A cDNA encoding tick chitinase was cloned from a cDNA library of mRNA from *Haemaphysalis longicornis* eggs and designated as CHT1 cDNA. The CHT1 cDNA contains an open reading frame of 2790 bp that codes for 930 amino acid residues with a coding capacity of 104 kDa. The deduced amino acid sequence shows a 31% amino acid homology to *Aedes aegypti* chitinase and a multidomain structure containing one chitin binding peritrophin A domain and two glycosyl hydrolase family 18 chitin binding domains. The endogenous chitinase of *H. longicornis* was identified by a two-dimensional immunoblot analysis with mouse anti-rCHT1 serum and shown to have a molecular mass of 108 kDa with a PI of 5.0. A recombinant baculovirus AcMNPV-CHT1-expressed rCHT1 is glycosylated and able to degrade chitin. Chitin degradation was ablated by allosamidin in a dose-dependent manner. The optimal temperature and pH for activity of the purified chitinase were 45 °C and pH 5–7. The CHT1 cDNA has an ELR motif for chemokine-mediated angiogenesis and appears to be a chitinase of the chemokine family. Localization analysis using mouse anti-rCHT1 serum revealed that native chitinase is highly expressed in the epidermis and midgut of the tick. AcMNPV-CHT1 topically applied to *H. longicornis* ticks exhibited replication. This is the first report of insect baculovirus infection of ticks. The importance of AcMNPV-CHT1 as a novel bio-.acaricide for tick control is discussed.

Chitin, the β-1,4-linked homopolymer of N-acetyl-D-glucosamine, is an insoluble structural polysaccharide that is important as a supporting element in extracellular structures. It is seen in the exoskeleton of arthropods where the chitin microfibrils complex with proteins to form the chitinous structures of the cuticle and the peritrophic membrane (PM) lining the gut (1, 2). The PM extracellularly surrounds the food bolus in the guts of most arthropods (3, 4). It has been shown to be important in preventing damage or clogging of microvilli by the luminal contents (5, 6) and compartmentalization of digestive events by acting as a permeability barrier for digestive enzymes (7) and to have a novel role for protecting *Leishmania* from the hydrolytic activities of the sand fly midgut (8). However, arthropods must be able to hydrolyze the chitin to allow degradation of the cuticle and development during the immature stages. During the molting cycle have been investigated biochemically and morphologically (9), and chitinase has been shown to be an essential component in the hydrolysis of chitin. Ticks are hemimetabolous in nature, implying they also require chitinolytic enzymes to remove old cuticle and allow synthesis of new cuticle for continued growth and development. Chitinase is induced by ecysoides to degrade the older chitin at the time of molting (10, 11). The chitinase inhibitor, allosamidin, isolated from culture broth of *Strepto- myces* sp. (12), has been used as an important tool to elucidate several essential features of insect chitinases (13).

Ticks are second only to mosquitoes as vectors of disease-causing agents in humans and are the most important arthropod transmitting pathogens to domestic and wild animals, *e.g.* Babesia and *Theileria* *protozoa, Borrelia* bacteria, and hemorrhagic and encephalomyelitis virus (14, 15). The hard tick, *Haemaphysalis longicornis*, is distributed mainly in East Asia and Australia, where it transmits these pathogens (16, 17). A variety of methods have been employed to suppress tick vector populations, including the application of biological control agents and the heavy reliance on chemical acaricides. However, the development of resistance to acaricides (18–20) and the increase in legislation to combat detrimental effects of residues of acaricides in the environment (21) emphasize the need to develop alternatives for tick vector control. Recently, we have shown that tropomycin (22) and peroxiredoxin (23) from *H. longicornis* may be candidates for control of ticks. Molting-associated molecules responsible for protecting from invasion of pathogens, control of the PM, and other necessary functions during blood feeding and molting in ticks may be candidates for tick control. Chitinase is a selective insect control protein and is safe as it is readily degradable in the environment (24).

In the present study, we report the results of the DNA cloning, identification, characterization, and expression of chitinase from the hard tick *Haemaphysalis longicornis*.

*This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank Data Bank with accession number(s) AB074977.

†† Supported by the program for Promotion of Basic Research Activities for Innovative Bioscience.

§ Supported by the 21st Century of Excellence program of the Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology. To whom correspondence should be addressed. Tel.: 81-155-49-5646; Fax: 81-155-49-5643; E-mail: fujisaki@obihiro.ac.jp.

1 The abbreviations used are: PM, peritrophic membrane; GST, glutathione S-transferase; AcMNPV, *A. californica* multiple nuclear polyhedrosis virus; 4MU-(GlcNAc)₃, 4-methylumbelliferyl-N-N′-tri-acetyl-β-D-glucosaminide; ACM, AcMNPV-CHT1 culture medium; PBS, phosphate-buffered saline.
Tick—The pathogenetic Okuyama strain of the hard tick *H. longicornis* (16) has been maintained by feeding on rabbits and mice in our laboratory since 1997.

**EXPERIMENTAL PROCEDURES**

**Tick Cytosine Deaminase**—The cDNA expression library was constructed in M13 reverse and universal phagemids (PerkinElmer Life Sciences). Sequence analysis was performed with the computer program MacVector (Oxford Molecular, Madison, WI).

**Expression of the H. longicornis Chitinase in Escherichia coli**—A 2790-bp PCR fragment from *H. longicornis* chitinase containing an open reading frame was inserted into the EcoRI site of an AcMNPV transfer vector, pBlueBac4.5 (25). The resulting recombinant virus, AcMNPV-ChT1, was propagated in *Sf9* cells infected with *Bombyx mori* expression supernatant. The culture supernatant containing recombinant virus was harvested and subjected to plaque purification. The expression of rCHT1 in the plaques was confirmed by indirect immunofluorescence antibody test with anti-rCHT1 serum. Positive plaques were selected, and after three cycles of purification a recombinant virus, AcMNPV-ChT1, was obtained.

**Production of rCHT1**—Female mice (BALB/c, 8 weeks old) were immunized intraperitoneally three times at 2-week intervals with 100 μg of the recombinant fusion protein in Freund’s incomplete adjuvant. Sera were collected from immunized mice 10 days after the last immunization.

**Two-dimensional Immunoblot**—Tick extracts were treated with an equal volume of urea mixture consisting of 9 g urea, 4% Nonidet P-40, 0.8% amphotine (pH 3.5–8; Amersham Biosciences), and 2% mercaptoethanol and then subjected to two-dimensional PAGE. Non-equilibrium pH gradient electrophoresis was performed in the first dimension using a rectangular gel electrophoresis apparatus (AE-6050 A; ATTO, Tokyo, Japan). After electrophoresis at 400 V for 2 h, the gels were prepared for the second dimension by excision capabilities of a rectangular gel electrophoresis apparatus (AE-6050 A; ATTO, Tokyo, Japan). After electrophoresis at 400 V for 2 h, the gels were prepared for the second dimension by excision of the desired zone and transferred to nitrocellulose membranes. Immunoblot analysis was carried out as previously described (26). Anti-mouse rCHT1 serum was used at a dilution of 1:500. The proteins bound to the secondary antibody were visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**Northern Blot Analysis**—Formaldehyde-denatured RNA (10 μg) extracted from eggs of *H. longicornis* incubated at 25 °C for 10 days was fractionated on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Biosciences), and hybridized to a probe alkaline phosphatase-labeled probe derived from the CHT1 cDNA. The presence of probe was detected using CDP-Star (Amersham Biosciences) by chemiluminescent technique. The hybridization technique was performed as described in the Alkphos Direct manual (Amersham Biosciences).

**Cells and Viruses**—*Spodoptera frugiperda* (Sf9) cells were propagated at 27 °C in TC-100 serum-free SF-900 II serum-free medium obtained from Invitrogen. *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and recombinant viruses were grown in Sf9 cells.

**Construction of Recombinant Baculovirus Expressing the H. longicornis Chitinase**—The 2790-bp PCR fragment from *H. longicornis* cDNA containing the open reading frame was inserted into the EcoRI site of an AcMNPV transfer vector, pBlueBac4.5/5/V5-His (Invitrogen) to produce the recombinant transfer plasmid pBlueBac4.5/5/V5-His-CHT1, and the recombinant protein was expressed by recombinant baculovirus as a protein fused with a COOH-terminal peptide (3 kDa) containing the V5 epitope and a His tag. Restriction enzyme analysis was performed to identify the construct containing the correct orientation. Sf9 cells were cotransfected with pBlueBac4.5/5/V5-His-CHT1 and linear AcMNPV DNA, Bac-N-Blue DNA (Invitrogen), by using Cellfectin reagent (Invitrogen). After 4 days of incubation at 27 °C, the culture supernatant containing recombinant virus was harvested and subjected to plaque purification. The expression of rCHT1 in the plaques was confirmed by indirect immunofluorescence antibody test with anti-rCHT1 serum. Positive plaques were selected, and after three cycles of purification a recombinant virus, AcMNPV-ChT1, was obtained.

**Purification of *H. longicornis* Chitinase**—Thirty ml of Sf9 cells culture supernatant infected with the recombinant virus (AcMNPV-ChT1) or control virus infected with the wild type virus (*AcMNPV*) were dialyzed against binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole (pH 7.4)) and concentrated by ultrafiltration (Concentrator 10/20, exclusion size: 30 kDa). Two ml of 50% nickel nitritolriacetic acid slurry (Invitrogen) was centrifuged (5 min at 500 × g) and washed twice with binding buffer. After incubation at 4 °C for 1 h, the slurry was centrifuged, and the supernatant was discarded. The pellet was washed at 4 °C with 20 ml of washing buffer (20 mM sodium phosphate, 500 mM NaCl (pH 6.0)) containing 50 mM imidazole. Elution was performed with 5 ml of elution buffer (pH 6.0) containing 350 mM imidazole. The final eluent containing the isolated protein was dialyzed against 20 mM sodium phosphate buffer (pH 6.0) at 4 °C and stored at −80 °C until further analysis.

**In Vitro Chitinase Activity Assay**—4-Methylumbelliferyl-N, N′-triacetyl-β-chitotrioside (Sigma) (4MU-(GlcNAc)3) was used for chitinase activity assay at a concentration of 0.8 mM in distilled water. Kinetic studies of chitinase were performed in a fluorescence spectrophotometer (excitation 350 nm, emission 450 nm) (Japan spectrophotometer FP-770, Tokyo, Japan) by continuously recording changes in fluorescence. Inhibition of chitinase activity was studied by using allosamidin at a final concentration of 0.01 and 0.05 μM in the reaction mixture. Allosamidin was obtained from S. Sakuda (12). Reactions were initiated by the addition of enzyme (1 μM). The enzymatic activity of the rCHT1 was quantified using 4MU-(GlcNAc)3 (31). The enzyme solutions (0.16 μg) were incubated with substrate (4MU-(GlcNAc)3) in a wide range buffer (pH 2.0–12.0) (32) at 37 °C for 15 min (final concentration 3.8 μM). Reactions were terminated by the addition of 1.2 ml of 1 M glycine buffer. In the chitinase activity assay, the enzyme solutions (0.16 μg) were incubated with substrate (4MU-(GlcNAc)3) in 20 mM sodium phosphate (pH 6.0) at 4, 15, 27, 37, 45, 50, 60, and 70 °C for 15 min (final concentration 3.8 μM). Reactions were terminated by addition of 1.2 ml of 1 M glycine buffer.

**Glycosylation Studies**—Glycosylation studies with tunicamycin were performed as described previously (24). Briefly, tunicamycin (Sigma) was added at a concentration of 10 μg/ml to the Sf9 cells 15 h after infection with AcMNPV-chT1. Purified rCHT1 was treated with N-glycosidase F under denaturing conditions as recommended by the manufacturer (N-glycosidase F deglycosylation kit, Roche Molecular Biochemicals) and analyzed by immobiloblotting with mouse anti-rCHT1 serum.

**Immunohistochemistry**—Immunohistochemistry was performed with a monospecific goat anti-mouse IgG secondary antibody as described previously (22). Molting nymphs 10 days after engorgement, unfed females, and tick tissues were handled as described previously (22).

**Infection with AcMNPV-ChT1 and Detection of rCHT1 in H. longicornis Infected with AcMNPV-ChT1**—Female *H. longicornis* larvae and adults. Ticks were collected 10 days after application. The salivary glands of adult ticks were dissected under a microscope (16). The dissected ticks were stored at −80 °C until use. Salivary glands were embedded in embedding medium (Tissue-Tek), Sakura.
Tokyo, Japan) and fixed using standard methods. Thin transverse sections of the ticks were then prepared using a microtome. The slides were blocked for 1 h with 3% skim milk (Wako, Tokyo, Japan) in PBS and then incubated for 4 h at room temperature with mouse anti-CHT1 serum diluted to 1:200 with PBS. The slides were rinsed thoroughly with PBS and reacted 1 h with red fluorescence-labeled FIG. 1. Nucleotide sequence and deduced amino acid sequence of H. longicornis CHT1 cDNA. The initial codon ATG and termination codon TAG are indicated in bold, and the putative signal peptide of the deduced amino acid sequence is underlined.

in PBS and then incubated for 4 h at room temperature with mouse anti-CHT1 serum diluted to 1:200 with PBS. The slides were rinsed thoroughly with PBS and reacted 1 h with red fluorescence-labeled.
mouse IgG secondary antibody (Alexa Flour 594® goat anti-mouse IgG (H+L), Molecular Probes, Eugene, OR). The slides were then rinsed thoroughly with PBS, covered with glass slips, and observed under a fluorescence microscope (ECLIPSE E600, Nikon, Tokyo, Japan). Larvae ticks topically treated with ACM were washed 3 times with PBS, covered with glass slips, and observed under a fluorescence microscope (ECLIPSE E600, Nikon, Tokyo, Japan). Larvae ticks topically treated with ACM were washed 3 times with PBS, covered with glass slips, and observed under a fluorescence microscope (ECLIPSE E600, Nikon, Tokyo, Japan).

RESULTS

DNA Probe, cDNA Library Screening, and DNA Sequence Analysis—Four degenerate primers based on a conserved region in the cDNAs of several chitinases were used to amplify the *H. longicornis* chitinase cDNA fragment. A PCR fragment of 291 bp was inserted into pBluescript SK(+) vector and sequenced. The nucleotide sequence of the PCR fragment from *H. longicornis* had a putative conserved chitinase domain. The

PCR fragment was used as a probe for screening the entire chitinase cDNA from the cDNA library of *H. longicornis* eggs. Five positive clones were isolated from the *H. longicornis* egg cDNA library, and no sequence heterogeneity was found among these clones. The clones appeared to be about 2.9, 4.1, 4.3, 5.5, and 6.5 kilobase pairs in size. One of the 5 cDNA clones of 6.5 kilobase pairs was designated CHT1. DNA sequencing revealed that the CHT1 cDNA with 6439 bp had a start codon at position 571 and codes 930 amino acid residues with a predicted molecular mass of about 104 kDa (Fig. 1). This deduced protein has six potential N-glycosylation sites (Fig. 1).

Analysis of *H. longicornis* egg cDNA library, and no sequence heterogeneity was found among these clones. The clones appeared to be about 2.9, 4.1, 4.3, 5.5, and 6.5 kilobase pairs in size. One of the 5 cDNA clones of 6.5 kilobase pairs was designated CHT1. DNA sequencing revealed that the CHT1 cDNA with 6439 bp had a start codon at position 571 and codes 930 amino acid residues with a predicted molecular mass of about 104 kDa (Fig. 1). This deduced protein has six potential N-glycosylation sites (Fig. 1). The mRNA corresponding to the cloned cDNA was confirmed by Northern blotting analysis of total RNA extracted from eggs of *H. longicornis* ticks to be about 7.5 kilobases in size (Fig. 2).
Procedures.

Data are presented as fluorescence units. Mean dase digestion at 35°C with a solution of 2% citrate, and subjected to lysyl endopeptidase previously (33). The spots were pooled from 20 gels, washed using the two-dimensional gels and processed as described duces, protein spots of interest were excised from tick extracts B). To determine the internal amino acid residues, protein sequencing system (Applied Biosystems, Foster, CA) an amino acid sequence except for a signal peptide. In addition, an putative mature protein (104 kDa) calculated from the CHT1 confirmed that it corresponded to the predicted size of the A having a molecular mass of 108 kDa with a pI of 5.0 (Fig. 4 B), ticks. Mouse anti-rCHT1 serum strongly reacted with a protein sional immunoblot analysis to identify endogenous CHT1 in s of the protein, peptides were collected by reverse phase high pressure liquid chromatography, and several peptides was an-

Enzyme activity was assayed using 4MU-(GlcNAc)3 as the substrate as described under “Experimental Procedures.” 

Detection of Endogenous CHT1—We performed two-dimen-
sional immunoblot analysis to identify endogenous CHT1 in ticks. Mouse anti-rCHT1 serum strongly reacted with a protein having a molecular mass of 108 kDa with a pI of 5.0 (Fig. 4A), confirming that it corresponded to the predicted size of the putative mature protein (104 kDa) calculated from the CHT1 amino acid sequence except for a signal peptide. In addition, an endogenous CHT1 was identified on silver-stained two-dimen-
sional gels on which more than 200 visible protein spots ap-
ppeared (Fig. 4A). To determine the internal amino acid residues, protein spots of interest were excised from tick extracts using the two-dimensional gels and processed as described previously (33). The spots were pooled from 20 gels, washed with a solution of 2% citrate, and subjected to lysyl endopeptidase digestion at 35°C for overnight (34). After in-gel digestion of the protein, peptides were collected by reverse phase high pressure liquid chromatography, and several peptides was analyzed for internal amino acid sequence in Procise 494 cLC protein sequencing system (Applied Biosystems, Foster, CA) (35, 36). The resultant amino acid sequences of four major reverse phase high pressure liquid chromatography purified peaks were identical to those of the deduced amino acid se-
quences encoded by CHT1 cDNA.

The H. longicornis Chitinase Secreted into the Medium of SF9 Cells Culture Is Glycosylated—Expression of chitinase in AcMNPV-CHT1-infected SF9 cells in the presence or absence of tunicamycin, an inhibitor of protein N-linked glycosylation, is shown in Fig. 5A. In the absence of tunicamycin, immunoreactive chitinase was released into the medium as a 116-kDa protein (Fig. 5A, lane 1). However, tunicamycin inhibited the secretion of this protein into the medium (Fig. 5A, lane 3). In extracts of cells not treated with tunicamycin, the anti-rCHT1 serum reacted with major proteins of ~107 and 116 kDa. Cells treated with tunicamycin exhibited an immunoreactive protein with an apparent molecular mass of about 107 kDa (Fig. 5A, lane 1). N-Glycosidase-treated rCHT1 protein exhibited a shift to a lower apparent molecular mass of about 107 kDa (Fig. 5B, lane 2), indicating the existence of N-linked glycan chains.

Purification of Recombinant H. longicornis CHT1 and In Vitro Chitinase Activity Assay of Purified rCHT1—The His-
tagged H. longicornis rCHT1 was separated from the endoge-
nous viral chitinases using affinity chromatography. In vitro chitinase activity assay using the purified rCHT1 showed a strong hydrolysis of 4MU-(GlcNAc)3 substrate (Fig. 6). In vitro inhibition assay of chitinase activity using 0.05 μM (final concentration) allosamidin with purified rCHT1 showed a strong inhibition of the chitinase activity (Fig. 6). The specific activity (fluorescence unit) of His6-tagged H. longicornis rCHT1 against 4MU-(GlcNAc)3 was optimal at pH 5–7 but was inactive under acidic (pH <3) or alkaline (pH >10) conditions (Fig. 7A). The enzymatic activities increased gradually as the temperature
was raised from 0 to 45–50 °C (Fig. 7B). The maximum activity was exhibited at 45 °C (Fig. 7B). The enzymatic activities started to decrease dramatically when the temperature was above 50 °C (Fig. 7B). The heat stability of the enzyme was tested after preincubation in 20 mM sodium phosphate buffer (pH 6.0) at 4, 15, 27, 37, 45, 50, 60, and 70 °C for 60 min. The results are shown in Fig. 7C. The rCHT1 protein was stable up to 45 °C and completely inactivated at 60 °C and higher temperatures.

**Localization of Native CHT1—**The location of native CHT1

**Fig. 9. Detection of rCHT1.** A, detection of rCHT1 expressed in adult *H. longicornis* salivary glands with mouse anti-rCHT1 serum by immunofluorescence assay. AcMNPV-HT1 was dropped as described under “Experimental Procedures.” The picture on the left was detected with mouse anti-rCHT1 fusion protein serum. The picture on the right was detected with mouse anti-GST serum. B, immunoblot analysis with mouse anti-V5-AP antibody (Fig. 9A). Existence of rCHT1 in infected *H. longicornis* larvae was also confirmed by immunoblot analysis with anti-V5-AP antibody (Fig. 9B). *H. longicornis* larvae lysate treated with ACM reacted with the 107-kDa band.

**DISCUSSION**

Chitinases are extremely important for the hydrolytic cleavage of the β-glycosidic linkages between GlcNAc residues of chitin to allow molting and growth in the arthropods. In general this hydrolysis can occur in one of two ways, either with retention of anomeric configuration in the product or with inversion (37). Several arthropod chitinases have been identified (25, 26, 38), but this is the first report for identification of chitinase in the arachnids, including ticks.

The complete sequence of a chitinase cDNA, CHT1, was obtained from a cDNA library of *H. longicornis* eggs. The open reading frame codes a protein with a predicted molecular mass of 104 kDa. The NH2-terminal sequences of the encoded protein contain numerous hydrophobic residues, characteristic of a leader peptide (Fig. 1). The putative cleavage site of the signal peptide was between residues 22 and 23 as determined by signalP prediction (39). Expression of the CHT1 cDNA in *S. frugiperda* cells infected with a recombinant baculovirus under the control of a polyhedrin promoter produced and secreted an enzymatically active protein with a molecular mass of 116 kDa. These results indicate the presence of a functional signal peptide.

*H. longicornis* CHT1 possesses potential N-glycosylation sites at the amino acid residues 115–118 (NPSL), 257–260 (NETT), 479–482 (NWSA), 729–732 (NLTD), 745–748 (NYTG) and 909–912 (NESV). Studies with tunicamycin indicated the existence of rCHT1 in *H. longicornis* (Fig. 5A). The open reading frame of the CHT1 cDNA in the recombinant virus directs the synthesis of a 116-kDa protein larger than the predicted

**Fig. 10. Comparison of conserved regions in amino acid sequences of *H. longicornis* CHT1 with other chitinases.** The chitinase sequences listed are *H. longicornis* (this work), *B. mori* (GenBank™ accession number U86876), *M. sexta* (GenBank™ accession number U02270), *A. aegypti* (GenBank™ accession number T14075), *Spodoptera litura* (GenBank™ accession number AB032107), *Chelonus* sp. (GenBank™ accession number U10422), *Brugia malayi* (GenBank™ accession number M73689), *Aphanocladium album* (GenBank™ accession number X64104). Numbers in parentheses list position in the amino sequence. Asterisks indicate highly conserved residues.
molecular mass of 104 kDa, implying that the *H. longicornis* rCHT1 undergoes posttranslational modification. Consistent with this are the observations that the mouse anti-rCHT1 serum in the presence of tunicamycin did not react with major constituent protein bands of 116 kDa in the media or the cells. Instead, mouse anti-rCHT1 serum detected a 107-kDa protein accumulating in the cells. Use of the enzyme N-glycosidase F is the most effective method of removing virtually all N-linked oligosaccharides from glycoproteins (40). Fig. 5B of N-glycosidase-treated CHT1 protein provides evidence that the CHT1 protein from the tunicamycin-treated cells of Fig. 5A is deglycosylated. These results verify that *H. longicornis* rCHT1 with N-glycosylation sites is glycosylated.

*H. longicornis* CHT1 possesses a chitin binding peritrophin A domain at amino acid residues 868–922 and 2 glycosyl hydrolase family 18 chitin binding domains at amino acid residues 44–389 and 471–817. These domains strongly suggest *H. longicornis* CHT1 plays an important role as a chitin hydrolase in the life cycle of ticks.

The deduced amino acid sequence showed low homology to other reported chitinase sequences, such as *Aedes aegypti* (GenBank™ accession number T14075; 31% amino acid homology) by BLASTN (prediction of protein localization site) and MacVector Ver. 6.5 programs (sequence analysis software). Comparison of conserved regions in the amino acid sequences of chitinases revealed *H. longicornis* CHT1 has two pairs of conserved domains similar to those of *A. aegypti* chitinase (Fig. 10). The low homology in chitinase sequences indicates a strong difference between species. Only the sequence of the actual catalytic center, region II (Fig. 10), which includes the amino acids Asp-164 and Asp-592 and Glu-168 and Glu-596 in *H. longicornis* CHT1, are highly conserved in all family 18 chitinases characterized so far. This catalytic center is indispensable for enzyme activity (41) and implies that *H. longicornis* CHT1 is a member of the family 18 chitinases.

*In vitro* chitinase activity assay using the purified baculovirus-expressed rCHT1 showed chitin hydrolysis, indicating rCHT1 is a *H. longicornis* chitinase (Fig. 6). All family 18 chitinases are inhibited by allosamidin (13), and the activity of rCHT1 is a member of the family 18 chitinases.

R. *Chitinase from the Hard Tick Haemaphysalis longicornis*

Chitinase is produced in molting fluid and gut tissues subsequent to feeding at the end of a larval instar in preparation for a molt (25, 45). Examination of flat sections showed a strong reactivity in the cuticle epidermis and midgut of *H. longicornis*, illustrating the *H. longicornis* chitinase is abundantly expressed there (Fig. 8). The PM formed in the midgut lumen of blood-sucking arthropods after a blood meal (3, 5) may act as a barrier for invasion of ingested microorganisms (46). Chitin is a key structural component of the PM (3, 47). In support of this, gut specific chitinases have been found, and PM shown to be stronger and more persistent in the guts of mosquitoes fed chitinase inhibitors (47, 48). The existence of PM in ticks has been demonstrated morphologically with *Babesia microti* passing through it in *Ixodes* ticks (49). The appearance of *H. longicornis* chitinase in the midgut (Fig. 8) suggests this chitinase may be involved in controlling turnover and porosity of the chitin-containing PM. The appearance of *H. longicornis* chitinase in the cuticle epidermis (Fig. 8) implies it acts as a molting enzyme. These results indicate *H. longicornis* chitinase is a very important enzyme for molting and control of turnover and porosity of the PM in ticks and, thus, is a major candidate as a bio-acaricide.

Baculoviruses have long been attractive biological agents for control of crop pest insect. However, limitations of their use have been a very narrow host range and slow killing rate, resulting in significant crop damage. Recently, viruses with increased host range and improved virulence are being engineered (50, 51). *S. frugiperda* larvae injected with a transformed AcNPV expressing *M. sexta* chitinase died more quickly than those injected with a wild type virus (24). In this study, *H. longicornis* became infected with AcNPV-CHT1 (Fig. 9), and rCHT1 was replicated. This is the first report of infection with recombinant insect-baculoviruses in ticks. *H. longicornis* adults treated with high concentration of ACM died (data not shown) as with *M. sexta* chitinase (24). Therefore, AcNPV-CHT1 is a candidate for a potential bio-acaricide. However, to facilitate the use of chitinases in an integrated pest management system as a new and highly selective approach to tick control we must gain much more knowledge.

This study represents the first report of the cloning, identification, and characterization of a tick chitinase, expression of the eDNA in an insect baculovirus, demonstration of the ability of the purified baculovirus expressed rCHT1 to degrade chitin, localization of chitinase in the epidermis and midgut of ticks, and infection of ticks with a recombinant insect-baculovirus. Further studies exploring the usefulness of *H. longicornis* chitinase as a vaccine and novel bio-acaricide against ticks need to be investigated extensively.

Acknowledgments—We thank Dr. Y. Ando of National Institute of Animal Health for excellent technical assistance. We thank Dr. S. Sakuda (Tokyo University) for providing allosamidin.

REFERENCES

1. Shao, L., Deavenport, M., and Jacobs-lorena, M. (2001) Arch. Insect Biochem. Physiol. 47, 119–125
2. de la Vega, H., Specht, C. A., Liu, Y., and Robbins, P. W. (1998) Insect Mol. Biol. 7, 233–239
3. Peters, W. (1992) in Zoophysiology: Peritrophic Membranes (Bradshaw, S. D., Burgreen, W., Heller, H. C., Ichii, S., Langer, H., Neuweiler, G., and Randall, D. J., eds) Vol. 3, Springer-Verlag, Berlin
4. Lehane, M. J. (1997) Ann. Rev. Entomol. 42, 525–550
5. Richards, A. G., and Richards, P. A. (1977) Ann. Rev. Entomol. 22, 219–240
6. Burner, R., Rudin, W., and Becker, H. (1983) J. Ultrastruct. Res. 83, 195–204
7. Terra, W. R. (1996) Annu. Rev. Entomol. 35, 181–200
8. Pimenta, P. F. P., Morigi, G. B., Pereira, S. T., Shahabuddin, M., and Sacks, D. L. (1997) Parasitology 115, 359–369
9. Reynolds, S. E., and Samuels R. I. (1996) Adv. Insect Physiol. 26, 157–232
10. Kimura, S. (1973) J. Insect Physiol. 19, 115–123
11. Koga, D., Fujimoto, H., Funakoshi, T., Minzaki, I., Ake, K., Kramer, K. J., Zen, K. C., Choi, H., and Muthukrishnan, S. (1992) Insect Biochem. Mol. Biol. 22, 305–311
12. Sakuda, S., Isoag, A., Matsumoto, A., Suzuki, A., and Koseki, K. (1986) Tetrahedron Lett. 27, 2475–2479
13. Spindler, K. D., and Spindler-Barth, M. (1999) in Chitin and Chitinases (Jolles, P., Muzzarelli R. A. A., ed.) pp. 201–209, Birkhaeuser Verlag, Basel, Switzerland
14. Balasahov, Y. S. (1972) Misc. Publ. Entomol. Soc. Am. 8, 161–176
15. Hoogstraal, H. (1995) Adv. Parasitol. 34, 135–238
16. Fujisaki, K. (1978) Natl. Inst. Anim. Health Quart. (Yokame) 18, 27–38
17. Fujisaki, K., Kawazu, S., and Kamio, T. (1994) Parasitol. Today 10, 31–33
18. Rand, K. N., Moore, T., Srikantha, A., Spring, K., Tellam, R., Willadsen, P., and Yatabe, Y. (1994) Parasitol. Today 10, 31–33
19. Fujisaki, K. (1978) Natl. Inst. Anim. Health Quart. (Yokame) 18, 27–38
20. Rand, K. N., Moore, T., Srikantha, A., Spring, K., Tellam, R., Willadsen, P., and Yatabe, Y. (1994) Parasitol. Today 10, 31–33
21. Fujisaki, K., Kawazu, S., and Kamio, T. (1994) Parasitol. Today 10, 31–33
22. Fujisaki, K. (1978) Natl. Inst. Anim. Health Quart. (Yokame) 18, 27–38
23. Fujisaki, K., Kawazu, S., and Kamio, T. (1994) Parasitol. Today 10, 31–33
24. Gopalakrishnan, B., Muthukrishnan, S., and Kramer, K. J. (1995) Insect Biochem. Mol. Biol. 25, 255–265
