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Molecular characterization of chitinase from polyphagous pest *Helicoverpa armigera*

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Abstract

Chitinase from a polyphagous pest, *Helicoverpa armigera*, has been cloned and expressed. The *Helicoverpa* chitinase cDNA is 2870 bp in length and contains an open reading frame of 1767 bp. The cDNA encodes a polypeptide of 588 residues with a predicted molecular weight of 66 kDa and a p*I* of 5.99. The polypeptide has distinct catalytic and substrate binding domains at the N- and the C termini, respectively. The two domains are held together by a proline, threonine rich linker region. The catalytic and the substrate binding domains shared a high level of homology with other lepidopteran chitinases, but the proline and threonine rich region is longer in *H. armigera* chitinase than in other lepidopteran chitinases. The transcription of chitinase at different developmental stages and in different tissues was analysed by RT-PCR. Chitinase transcript was found in the integument, gut, and fat bodies but was absent in the haemocytes. The levels of chitinase mRNA were abundant at the moulting stages and a basal level of transcript was maintained throughout the development of the insect. Interestingly, Western blot analysis of total proteins from the integument and the gut showed the presence of chitinase in the moulting stages but was absent in the intermoult periods, suggesting post-transcriptional control. The chitinase cDNA was expressed in bacteria and in insect cells. The insect cell expressed chitinase was glycosylated and catalytically active against the simple and complex substrates. The chitinase gene spans about 6.8 kb of genomic DNA and is organized into 10 exons and 9 introns. The 6.8 kb genomic clone of chitinase revealed a high degree of conservation in the position and size of the exons with other lepidopteran insects.

Keywords: Chitinase; Genomic organization; Developmental expression; *Helicoverpa armigera*

Chitin is a linear homopolymer of *N*-acetylglucosamine in a β-1,4 linkage. It is found in lower organisms as a structural polysaccharide where it imparts rigidity and mechanical strength to the structural elements such as cell walls and exoskeleton in fungi and arthropods, respectively [1]. Chitin is hydrolysed by chitinases (EC 3.2.1.14), which are found ubiquitously, even in organisms that do not contain chitin. Chitinases are produced in bacteria for nutrient assimilation and, in fungi and invertebrates chitinases are responsible for chitin turnover and metabolism [2]. In higher vertebrates like humans, chitinases are produced as an immune response towards fungal pathogens [3]. In plants, chitinases are a component of pathogenesis-related proteins [4].

In insects, the cuticle and the peritrophic matrix contain chitin that fulfils the role of providing tensile strength and impermeability to the exoskeleton, but unlike the endoskeleton lacks growth capability [1]. To grow, the insect has to undergo repeated cycles of moulting which are brought about by chitinases and *N*-acetylglucosaminidases. Chitinase expression therefore coincides with the moulting process and is developmentally regulated in insects. Chitinase expression is under hormonal control and is induced by ecdysone and repressed by the juvenile hormone, fenoxycarb [5]. Since chitin is a structural component in insects, chitin synthesis and hydrolysis are considered to be potential targets for pest management. A detailed understanding of chitin metabolism in target pests is necessary for the development of chitinase-based insecticides. Chitinases have been studied in lepidopteran insects *Manduca sexta* [6] and *Bombyx mori* [7] and the genes encoding

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chitinases have been cloned from *M. sexta* [8], *B. mori*, *Hyphantria cunea* [9], *Spodoptera litura* [10], and *Choristoneura fumiferana* [11]. The genomic structure of chitinase genes has been described in *M. sexta* [12] and *B. mori* [13]. Chitinase from *M. sexta* has been demonstrated to be toxic to the merchant grain beetle, *Oryzaephilis mercator* [14], and blocked the development of the tobacco hornworm, *Helicoverpa virescens* when introduced in transgenic plants [15]. *M. sexta* chitinase also enhanced the insecticidal activity of a recombinant AcMNPV against *Spodoptera frugiperda* larvae [16].

We report here the cloning, genomic organization, and expression of a chitinase gene from the cotton bollworm, *Helicoverpa armigera*, a polyphagous pest that attacks many agriculturally important crops and inflicts heavy damage.

Materials and methods

Insect rearing and tissue collection. The culture of *H. armigera* was maintained in the laboratory under controlled conditions of temperature 25 °C, 70% relative humidity, and a photoperiod of 12 h light:12 h dark. The larvae were reared on a semi-synthetic diet. The larvae were dissected under insect physiological saline and integuments, midguts, and fat bodies were collected. Haemocytes were separated from the haemolymph by centrifuging at 7500*g*. Moulting fluid was extracted from the prepharate pupae as described in [6]. All tissues and fluids were snap-frozen in liquid nitrogen and stored at −80 °C until further use.

Isolation of the H. armigera chitinase cDNA. Total RNA was isolated from the pupal integuments with the Trizol reagent (Life Technologies). Ten micrograms of total RNA was used to synthesize first strand cDNA with Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Degenerate forward (5′KCGGACAGCARAGCGCGCAT3′) and reverse primers (5′CARTTCATCTTGATYTCCAC) were designed from the conserved regions of lepidopteran chitinases and were used to amplify a specific product. The amplified product was cloned into pGEMT-easy (Promega) and sequenced completely and gene specific forward primers (5′CGCAGTGTGGAAAATAAGATGAACTGGATC3′) and reverse primers (5′CATTCTTATCTACGTCCAACTCAGGA TC3′) were synthesized based on the acquired sequence. These primers were used for 5′ and 3′ RACE, respectively. The first strand pupal cDNA was subjected to 5′ and 3′ RACE to obtain the flanking regions of the cDNA using the 5′ and 3′ kits (Life Technologies) and performed according to the manufacturer's instructions. The sequence analysis was carried out with Mac Vector 7.0 software. Sequence homology was identified using the BLAST application at the National Centre for Biotechnology Information (NCBI). The *H. armigera* chitinase cDNA sequence has been submitted in GenBank under Accession No. AY325496.

Isolation of H. armigera chitinase genomic fragments. High molecular weight genomic DNA from the gut of fifth instar larvae was prepared as described in [17]. The entire gene was amplified in four overlapping fragments with cDNA specific primers in the primer combinations 5′AAATGAGAGTGATACTAGCGACG3′ and 5′TTG GCAAAGATCAGGTACGTGGTA3′, 5′GGTTTGGACTTGGATT GGGAGTAC3′ and 5′CTTGTTGATGTAGGTACCAAGTCC3′, 5′TTCTACGGTCGCTCATTCACTCTG3′ and 5′CGGAGCTCAG TCTACAGCTCGCAGTC3′, and 5′GAAATAGATAATCACGAC GTTTGC3′ and 5′TTTAAGCGAGCGACCACCCGCGAC3′. The amplification reaction included 100 ng of genomic DNA as template, 150 ng of forward and reverse gene specific primers, 200 μM each dNTP, and 5 U *Taq* polymerase (Roche) in a total volume of 50 μl. The reaction conditions were initial denaturation at 94 °C for 2 min, 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 3 min for 32 cycles and a final extension at 72 °C for 10 min. The amplified products were cloned in pGEMT-easy and sequenced completely. The sequence of the *H. armigera* chitinase genomic clone has been submitted in GenBank under Accession No. AY326455.

Transcript analysis in different tissues and at different developmental stages. Integument and guts of insects were collected each day from the fourth instar to the pupal stages in *H. armigera*. Total RNA was extracted from the tissues using the Trizol reagent (Life Technologies) and 1 μg of total RNA was used as template in one step RT-PCR, performed according to the manufacturer's instructions (Qiagen). Forward (5′AGGAACTTCACAGCTCTTCG3′) and reverse (5′CTCATAAGCCCACTGACATG3′) gene specific primers were used to amplify a specific product. β-Actin was amplified in the same total RNA samples with the β-actin forward (5′CAGATCATG TTTGAGACCTTCAAC3′) and β-actin reverse (5′GSCACATGTTT GAGACCTTCAAC 3′) primers [18].

Chitinase levels in the integuments and the midguts were estimated by Western blotting the insect proteins at different stages of development and probing with anti-chitinase antibodies. The integuments and midguts of insect larvae were frozen and ground to a fine powder under liquid nitrogen. One millilitre of 50 mM phosphate buffer, pH 7.0, containing protease inhibitor minitablet (Roche) was added and the homogenate was centrifuged at 4 °C at 12,000*g* for 15 min. The supernatant containing proteins was collected and 15 μg of total proteins was loaded on a 7.5% SDS–PAGE for Western blotting. Estimation of total proteins was done by the Bradford reagent (Bio-Rad), using bovine serum albumin as the standard.

Heterologous expression of H. armigera chitinase. The cDNA fragment encoding the putative chitinase open reading frame was amplified by RT-PCR using pupal total RNA as template with forward (5′AAAATGAGAGCGATACTGGCG3′) and reverse (5′AGG CGCCTGTTCATGAGCCG3′) gene specific primers designed from the start site and the stop site of the chitinase cDNA. The fragment was cloned in pGEMT-easy and sequenced to confirm sequence congruence. It was subcloned in the *Eco*RI site of pET28b (Novagen) in-frame with a C-terminal 6× Histidine tag and transformed in the expression host BL21 (DE3). Induction was performed with IPTG at a final concentration of 1 mM and the culture was allowed to grow for 2 h at 37 °C with shaking. The cells were collected by centrifugation, resuspended, and sonicated to disrupt the cell membranes. The soluble and the membrane fractions were separated by centrifugation at 12,000*g* and the induced proteins were resolved on 7.5% SDS–PAGE. Affinity purification of 6× His recombinant chitinase from the soluble fraction was carried out with Ni–NTA agarose (Qiagen) under native conditions, according to the manufacturer's instructions. The purified protein was used for chitinase assays and for raising polyclonal antibodies against chitinase.

For expression in insect cells, the chitinase ORF amplified by RT-PCR, as described above, was cloned in the transfer vector, pBlueBac 4.5/V5 His TOPO (Invitrogen). The recombinant transfer vector was cotransfected with linear AcMNPV DNA, Bac-N-Blue DNA (Invitrogen) into Sf21 cells by using Bacfectin reagent (Gibco-BRL). After 4 days of incubation at 27 °C, the culture supernatant containing recombinant virus was harvested and subjected to plaque purification. The expression of recombinant chitinase in the plaques was confirmed by anti-chitinase serum. Positive plaques were selected and after three cycles of purification a recombinant virus was obtained. *Trichoplusia ni* cells were infected with the recombinant virus for expression studies.

Carbohydrate staining. The glycosylation status of the insect cell expressed chitinase was examined and compared to the *Escherichia coli* expressed chitinase and to the native chitinase from the moulting fluid. The purified insect cell chitinase, along with the moulting fluid and the induced *E. coli* lysate, was separated on a 7.5% SDS–PAGE and

electroblotted on the nitrocellulose membrane. After blocking with 3% BSA, the blot was overlaid with biotin labelled concanavalin A (Sigma), 5 μg/ml in 1× PBS + 1% BSA. The blot was washed with 1× PBS + 0.05% Tween, probed with streptavidin labelled alkaline phosphatase (Sigma) 1:5000 K, and developed with NBT-BCIP.

Chitinase assay. Fluorometric substrates 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotrioside and 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside were obtained from Sigma. The reaction mixture contained in a total volume of 100 μl, 50 μM of the substrate, 50 mM phosphate buffer, pH 7.0, and an appropriate amount of the enzyme. To determine the optimum pH of the reaction, the following ranges of buffers were used: 50 mM sodium acetate buffer, pH 4.0–5.5, 50 mM sodium phosphate buffer, pH 6.0–7.5, 50 mM Tris–HCl, pH 8.0–9.0, and sodium carbonate buffer, pH 10–11. The reaction mixture was incubated at 30 °C for 20 min and stopped by adding 2.4 ml of 150 mM glycine–NaOH buffer, pH 10.5. The release of free 4-methylumbelliferone was monitored by fluorescence spectroscopy with excitation at 360 nm and measuring emission at 450 nm. The fluorimetric intensity was converted to nmol of 4-methylumbelliferone released by preparing a standard curve of 4-methylumbelliferone. The activity has been expressed as nmol of 4-methylumbelliferone released min^{-1} mg^{-1} protein.

Results

Cloning and sequence analysis of chitinase cDNA

A 1 kb fragment was amplified when degenerate primers, synthesized from the conserved regions ADSKRI and VEIKMNW of lepidopteran chitinases, were used with pupal cDNA as template. The fragment was cloned into the PCR cloning vector and the sequence was subjected to the BLAST application at the NCBI server. It showed extensive homology with *S. litura* chitinase and other lepidopteran chitinases. To amplify the flanking 5′ and 3′ regions of the chitinase cDNA, 3′ and 5′ RACE was conducted and a 1.6 and a 0.3 kb specific product was obtained which shared the expected homology with the partial gene fragment.

The *Helicoverpa* chitinase cDNA is 2870 bp in length and contains an open reading frame of 1767 bp. The cDNA has a GC rich 5′ untranslated region of 47 bp and a 3′ untranslated region of 1057 bp. The sequence of translation initiation codon CAAAATGA agrees with the *Drosophila* start site sequence, AAACATG [19], more closely than the Kozak sequence, GCCATGG [20]. A putative polyadenylation signal sequence is present at the position 2832 within the 3′ UTR. The *Helicoverpa* ORF encodes a polypeptide of 588 residues with a predicted molecular weight of 65.72 kDa and a p*I* of 5.99. The SIGNAL P program predicted a hydrophobic signal peptide of 20 residues, with the cleavage between A20 and D21. The domain search program at the NCBI server identified two domains, the glycosyl hydrolase_18 conserved domain, FDGLDLDWE, and the conserved peritrophin chitin binding region at the C-terminus which contained six conserved cysteines. Three putative N-glycosylation sites were identified by Swiss Prot at the sites 86–88, 304–306, and 408–410. A region rich in proline and threonine was present at sites 393–516 which contains 26 prolines and 28 threonines and is 124 residues in length (Fig. 1). The sequence of *H. armigera* chitinase is well conserved at the catalytic and the chitin binding domains but the proline, threonine region is variable and longer in *Helicoverpa* chitinase than other lepidopteran chitinases.

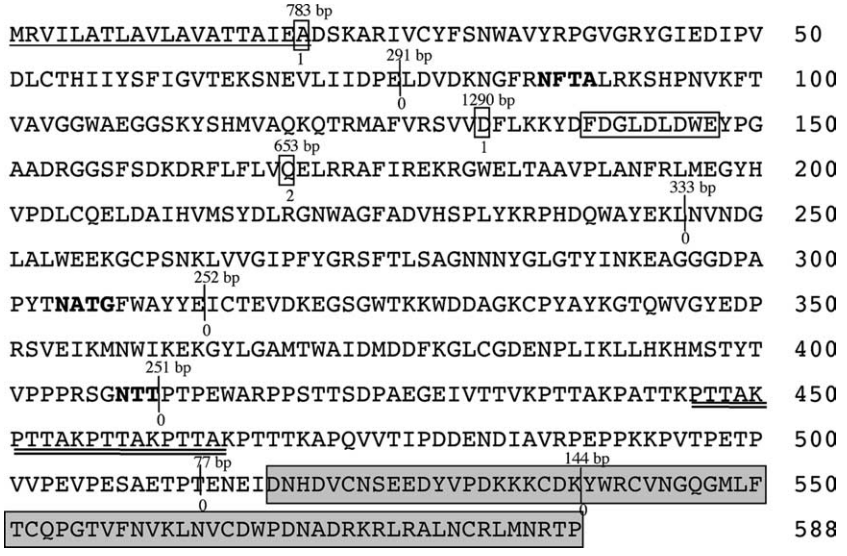

Fig. 1. The deduced amino acid sequence of *H. armigera* chitinase. The secretory signal peptide is underlined and the conserved family 18 chitinase signature sequence is boxed. The putative N-glycosylation sites are shown in bold lettering. The repeat sequences within the linker region are shown with double underline. The chitin binding region is shown in a grey box. The positions of the introns are shown within the sequence, the introns disrupting the amino acids are boxed, and the introns which lie between two residues are shown with a vertical line. Numbers above and below denote the size and the phase of the intron, respectively.

Gene organization of H. armigera chitinase

The genomic organization of *H. armigera* chitinase was studied by amplifying the genomic DNA with cDNA specific primers. The entire genomic clone was obtained in four overlapping fragments that were cloned and sequenced completely. The genomic sequence was compared with its corresponding cDNA sequence and intervening sequences, that disrupted the coding regions and were bordered by the splice signals, were identified as the introns. The chitinase gene in *H. armigera* spans about 6.8 kb of genomic DNA and is organized into 10 exons and 9 introns. The *M. sexta* [12] and the *B. mori* [13] chitinase genes also have a similar gene organization. The introns are distributed throughout the coding sequence and follow the GT–AG dinucleotide consensus splice sites for eukaryotes. The phase of the intron, which defines the nucleotide of a codon at which the intron is inserted [21], as well as the position of intron insertion, is conserved in *H. armigera*, *M. sexta*, and *B. mori* except for the position of intron 6 in *M. sexta* that is inserted between the residues YY instead of EI. The sizes of the exons are also conserved in the three species except for the exon 8, which is longest in *H. armigera* and reflects the extended linker region. However, the size of the intron varies considerably among the three species. The total length of introns is 4075 bp in *H. armigera*, 8402 bp in *M. sexta*, and 12,213 bp in *B. mori*.

Developmental and tissue specific expression of chitinase

The tissue specific expression of chitinase transcript was studied in the integument, gut, haemocytes, and the fat bodies by RT-PCR in a nonmoulting fifth instar larvae. The gene specific primers chosen for RT-PCR flanked introns 3 and 4 in the chitinase gene to distinguish any amplification arising from genomic DNA. The relative abundance of mRNA was normalized by using β-actin, which expresses constitutively, as an internal control. An amplification of 500 bp demonstrated the presence of the chitinase in the integument, gut, and fat bodies whereas it was absent in the haemocytes (Fig. 2A).

The developmental expression of chitinase was studied in the integument and the midgut by RT-PCR. The integuments and midguts of larvae were collected for seven days that included moultings from fourth to fifth instar and from fifth to the pupal stage. A specific product was obtained at all stages under study but the relative intensity of the band varied at each stage, indicating varying mRNA levels. A high level of transcript was present at the stages where the larvae were moulting into the next instar or a pupa. However, a detectable level of chitinase transcript was also present at the stages when the larvae were not moulting, at the second, third, and fourth days of the fifth instar showing that the chitinase transcript was present during the intermoulting stages as well (Fig. 2B). Identical expression patterns were obtained in the integument and the midgut (Fig. 2C).

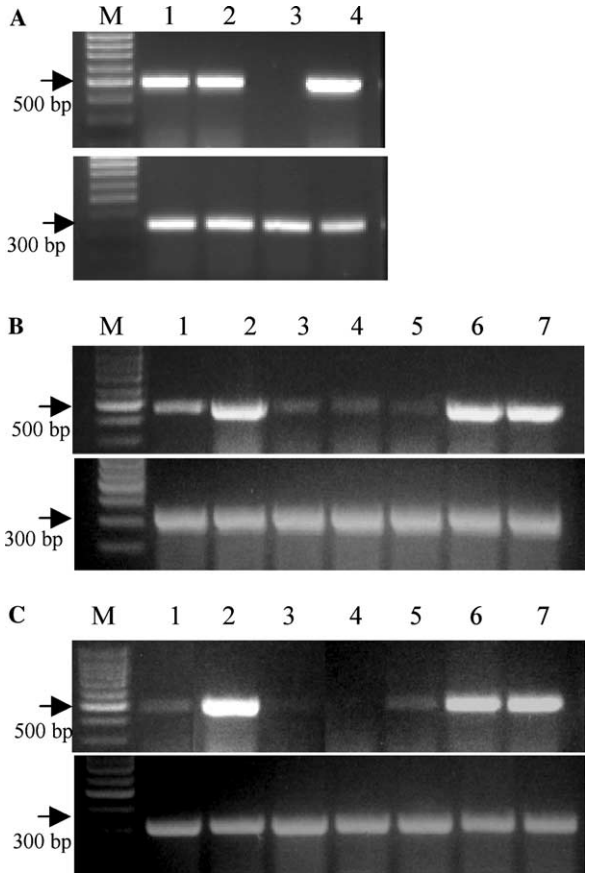

Fig. 2. Transcript analysis of *H. armigera* chitinase. One microgram of total RNA was used as a template for amplification with the chitinase specific or β-actin specific primers. (A) RT-PCR in different tissues of *H. armigera*. Lane 1, midgut; lane 2, fat bodies; lane 3, haemocytes; and lane 4, integument. (B) RT-PCR during different developmental stages in the integument. Upper panel: RT-PCR with chitinase specific primers. Lane 1, marker; lane 2, fourth instar larva, second day; lane 3, fifth instar larva, first day; lane 4, fifth instar, second day; lane 5, fifth instar, third day; lane 6, fifth instar, fourth day; lane 7, fifth instar, fifth day; and lane 8, prepupa. Lower panel: corresponding β-actin controls. (C) RT-PCR during different developmental stages in the midgut. Upper panel: RT-PCR with chitinase specific primers. Lane 1, marker; lane 2, fourth instar larva, second day; lane 3, fifth instar larva, first day; lane 4, fifth instar, second day; lane 5, fifth instar, third day; lane 6, fifth instar, fourth day; lane 7, fifth instar, fifth day; and lane 8, prepupa. Lower panel: corresponding β-actin controls.

To examine the level of chitinase in the total protein extract at different developmental stages, Western blot analysis of the total proteins was done with anti-chitinase antibodies. Western blot analysis showed the presence of a reactive band only at the stages where the insect was moulting from the fourth instar to the fifth instar and from the fifth instar to the pupal stage. Unlike the chitinase mRNA, the protein was absent at the stages at the intermoulting stages of the insect.

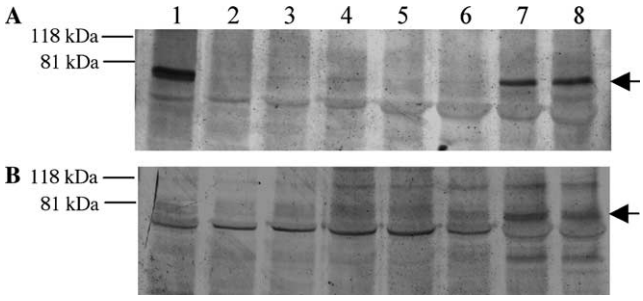

Fig. 3. Western blot analysis of total proteins at different developmental stages in *H. armigera*. Fifteen micrograms of total protein extracts was loaded on a 7.5% polyacrylamide gel, electroblotted to nitrocellulose membrane, and probed with anti-chitinase antibodies. (A) Developmental pattern of chitinase in the integument. Lane 1, fourth instar larva, second day; lane 2, fifth instar larva, first day; lane 3, fifth instar, second day; lane 4, fifth instar, third day; lane 5, fifth instar, fourth day; lane 6, fifth instar, fifth day; lane 7, prepupa; and lane 8, pupa. (B) Developmental pattern of chitinase in the midgut. Lane 1, fourth instar larva, second day; lane 2, fifth instar larva, first day; lane 3, fifth instar, second day; lane 4, fifth instar, third day; lane 5, fifth instar, fourth day; lane 6, fifth instar, fifth day; lane 7, prepupa; and lane 8, pupa.

The presence of chitinase protein also followed a similar pattern in the integument and the midgut (Fig. 3).

Heterologous expression of chitinase

The PCR amplified coding region of the *H. armigera* chitinase cDNA was cloned in the expression vector, pET28b, under the control of the T7 promoter. An induced band at ~70 kDa was observed in the induced cultures and it migrated higher than its predicted molecular size on a 7.5% SDS–PAGE. The recombinant chitinase expressed in *E. coli* was purified under non-denaturing conditions from the soluble fraction by affinity Ni chelating chromatography. The *E. coli* expressed chitinase was catalytically active and hydrolysed the fluorometric oligosaccharide substrates but the specific activity of the *E. coli* expressed chitinase was very low as compared to those of the insect cell expressed and the native moulting fluid chitinase.

To express *H. armigera* chitinase in insect cells, the chitinase cDNA was cloned in the transfer vector, pBlueBac 4.5/V5 His TOPO. The recombinant virus thus generated was used to infect *T. ni* cells and the production of recombinant protein was analysed by Western blotting the culture medium and probing with anti-chitinase antibodies at 24, 48, 72, and 96 h post-infection. The reactive band obtained in the medium infected with the recombinant virus was absent in the culture medium of cells infected with the wild type Ac-MNPV and in the uninfected cells. The recombinant chitinase appeared 48 h post-infection and increased until 96 h post-infection (Fig. 4).

The glycosylation status of chitinase produced in insect cells was compared with those of the native chitinase from the moulting fluid and the *E. coli* expressed chitinase using biotin labelled concanavalin A and streptavidin conjugated alkaline phosphatase. The insect cells expressed chitinase as well and the moulting fluid chitinase showed a band at the expected size while the *E. coli* expressed chitinase did not react with concanavalin A. A parallel blot was allowed to react with the anti-chitinase antibodies and it showed the presence of chitinase at identical positions as the blot developed with biotinylated concanavalin A. This indicated that the chitinase expressed in insect cells was glycosylated and the *E. coli* expressed chitinase lacked any post-translational modifications (Fig. 5).

The recombinant chitinase was purified from the culture medium of *T. ni* cells by ion exchange chromatography and it displayed high activity against the polymeric substrate on a glycol chitin activity gel and the fluorometric oligosaccharide substrates. The pH and the temperature optimum were investigated using the substrate 4-MU $(GlcNAc)_3$. The recombinant insect cell expressed chitinase showed bimodal pH of 6.0 and 7.5 and was active over a wide range of pH (Fig. 6A). Maximum activity of recombinant chitinase was obtained at 45 °C and the enzyme was not active at high temperatures (Fig. 6B). The enzyme preferred the trisaccharide substrate as compared to the disaccharide

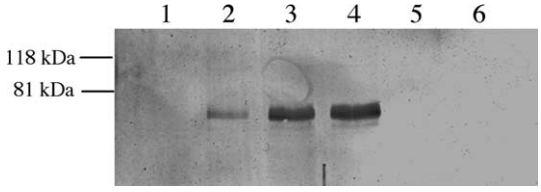

Fig. 4. Time course of chitinase expression in *T. ni* cells. The culture medium was harvested at different time periods and resolved on 7.5% polyacrylamide gel, electroblotted on nitrocellulose, and probed with anti-chitinase antibodies. Lane 1, 24 h; lane 2, 48 h; lane 3, 72 h; lane 4, 96 h; lane 5, culture medium from cells infected with the wild type virus after 96 h; and lane 6, culture medium from uninfected cells.

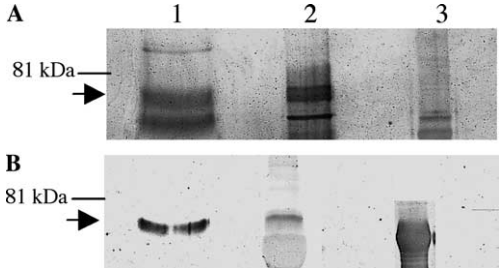

Fig. 5. Glycosylation status of *H. armigera* chitinase from different sources. (A) The proteins were electroblotted on nitrocellulose and were allowed to react with biotinylated concanavalin A. The complex was developed with streptavidin conjugated alkaline phosphatase. Lane 1, culture medium from *T. ni* cells infected with the recombinant virus; lane 2, moulting fluid from *H. armigera*; and lane 3, lysate from induced *E. coli* cells expressing chitinase. (B) A parallel blot developed with anti-chitinase antibodies.

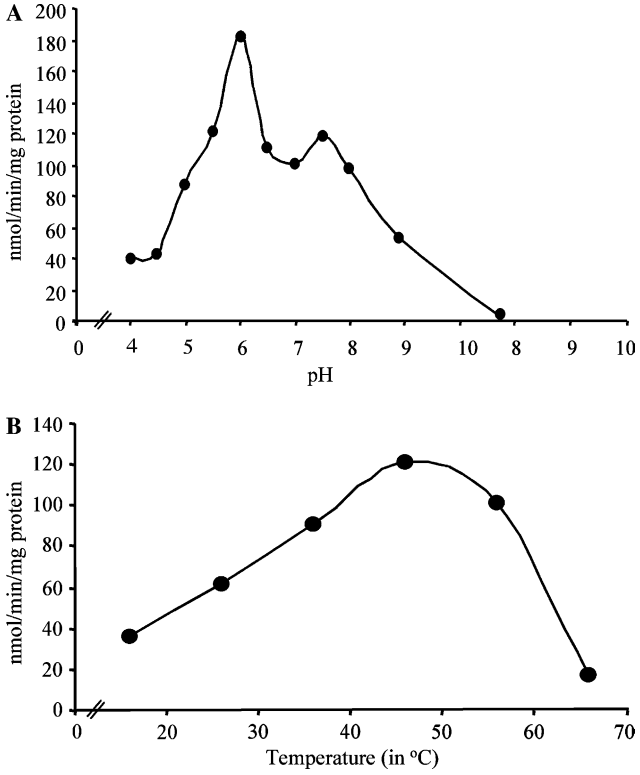

Fig. 6. (A) pH optimum of *H. armigera* chitinase. Activity of chitinase was measured with 4-MU $(GlcNAc)_3$ final concentration 50 μM, in sodium acetate buffer, pH 4.0–5.0, sodium phosphate buffer, pH 6.0–7.5, Tris–HCl, pH 8.0–9.0, and carbonate buffer, pH 10–11, at 30 °C for 30 min. (B) Temperature optimum of *H. armigera* chitinase. Activity of chitinase was measured with 4-MU $(GlcNAc)_3$ or 4-MU$(GlcNAc)_2$, final concentration 50 μM, in 50 mM sodium phosphate buffer, pH 6.5, at 5–65 °C for 30 min.

substrate, it can therefore be classified as an endochitinase. The K_m and the V_{max} for the fluorometric trisaccharide substrate were calculated to be 10 μM and 215 nmol/min/mg protein.

Discussion

Chitinase cDNA and its genomic organization

The cloned chitinase cDNA showed a high level of homology with other lepidopteran chitinases from *M. sexta* [8], *B. mori*, *Hy. cunea* [9], *S. litura* [10], and *C. fumiferana* [11]. As in other species, the *H. armigera* chitinase cDNA showed a modular structure consisting of a catalytic domain, a chitin-binding region, and a connecting linker. The presence of conserved motif in the catalytic domain that has the consensus, [LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-x-E motif, where E is the catalytic residue, suggested that it belonged to the family of 18 glycosyl hydrolases [22]. The C terminus of the protein contains a six cysteine domain, which conforms to the consensus C-(X11)–C-(X5)–C-(X9)–C-(X12)–C-(X7)–C [23] except that there are 12 residues between the first and the second cysteine instead of 11. The proposed function of the chitin binding domain is to anchor chitin but deletion experiments with *M. sexta* chitinase showed that the chitinase was active on polymeric substrates like glycol chitin, even in the absence of the chitin-binding domain although lesser activity in chitin binding domain deleted chitinase was observed when colloidal chitin was used [24].

The linker region connects the catalytic domain to the chitin-binding domain. Among the lepidopteran chitinases reported until now, the *H. armigera* chitinase contains the longest linker. Careful analysis of the *H. armigera* chitinase protein sequence reveals that a stretch of five residues PTTAK within the linker region is repeated in tandem four times and this stretch is absent in other lepidopteran chitinases. It is possible that a segment of DNA duplicated within the chitinase gene in *Helicoverpa* increasing the length of the linker. The linker region is rich in proline, threonine, and serine residues and is commonly found in chitinases. Abdel-Banat and Koga et al. [13] have suggested that this region has similarities to the PEST domain, being rich in these residues. It is now known that the linker region is responsible for the stability of the protein in the presence of gut proteases and is also extensively O-glycosylated [25]. It is also possible that the PEST sequences are involved in the proteolytic degradation of the protein and C-terminal truncated products are known to occur in *B. mori* and *M. sexta.*

The genomic organization of chitinase is highly conserved in lepidopteran insects *H. armigera*, *M. sexta*, and *B. mori*. The three genes have an identical exon-intron structure, the only difference being in the total length of the introns. *H. armigera* chitinase gene spans about 6.8 kb of genomic DNA while the *M. sexta* and *B. mori* genes span about 11 and 15 kb of genomic DNA, respectively [13,14]. The phase of the intron, which defines the base of a codon at which the intron is inserted, is also conserved. It is interesting to note that the introns at the 3′ end of the gene are phase 0 type, which means that the intron is inserted between two codons. The phase 0 introns are amenable to alternative splicing and may give rise to various mRNA products of different lengths [26]. Also, C-terminal truncated products of chitinases have been reported in *M. sexta* and *B. mori* [6,7].

Developmental expression of chitinase

The presence of the chitinase transcript was studied in the integument, midgut, haemocytes, and the fat bodies by one-step RT-PCR. Earlier reports have demonstrated the presence of the chitinase transcript in integument and midgut [8,9]. These experiments were done by

Northern blot hybridizations. We employed a more sensitive technique of analysis by one step RT-PCR using primers internal to the cDNA that encompassed introns to differentiate any contaminating genomic DNA. Since RT-PCR is more sensitive than Northern blot hybridizations and requires very little amount of RNA, it was possible to detect a low level of transcript. The tissue specific expression of chitinase showed that it was expressed in the integument, gut, and the fat bodies in *H. armigera.* Chitinase transcript was not found in the haemocytes. Expression of chitinase in the integument and gut has been shown in many lepidopteran insects, like *M. sexta*, *B. mori*, *Hy. cunea*, and *C. fumiferana* [8,9,11]. Chitinase expression in fat bodies was described in *C. fumiferana* [11], although it was absent in *M. sexta* [8]. The role of chitinase in the integument is to degrade cuticular chitin as suggested by its expression at the moulting stages. Chitinase expression also follows a similar pattern in the gut. The peritrophic membrane surrounding the gut also contains chitin, so the function of gut chitinase could be to degrade the peritrophic membrane chitin. Chitinases in the midgut could also have a protective function and could be related to the immune response against fungal pathogens. Chitinase expression in fat bodies was described in *C. fumiferana*, whereas it was absent in the fat bodies in *M. sexta.* The role of chitinase transcript in fat bodies is not clear, although Zheng et al. [11] have suggested that protein synthesized in the fat bodies is transported to the epidermis or gut where it plays a role in degradation. Chitinase transcript was not found in the haemocytes in this study. It is possible that chitinases produced elsewhere are secreted into the haemolymph, since the protein has been found to be present in the larval and pupal haemolymphs [6].

To study the developmental expression of chitinase in the integument and the gut, RT-PCR was performed for seven consecutive days during which the insect moulted twice. We found in this study that a basal level of transcript was maintained in the insect throughout the growth period examined and was upregulated during moulting. This differs from the pattern of chitinase expression described in *M. sexta*, *B. mori*, *Hy. cunea*, and *C. fumiferana* where chitinase expression was found only during larval–larval and larval-pupal transformations [8,9,11]. The low transcript level during the intermoulting period was not detected probably because Northern blot hybridizations were used which are less sensitive than RT-PCR. The Western blot analysis of integument and midgut showed that the chitinase enzyme was present only at the moulting stages and was absent in the intermoult periods. It is possible that the chitinase expression is regulated post-transcriptionally and the mRNA is translated only at the moulting stages when the enzyme activity is required. In *Choristoneura fumiferana*, the chitinase enzyme appeared 12 h before degradation of the old cuticle and disappeared rapidly after the moult [11].

Heterologous expression of chitinase

Chitinase was expressed in *E. coli* in a catalytically active form but migrated higher than its predicted molecular weight. The observed shift has been attributed to the presence of a high degree of secondary structure such as α-helix and β-sheets [11] since post-translational modifications are absent in *E. coli*. The specific activity of the *E. coli* expressed chitinase was very low as compared to those of the insect cell expressed and the native moulting fluid chitinase. Low activity of chitinase expressed in bacterial systems has been observed in *S. litura* [10] and *C. fumiferana* [11] as well.

The *H. armigera* chitinase cDNA was also expressed in insect cells. The recombinant chitinase was secreted into the culture medium due to the secretory signal peptide in the protein. Chitinases from *M. sexta* [16], *C. fumiferana* [11], *Haemaphysalis longicornis* [27], and *G. morsitans morsitans* [28] have also been expressed in insect cells and were secreted into the culture medium. The recombinant chitinase produced by insect cells had a high specific activity against the fluorogenic substrates that was nearly 800 times higher than that obtained in *E. coli*, showing that recombinant chitinase in insect cells was produced in a catalytically active and properly folded form (data not shown). The catalytic activity of insect cell expressed chitinase was compared with that expressed in *E. coli* in *S. litura* chitinase and the activity of the insect cell expressed protein was reported to be a thousandfold more active [10].

One of the reasons for the poor catalytic activity of *E. coli* expressed chitinase could be that the protein lacks post-translational modifications. The carbohydrate staining of the recombinant chitinase, moulting fluid chitinase, and the chitinase expressed in *E. coli* demonstrated that the chitinase produced in insect cells was glycosylated while that produced in *E. coli* was not post-translationally modified. Arakane et al. [25] have recently demonstrated that in the *M. sexta* chitinase expressed in insect cells, the linker region is extensively O-glycosylated while the catalytic domain is moderately N-glycosylated. In *Ha. longicornis* chitinase, deglycosylation with PNGase F resulted in a protein with a predicted molecular weight [27], showing that the insect cell produced chitinase is glycosylated. Similar results have also been obtained when a N-glycosylation inhibitor, tunicamycin, was added to the culture medium [16,11].

It is interesting that the recombinant chitinase expressed in insect cells has bimodal pH optima. The *C. fumiferana* chitinase also showed two peaks of activity, at acidic and at alkaline pH [11]. The *S. litura* chitinase is active at pH 5–8 [10]. It is therefore suggested that chitinase has a broad pH range so that it can function in

the slightly acidic environment of the haemolymph and the alkaline environment of the gut [11]. Availability of recombinantly expressed chitinase will mow permit a detailed investigation of putative protective role believed to be played by this enzyme in the development of insects.

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References

[1] K.J. Kramer, D. Koga, Insect chitin: physical state, synthesis, degradation and metabolic regulation, Insect Biochem. 16 (1986) 851–877.
[2] R. Cohen-Kupiec, I. Chet, The molecular biology of chitin digestion, Curr. Opin. Biotechnol. 9 (1998) 270–277.
[3] R.G. Boot, G.H. Renkema, A. Strijland, A.J. van Zonneveld, J.M. Aerts, Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages, J. Biol. Chem. 270 (1995) 26252–26256.
[4] D.B. Collinge, K.M. Kragh, J.D. Mikkelsen, K.K. Nielsen, U. Rasmussen, K. Vad, Plant chitinases, Plant J. 3 (1993) 31–40.
[5] K.J. Kramer, S. Muthukrishnan, Insect chitinases: molecular biology and potential use as biopesticides, Insect Biochem. Mol. Biol. 27 (1997) 887–900.
[6] D. Koga, D. Joseph, K.J. Kramer, Insect endochitinases: glycoproteins from the moulting fluid, integument and pupal haemolymph of *Manduca sexta*, Insect Biochem. 13 (1983) 295–305.
[7] D. Koga, Y. Sasaki, Y. Uchiumi, N. Hirai, Y. Arakane, Y. Nagamatsu, Purification and characterization of *Bombyx mori* chitinases, Insect Biochem. Mol. Biol. 27 (1997) 757–767.
[8] K.J. Kramer, L. Corpuz, H. Choi, S. Muthukrishnan, Sequence of cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*, Insect Biochem. Mol. Biol. 23 (1993) 691–701.
[9] M.G. Kim, S.W. Shin, K.S. Bae, S.C. Kim, H.Y. Park, Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori* and the fall webworm, *Hyphantria cunea*, Insect Biochem. Mol. Biol. 28 (1998) 163–171.
[10] T. Shinoda, J. Kobayashi, M. Matsui, Y. Chinzei, Cloning and functional analysis of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a recombinant baculovirus lacking the virus encoded chitinase gene, Insect Biochem. Mol. Biol. 31 (2001) 521–532.
[11] Y. Zheng, S. Zheng, X. Cheng, T. Ladd, E.J. Lingohr, P.J. Krell, B.M. Arif, A. Retnakaran, Q. Feng, A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*, Insect Biochem. Mol. Biol. 32 (2002) 1813–1823.
[12] H.K. Choi, K.H. Choi, K.J. Kramer, S. Muthukrishnan, Isolation and characterization of a genomic clone for the gene of an insect molting enzyme, chitinase, Insect Biochem. Mol. Biol. 27 (1997) 37–47.
[13] B.M. Abdel-Banat, D. Koga, A genomic clone for a chitinase gene from the silkworm, *Bombyx mori*: structural organization identifies functional motifs, Insect Biochem. Mol. Biol. 31 (2001) 497–508.
[14] X. Wang, X. Ding, B. Gopalakrishnan, T.D. Morgan, L. Johnson, F. White, S. Muthukrishnan, K.J. Kramer, Characterization of a 46 kDa insect chitinase from transgenic tobacco, Insect Biochem. Mol. Biol. 26 (1996) 1055–1064.
[15] X. Ding, B. Gopalakrishnan, L.B. Johnson, F.F. White, X. Wang, T.D. Morgan, K.J. Kramer, S. Muthukrishnan, Insect resistance of transgenic tobacco expressing an insect chitinase gene, Transgenic Res. 7 (1998) 77–84.
[16] B. Gopalakrishnan, S. Muthukrishan, K.J. Kramer, Baculovirus mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein, Insect Biochem. Mol. Biol. 25 (1995) 255–265.
[17] J.M. Henry, A.K. Raina, R. Ridgway, Isolation of high molecular weight DNA from insects, Anal. Biochem. 185 (1990) 147–150.
[18] R.L. Tellam, T. Vuocolo, S.E. Johnson, J. Jarmey, R.D. Pearson, Insect chitin synthase cDNA sequence, gene organisation and expression, Eur. J. Biochem. 267 (2000) 6025–6042.
[19] D.R Cavener, Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates, Nucleic Acids Res. 15 (1987) 1353–1361.
[20] M. Kozak, Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs, Nucleic Acids Res. 12 (1984) 857–872.
[21] T. Endo, A. Federov, S.J. de Souza, W. Gilbert, Do introns favour or avoid regions of amino acid conservation?, Mol. Biol. Evol. 19 (2002) 521–525.
[22] A.C. Terwisscha van Scheltinga, M. Hennig, B.W. Dijkstra, The 1.8 Å resolution structure of hevamine, a plant chitinase/lysozyme, and analysis of the conserved sequence and structure motifs of glycosyl hydrolase family 18, J. Mol. Biol. 262 (1996) 243–257.
[23] V. Royer, S. Fraichard, H. Bouhin, A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis, Biochem. J. 366 (2002) 921–928.
[24] X. Zhu, H. Zhang, T. Fukamizo, S. Muthukrishnan, K.J. Kramer, Properties of *Manduca sexta* chitinase and its C-terminal deletions, Insect Biochem. Mol. Biol. 31 (2001) 1221–1230.
[25] Y. Arakane, Q. Zhu, M. Matsumiya, S. Muthukrishnan, K.J. Kramer, Properties of catalytic, linker and chitin-binding domains on insect chitinase, Insect Mol. Biol. Biochem. 33 (2003) 31–648.
[26] C. Burge, S. Karlin, Prediction of complete gene structures in human genomic DNA, J. Mol. Biol. 268 (1997) 78–94.
[27] M. You, X. Xuan, N. Tsuji, T. Kamio, D. Taylor, N. Suzuki, K. Fujisaki, Identification and molecular characterization of a chitinase from the hard tick *Haemaphysalis longicornis*, J. Biol. Chem. 278 (2003) 8556–8563.
[28] J. Yan, Q. Cheng, S. Narashimhan, C.B. Li, S. Aksoy, Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans*, Insect Biochem. Mol. Biol. 32 (2002) 979–989.