.0% agarose gel. DNA from the gel was transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in hybridization buffer containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. The *AgChi* cDNA clone was labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, USA) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) for its use as a hybridization probe. After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and exposed to autoradiography film.

Production of recombinant protein and antibody

A baculovirus expression vector system (Je et al., 2001), using the *Autographa californica* nucleopolyhedrovirus (AcNPV) and the insect cell line Sf9, was employed for the production of recombinant AgChi protein. A cDNA fragment containing the full *AgChi* ORF was excised from pBlueScript-*AgChi* by digestion with *Bam*HI and *Xho*I and inserted into the same sites of the transfer vector pBacPAK9 (Clontech, Palo Alto, CA, USA) to express AgChi under the control of the AcNPV polyhedrin promoter. Five hundred nanograms of the construct (pBacPAK9-*AgChi*) and 100 ng of the AcNPV viral DNA (bApGOZA) (Je et al., 2001) were co-transfected into 1.0–1.5 × 10^6 Sf9 cells for 5 h using the Lipofectin reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultivated in TC100 medium (Gibco BRL) at 27°C for 5 days. The recombinant AcNPV was propagated in Sf9 cells, and the titer was expressed as plaque forming units (PFU) per milliliter as per standard methods (O'Reilly et al., 1992).

Insect Sf9 cells were infected with the recombinant AcNPV expressing polyhistidine-tagged AgChi at a MOI of 5 PFU per cell. Recombinant AgChi was purified from the recombinant AcNPV-infected Sf9 cells using the FastBreak™ Cell Lysis Reagent and the MagneHis™ Protein Purification System, according to the manufacturer's instructions (Promega).

The purified recombinant AgChi (~5 µg) was mixed with an equal volume of Freund's complete adjuvant (a total of 200 µl) and injected into Balb/c mice (Lee et al., 2005). Two successive injections were administered with antigens mixed with equal volumes of Freund's incomplete adjuvant (a total of 200 µl) at 1-week intervals beginning one week after the first injection. Blood was collected three days after the last injection with antigens only and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant antibodies were stored at –70°C until used.

Western blot analysis

Western blot analysis was carried out using an ECL Western blotting analysis system (Amersham Biosciences, Piscataway, NJ, USA). The protein samples were mixed with sample buffer, boiled for 5 min and loaded on a 10% SDS-PAGE gel. The proteins were blotted to a sheet of nitrocellulose transfer membrane (Schleicher & Schuell). After blotting, the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution, incubated with antiserum solution (1 : 1000 v/v) at room temperature for 1 h and washed in TBST [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) Tween-20]. The membrane was then incubated with anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiography film.
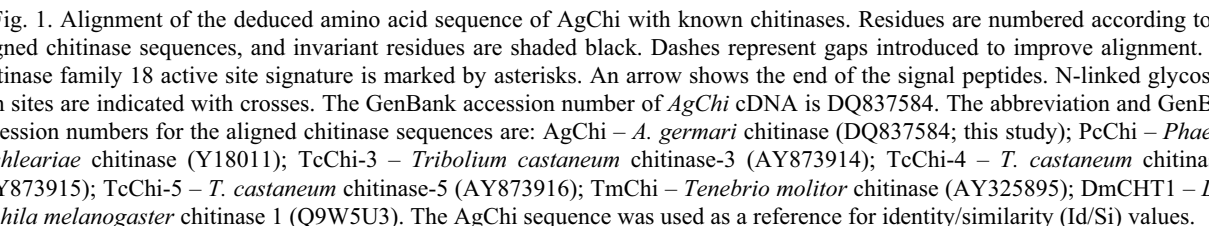
Determination of chitinase activity

Chitinase activity was detected from the gel after SDS-PAGE by the method of Trudel & Asselin (1989). The gel was incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min, and was then put on a glass plate and covered with a 7.5% polyacrylamide overlay gel containing 0.01% (w/v) glycol chitin in 100 mM sodium acetate buffer (pH 5.0). The gel was incubated at 37°C for 1 h under moist conditions. Lytic zones were visualized by placing the gels on a UV illuminator and were photographed.

Tunicamycin treatment

The addition of N-linked carbohydrate by infected insect cells was tested by culture in the presence of tunicamycin (5 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) to prevent the addition of N-linked carbohydrate (Wei et al., 2006). Sf9 cells were infected with recombinant AcNPV in a 35-mm diameter dish (1 × 10^6 cells) and incubated for 2 h at 27°C. The supernatants were replaced with 5 ml of supplemented TC100 medium containing 5 µg tunicamycin per ml of medium. After incubation at 27°C, total cellular lysates were harvested from infected cells at 24, 48 and 72 h post-infection (p.i.). Total cellular lysates were subjected to 10% SDS-PAGE containing 0.01% glycol chitin. The proteins with chitinolytic activity were identified as dark lytic zones under UV illumination (Trudel & Asselin, 1989).

RNA isolation and Northern blot analysis

A. germari larvae in the early stage of the sixth instar, which lasts for ~20 days (Yoon & Mah, 1999), were dissected on ice under a stereo-microscope (Zeiss, Jena, Germany). Several tissues, such as the epidermis, fat body, and gut (foregut, midgut, and hindgut, respectively), were collected and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4). Total RNA was isolated from the tissues of *A. germari* larvae using a Total RNA Extraction Kit (Promega). Total RNA (5 µg/lane) was separated by 1.0% formaldehyde agarose gel electrophoresis, transferred onto a nylon blotting membrane (Schleicher & Schuell) and hybridized at 42°C with a probe in hybridization buffer. Hybridization conditions, fragment labeling, and filter washing were as described for Southern blot analysis.

Fig. 1. Alignment of the deduced amino acid sequence of AgChi with known chitinases. Residues are numbered according to the aligned chitinase sequences, and invariant residues are shaded black. Dashes represent gaps introduced to improve alignment. The chitinase family 18 active site signature is marked by asterisks. An arrow shows the end of the signal peptides. N-linked glycosylation sites are indicated with crosses. The GenBank accession number of *AgChi* cDNA is DQ837584. The abbreviation and GenBank accession numbers for the aligned chitinase sequences are: AgChi – *A. germari* chitinase (DQ837584; this study); PcChi – *Phaedon cochleariae* chitinase (Y18011); TcChi-3 – *Tribolium castaneum* chitinase-3 (AY873914); TcChi-4 – *T. castaneum* chitinase-4 (AY873915); TcChi-5 – *T. castaneum* chitinase-5 (AY873916); TmChi – *Tenebrio molitor* chitinase (AY325895); DmCHT1 – *Drosophila melanogaster* chitinase 1 (Q9W5U3). The AgChi sequence was used as a reference for identity/similarity (Id/Si) values.
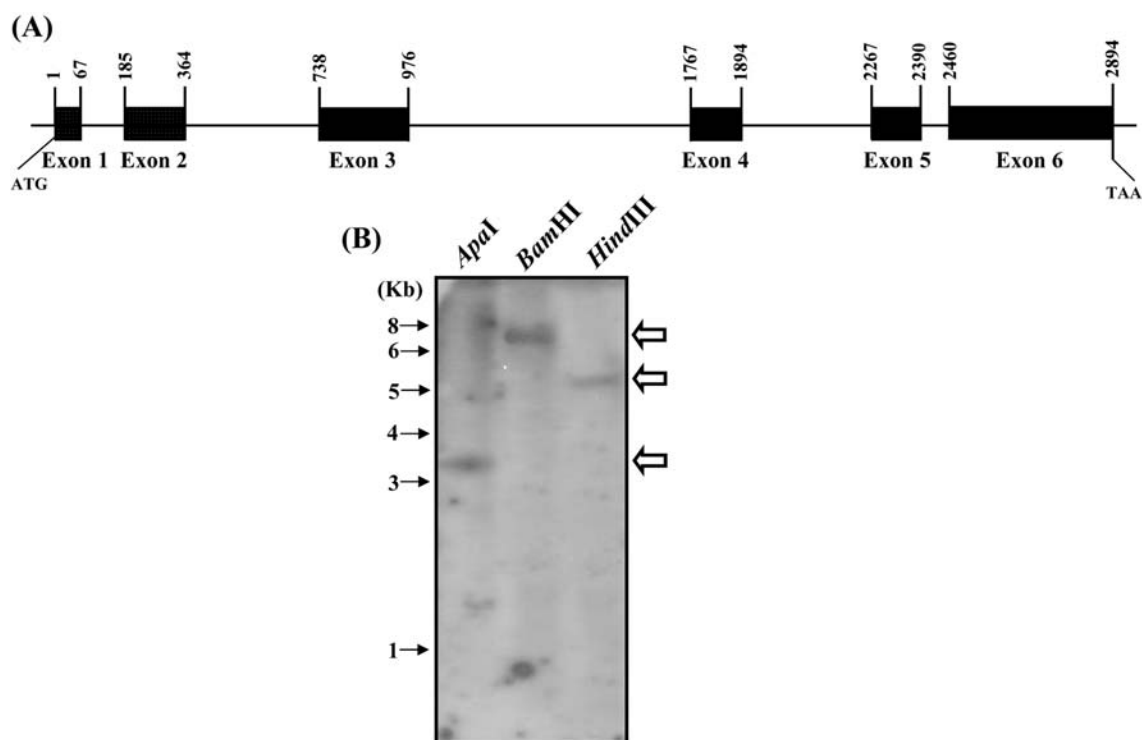
Fig. 2. Genomic organization and copy number of the *AgChi* gene. (A) Organization of the *AgChi* gene. Numbers indicate the position in the genomic sequences. The GenBank accession number of *AgChi* genomic DNA is DQ837585. (B) Southern blot analysis of *A. germari* genomic DNA for the *AgChi* gene. Genomic DNA was digested with three restriction enzymes, *Apa*I, *Bam*HI and *Hind*III, respectively, and hybridized with radiolabeled *AgChi* cDNA. Size markers are shown on the left. Open arrows indicate hybridized bands.

RESULTS

Cloning, sequencing and analysis of *AgChi* gene

The *AgChi* cDNA was isolated from searches of *A. germari* ESTs that encode a protein of 390 amino acids (GenBank accession number DQ837584). A comparison of the deduced amino acid sequence of the *AgChi* gene with that of other chitinase sequences is shown in Fig. 1. AgChi conserved the chitinase family 18 active site signature, which is a consensus sequence, [LIVMFY]-[DN]-G--[LIVMF]-[DN]-[LIVMF]-[DN]-X-E (Van Scheltinga et al., 1994). The deduced AgChi N-terminal sequence contained a highly hydrophobic amino acid stretch that is likely to function as a signal peptide. Using von Heijne's rule (Von Heijne, 1986), we predicted that the signal peptide is cleaved after Ala-19. AvChi possessed three putative N-glycosylation sites at the amino acid residues 113–116 (NFSA), 154–157 (NGSD) and 241–244 (NATL). Based on the sequence and predicted protein structure similarity, AgChi showed the closest amino acid identity (57%) with a gut-specific chitinase of the mustard beetle *Phaedon cochleariae* (Chrysomelidae). On the basis of these characteristics, we propose that AgChi is a member of the same family as all other insect chitinases identified to date.

To characterize the genomic structure of the *AgChi* gene, a primer set based on the sequences of the *AgChi* cDNA was designed and the PCR product was cloned and sequenced. The organization of the gene is illustrated in Fig. 2A. Comparison of the genomic sequence with the sequence of the cDNA revealed six exons and five introns in the *AgChi* gene. The sequences at the exon-intron boundaries conformed to consensus eukaryotic splice sites, including an invariant GT at the intron 5' boundary and an invariant AG at its 3' boundary. The genomic DNA size from translation start codon to stop codon was 2894 bp for *AgChi*.

The copy number of the *AgChi* gene in the *A. germari* genome was determined by Southern blot analysis. The genomic DNA was digested with restriction enzymes that do not cut within the *AgChi* gene, blotted, and then hybridized with the *AgChi* cDNA. A single hybridizing band was detected with each enzyme, indicating that the *AgChi* is present as a single copy in the genome of *A. germari* (Fig. 2B).

Expression of AgChi in baculovirus-infected insect cells

To assess the expression of *AgChi* cDNA, the 1170 bp of *AgChi* cDNA was inserted into a baculovirus transfer vector. The baculovirus transfer vector was used to generate a recombinant virus expressing AgChi. The transfer vector pBacPAK9-*AgChi* was constructed by insertion of *AgChi* cDNA under the control of an AcNPV polyhedrin promoter of pBacPAK9 (data not shown). Recombinant AcNPV, which we have termed AcNPV-AgChi, was produced in insect Sf9 cells by co-transfection with wild-type AcNPV DNA and the transfer vector.

Recombinant AgChi protein synthesis in Sf9 cells infected with the recombinant virus AcNPV-AgChi was
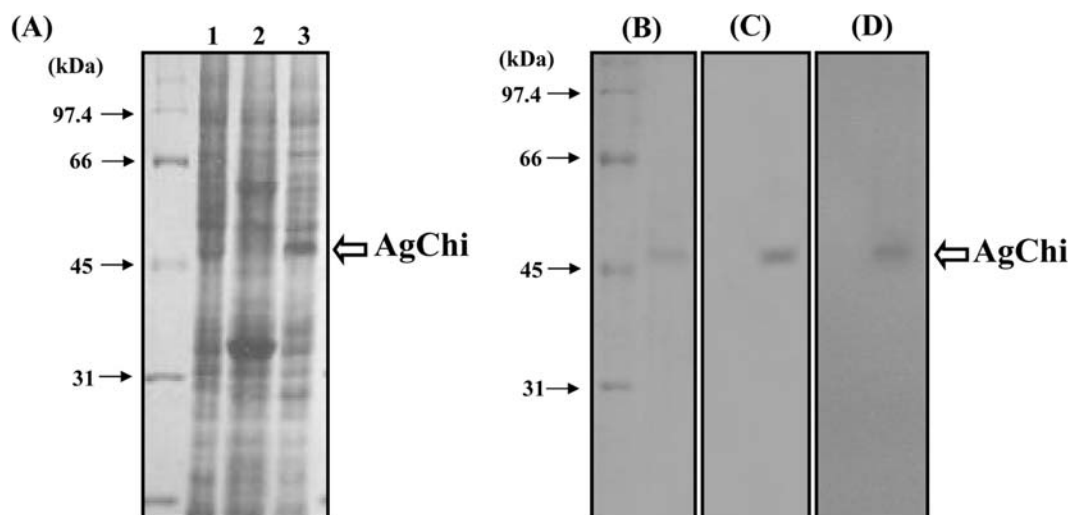
Fig. 3. Expression, purification and chitinolytic activity assay of recombinant AgChi in baculovirus-infected insect cells. (A) SDS-PAGE of the recombinant AgChi expressed in baculovirus-infected insect cells. Sf9 cells were mock-infected (lane 1) or infected with wild-type AcNPV (lane 2) and recombinant AcNPV (lane 3) at a MOI of 5 PFU per cell. Cells were collected at 2 days p.i. Total cellular lysates were subjected to 10% SDS-PAGE. The recombinant AgChi is indicated by open arrow on the right side of the panel. Molecular weight standards were used as size markers. (B–D) Purification and identification of recombinant AgChi. Recombinant AgChi purified from baculovirus-infected insect cells was subjected to 10% SDS-PAGE (B), electroblotted and incubated with recombinant AgChi antibody (C). The same recombinant AgChi as in (B) was separated by 10% SDS-PAGE containing 0.01% glycol chitin. The chitinolytic activity of recombinant AgChi was assayed as dark lytic zones under UV illumination (D).

confirmed by SDS-PAGE (Fig. 3A). The recombinant AgChi was present as a single polypeptide band of 46 kDa in cells infected with the recombinant virus, but not in cells infected with the wild-type AcNPV or mock-infected cells.

In order to characterize the recombinant AgChi expressed in baculovirus-infected insect cells, recombinant AgChi with a molecular mass of 46 kDa was purified from the recombinant baculovirus (AcNPV-AgChi)--infected Sf9 cells. The purified recombinant AgChi was identified as a single band of 46 kDa by SDS-PAGE (Fig. 3B) and confirmed by Western blot analysis using the antibody against recombinant AgChi (Fig. 3C). The protein bands with chitinolytic activity were directly detected as dark lytic zones under UV illumination in SDS-polyacrylamide gels containing 0.01% glycol chitin. Chitinolytic activity was detected for purified recombinant AgChi of 46 kDa from recombinant virus-infected insect cells (Fig. 3D).

N-linked glycosylation of AgChi

In order to determine whether the potential N-linked glycosylation sites are being utilized, recombinant baculovirus (AcNPV-AgChi)-infected cells were incubated with or without tunicamycin, which is a specific inhibitor of N-linked oligosaccharides addition (Elbein, 1984). Then, total cellular lysates were subjected to SDS-PAGE (Fig. 4A), Western blot analysis (Fig. 4B) and chitinolytic activity assays (Fig. 4C).

Fig. 4 shows an apparent shift in the molecular weight of the recombinant AgChi in tunicamycin-treated Sf9 cells. SDS-PAGE and Western blot analysis revealed two bands, one of 46 kDa and one of 42 kDa, corresponding to the N-linked glycosylated and non-glycosylated recom-

binant AgChi, respectively, suggesting that AgChi is N-linked glycosylated. The significance of N-linked glycosylation was further tested by measuring chitinolytic activity of recombinant AgChi (Fig. 4C). The chitinolytic activity was detected in the protein bands of both N-linked glycosylated and non-glycosylated AgChi, indicating a non-essential role of the carbohydrate moieties for AgChi activity.

Tissue distribution of AgChi

AgChi distribution among *A. germari* larval tissues was investigated at both the transcriptional and translational levels. First, the expression of *AgChi* was determined by Northern blot analysis of epidermis, fat body, and gut. Northern blot analysis showed that a hybridization signal was present as a single band in the gut, indicating the gut as a specific site for AgChi synthesis (Fig. 5A). The expression of *AgChi* was determined by Northern blot analysis of three different gut regions (Fig. 5B). *AgChi* was expressed in all of them, although expression in the hindgut was relatively low. Next, the distribution of AgChi was analyzed from protein samples of three gut regions using antiserum against recombinant AgChi (Fig. 5C). A signal band of 46 kDa corresponding to AgChi was detected from three gut regions by Western blot analysis. Consistent with Northern blot analysis, the results of Western blot analysis revealed that AgChi was observed in the three gut regions tested.

Furthermore, the tissue-specific expression of AgChi was analyzed using protein samples of epidermis, fat body, and gut of *A. germari* larvae. Each protein sample was subjected to 10% SDS-PAGE (Fig. 6A) and Western blot analysis (Fig. 6B). A 46 kDa band was detected specifically in the gut by Western blot analysis, but not in the
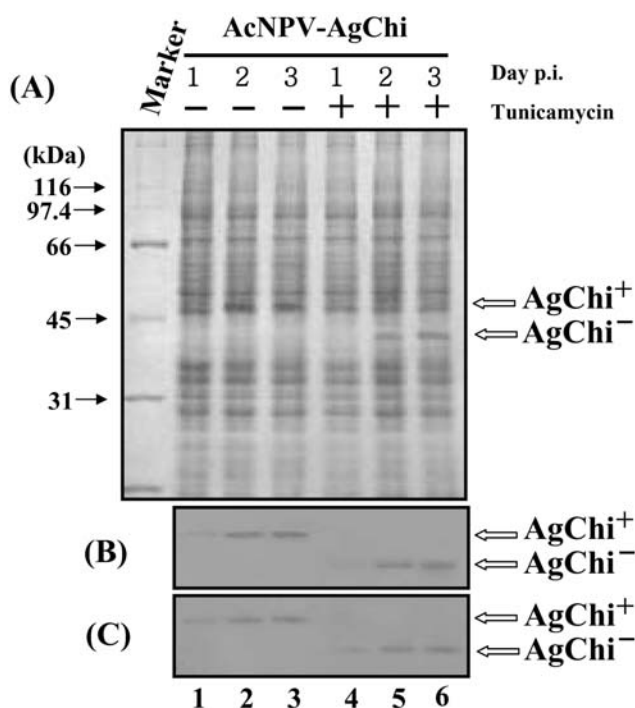
Fig. 4. N-linked glycosylation of recombinant AgChi by tunicamycin treatment. Sf9 cells were infected with recombinant AcNPV at a MOI of 5 PFU per cell. Cells were treated without (lanes 1, 2 and 3) or with (lanes 4, 5 and 6) tunicamycin (5 μg/ml). Total cellular lysates were collected at 1 (lanes 1 and 4), 2 (lanes 2 and 5) and 3 (lanes 3 and 6) days p.i., respectively. Total cellular lysates were subjected to 10% SDS-PAGE (A), electroblotted and incubated with recombinant AgChi antibody (B). The same cellular lysates as in (A) were separated by 10% SDS-PAGE containing 0.01% glycol chitin. The proteins with chitinolytic activity are identified as dark lytic zones under UV illumination (C). The N-linked glycosylated AgChi (AgChi+) and carbohydrate-deficient AgChi (AgChi−) are indicated by arrows on the right side of the panel.

epidermis and fat body protein samples, suggesting that the gut is the primary site for AgChi synthesis. The protein band of 46 kDa showed chitinolytic activity (Fig. 6C). The result was in good agreement with Northern blot hybridization results that showed AgChi tissue-specific expression in the gut. However, a protein band of 44 kDa from gut extracts also displayed chitinolytic activity in the enzyme assay (Fig. 6C). Thus, the chitinolytic activity assay suggested that *A. germari* has an additional chitinase in the gut. On the other hand, the chitinolytic activity in the enzyme assay was detected in both epidermis and fat body (Fig. 6C). This result suggests that *A. germari* has other chitinase genes in both the epidermis and the fat body, as well as in the gut.

DISCUSSION

We described the cloning and characterization of the beetle *A. germari* chitinase gene, which shows sequence similarity to members of family 18 of the glycosyl hydrolase superfamily. Family 18 glycosyl hydrolases possess a consensus pattern in their primary amino acid sequence, (LIVMEY) (DN) G (LIVMF) (DN) (LIVMF) (DN) XE,
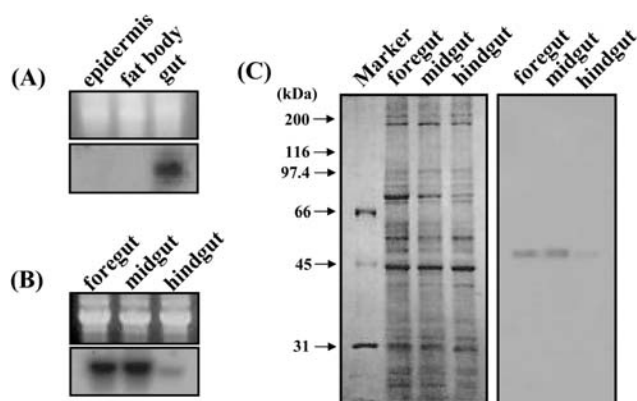

Fig. 5. Distribution of AgChi in *A. germari* larva. (A, B) Northern blot analysis of the *AgChi* gene. Total RNA was isolated from the epidermis, fat body, and gut (foregut, midgut, and hindgut), respectively. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panels), transferred onto a nylon membrane, and hybridized with radiolabelled *AgChi* cDNA (lower panels). (C) Western blot analysis of AgChi. The protein sample was collected from foregut, midgut and hindgut of *A. germari* larvae. The protein samples were subjected to 10% SDS-PAGE (left panel), electroblotted and incubated with recombinant AgChi antibody (right panel).

in which glutamic acid is the critical active site residue. AgChi, with 390 amino acid residues, exhibits homology to chitinases characterized from other coleopteran insects (Girard & Jouanin, 1999), and the consensus pattern of family 18 glycosyl hydrolases is present in AgChi. AgChi also has conserved catalytic site residues, although the cysteine-rich 3'-end domain associated with chitin binding and the serine/threonine rich domain are missing. Recent studies have reported that some chitinases lack the serine/threonine rich domain (Yan et al., 2002) or both
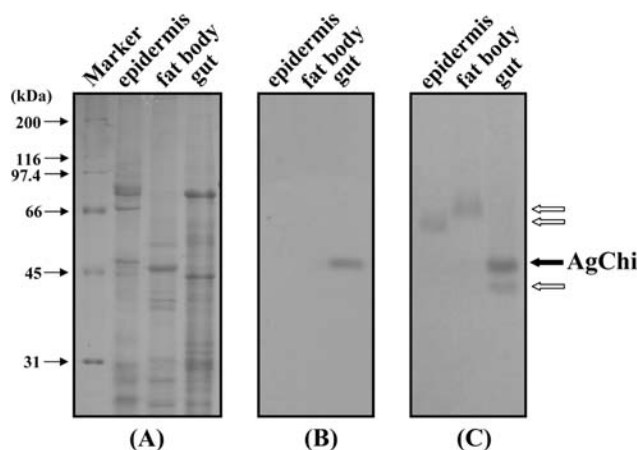

Fig. 6. Tissue-specific expression of AgChi. The protein samples were collected from epidermis, fat body, and gut of *A. germari* larvae. The protein samples were subjected to 10% SDS-PAGE (A), electroblotted and incubated with recombinant AgChi antibody (B). In addition, the protein samples as in (A) were separated by 10% SDS-PAGE containing 0.1% gelatin and the proteins with proteolytic activity were identified as lytic zones (C). The AgChi (solid arrow) and signal bands showing proteolytic activity (open arrows) are indicated on the right side of the panel.

the serine/threonine and chitin binding domains (De la Vega et al., 1998; Girard & Jouanin, 1999; Han et al., 2005). Based on the sequence and predicted protein structure similarity, the AgChi is similar to the beetle chitinase from *P. cochleariae*, which also lacks for both the serine/threonine and chitin binding domains, but is still enzymatically active (Girard & Jouanin, 1999).

The expression of AgChi revealed that it displays tissue-specific expression in the gut. Consistent with this, a chitinolytic activity assay confirmed the presence of a 46 kDa AgChi polypeptide in the gut. It is likely that the gut is the main tissue for AgChi synthesis. In the distribution of AgChi among three gut regions, AgChi was expressed in all three regions, but was mainly expressed in the foregut and midgut, while the hindgut showed a relatively low expression. Thus, the simultaneous expression of AgChi in three gut regions of *A. germari* larvae suggests that it is involved in the degradation of chitin in the gut. Chitin is one of the vital components of the lining of the digestive tract of insects; in the foregut and hindgut, chitin is found in a cuticular lining, whereas in the midgut, it is present in the peritrophic membranes (Kramer & Koga, 1986; Filho et al., 2002). It is known that insect chitinases are involved in the degradation of chitin in the gut and the cuticle during the molting process in insects (Merzendorfer & Zimoch, 2003).

The genomic organization of the *AgChi* gene in *A. germari* analyzed by Southern blot hybridization suggests that the *AgChi* gene exists as a single copy in the genome of *A. germari*. However, the protein bands displaying chitinolytic activity in the enzyme assay were detected in both epidermis and fat body. This result strongly suggests that *A. germari* has other chitinase genes in both the epidermis and fat body as well as in the gut. In addition, the result suggests the possible presence of another chitinase in the gut that was recognized by the chitinolytic activity assay. Chitinases belonging to multi-gene families have been found in *Aedes aegypti*, *Anopheles gambiae*, and *Drosophila* (De la Vega et al., 1998). Several arthropod chitinase genes have been identified from midgut (Shen & Jacobs-Lorena, 1997; Girard & Jouanin, 1999; Ramalho-Ortigao & Traub-Cseko, 2003), epidermis (Fitches et al., 2004), and fat body (Yan et al., 2002; Han et al., 2005). Taken together, our present results show that the genome of the beetle *A. germari*, includes at least four chitinase genes and suggest that chitinases are involved in a wide range of physiological functions through tissue-specific expression (Flach et al., 1992; Kramer & Muthukrishnan, 1997; Yan et al., 2002; Merzendorfer & Zimoch, 2003).

The recombinant AgChi is expressed as a 46 kDa polypeptide in baculovirus-infected insect cells. However, the size of recombinant AgChi observed by SDS-PAGE (46 kDa) did not correspond to the calculated molecular mass of AgChi (42 kDa). This possibly may be caused by differences at the level of post-translational modification such as glycosylation (Kramer et al., 1993; Kim et al., 1998; Shinoda et al., 2001; Wu et al., 2001; Zheng et al., 2002; You et al., 2003; Han et al., 2005). In order to assess whether the expected addition of an N-linked carbohydrate moiety in the potential N-linked glycosylation sites at Asn113, Asn154 and Asn241 is being accomplished, recombinant virus-infected cells were incubated in the presence of tunicamycin, which is a specific inhibitor of the addition of N-linked oligosaccharides. An apparent shift in the molecular weight of the recombinant AgChi in Sf9 cells treated with tunicamycin suggests that the 46 kDa polypeptide and 42 kDa polypeptide, respectively, correspond to N-linked glycosylated and non-glycosylated recombinant AgChi. This result indicates that AgChi is truly N-linked glycosylated. Furthermore, the chitinolytic activity between N-linked glycosylated and non-glycosylated recombinant AgChi revealed no substantial difference in enzymatic activity, suggesting that the carbohydrate moieties are not essential for AgChi chitinolytic activity.

In conclusion, the results reported here describing the gene cloning and expression of a novel gut-specific chitinase in *A. germari* provide a background for further investigation of the molecular physiology that elucidates AgChi function in the beetle *A. germari*. Two observations deserve attention: the functional role of AgChi through RNA interference-mediated knock-down and the enzymatic property of AgChi through kinetic analysis. Thus, additional studies are needed to elucidate the role as a digestive enzyme as well as chitin degradation in the gut during the molting process.

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