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A novel chitin-binding protein identified from the peritrophic membrane of the cabbage looper, *Trichoplusia ni*

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Abstract

A novel midgut peritrophic membrane (PM) protein, TnPM-P42, was identified from the cabbage looper, *Trichoplusia ni*. TnPM-P42 was shown as a 42 kDa protein by SDS-PAGE analysis and appeared to be associated with the PM throughout its entire length. In *T. ni* larvae, the midgut is the only tissue where TnPM-P42 could be detected during the feeding period of the larvae. TnPM-P42 has chitin-binding activity and is strongly associated with the PM, which is similar to the currently known peritrophin type PM proteins. However, TnPM-P42 represents a unique family of proteins distinctly different from the peritrophin type PM proteins in its sequence characteristics. TnPM-P42 does not contain the peritrophin domain which is present in all the currently known PM proteins, but instead has a chitin deacetylase-like domain. Sequence similarity search of the GenBank database did not result in identification of any known proteins with a significant overall sequence similarity to the TnPM-P42. However, expressed sequence tags (ESTs) from various arthropods were identified to code for proteins with high sequence similarities to TnPM-P42, indicating the presence of TnPM-P42 homologs in other arthropods. Consistent with the identification of various ESTs from arthropods, Western blot analysis demonstrated the presence of a TnPM-P42-like protein in the PMs from *Heliothis virescens* and *Helicoverpa zea* larvae. The sequence characteristics of TnPM-P42 indicate that TnPM-P42 represents a novel family of insect proteins. However, its biochemical and physiological functions require further investigation.
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Keywords: Peritrophic membrane; Chitin-binding protein; Chitin deacetylase-like protein; Midgut; *Trichoplusia ni*; TnPM-P42

1. Introduction

Insect peritrophic membranes (PMs) are composed of chitin and proteins. They play multiple physiological functions and serve as the first line of defense in the midgut (Peters, 1992; Lehane, 1997; Terra, 2001). Current understanding of the PM biochemistry suggests that PM proteins are important determinants of the PM structure and that binding of PM proteins to chitin plays an important role for the PM formation (Tellam et al., 1999; Wang and Granados, 2001; Wang et al., 2004; Shi et al., 2004).

At present, four classes of PM proteins have been suggested based on the solubility of the proteins from the PM structure under different extraction conditions (Tellam et al., 1999). Class 1 PM proteins are those that can be removed by washing with physiological buffers, Class 2 represents the PM proteins extractable by mild detergents, Class 3 includes those only extractable by strong denaturants, and Class 4 PM proteins are not extractable by strong denaturants. Class 3 proteins are the most abundant proteins extractable from the PMs. To date, 18 PM proteins or putative PM proteins have been identified from several insect species, including

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Lucilia cuprina (Elvin et al., 1996; Schorderet, et al., 1998; Casu et al., 1997; Wijffels et al., 2001; Tellam et al., 2003), *Trichoplusia ni* (Wang and Granados, 1997b; Wang et al., 2004), *Anopheles gambiae* (Shen and Jacobs-Lorena, 1998; Devenport et al., 2005), *Chrysomya bezziana* (Vuocolo et al., 2001; Wijffels et al., 2001), *Glossina morsitans morsitans* (Hao and Aksoy, 2002), *Plutella xylostella* (Sarauer et al., 2003) and *Mamestra configurata* (Shi et al., 2004). These identified PM proteins all fall into the category of Class 3 PM proteins. These PM proteins have chitin-binding activities conferred by their characteristic chitin-binding domains, or the peritrophin domains (Tellam et al., 1999). The peritrophin domains contain 60–75 amino acid residues and are characterized by a conserved register of cysteine residues and a number of aromatic amino acid residues (Tellam et al., 1999). The conserved cysteine residues are suggested to form intradomain disulfide bonds that contribute to the protein stability in the protease-rich gut environment (Wang and Granados, 1997b, 2001; Shen and Jacobs-Lorena, 1999; Tellam et al., 1999). Recently, the chitin-binding activity of the peritrophin-type chitin-binding domain sequence from *T. ni* has been experimentally confirmed (Wang et al., 2004). With the exception of peritrophin-15 from *L. cuprina* and *C. bezziana* (Wijffels et al., 2001) and the recently identified Ag-Aper14 from *A. gambiae* (Devenport et al., 2005), the identified PM proteins contain multiple chitin-binding domains. With the known biochemical and molecular characteristics of the identified PM proteins, it has been proposed that the presence of multiple chitin-binding domains in PM proteins allows cross-linking of chitin fibrils by the proteins for PM formation (Wang and Granados, 1997b, 2001; Shen and Jacobs-Lorena, 1999; Tellam et al., 1999). The presence of a large number of chitin-binding domains in the PM proteins also allows the partially degraded protein fragments to retain multiple chitin-binding domains and, thus, their function to cross-link chitin fibrils in PM formation in the extremely proteinase-rich environment (Wang et al., 2004). The 15 kDa protein identified from the PMs of *L. cuprina* and *C. bezziana* contains only one chitin-binding domain and its function was proposed to cap the chitin molecules in the PM to protect chitin from degradation by exochitinase or to control the length of the chitin polymer (Wijffels et al., 2001). A similar PM protein with a single chitin-binding domain has also been identified from the mosquito *A. gambiae* (Devenport et al., 2005).

One unique type of PM proteins is the insect intestinal mucin (IIM). The IIMs contain both highly O-glycosylated mucin domains and multiple chitin-binding domains, suggesting that the PM is a mucus analogue reinforced by chitin (Wang and Granados, 1997a, b). IIMs have been identified from both types I and II PMs (Wang and Granados, 1997b; Tellam et al., 1999; Sarauer et al., 2003; Shi et al., 2004).

So far, all of the PM proteins that have been identified and characterized invariably show chitin-binding activities with the characteristic peritrophin domains. In this paper, we report the identification of a novel PM protein, TnPM-P42, from the cabbage looper, *T. ni*. TnPM-P42 exhibits a strong chitin-binding activity, so it belongs to the PM protein Class 3. However, this protein contains a chitin deacetylase-like domain, instead of a peritrophin domain.

2. Materials and methods

2.1. Insect larvae

Larvae of *T. ni*, *Heliothis virescens* and *Helicoverpa zea* were obtained from laboratory colonies maintained at the New York State Agricultural Experiment Station (Cornell University, Geneva, NY, USA) and reared on a high wheat germ artificial diet (Bell et al., 1981). Fifth instar larvae were used for dissection to isolate the PMs and various tissues for analyses.

2.2. Cloning and sequencing of a cDNA coding for the T. ni PM protein TnPM-P42

A *T. ni* midgut cDNA expression library (Wang and Granados, 1997b) was screened by subtractive immunoscreening with antibodies made against a collection of *T. ni* PM chitin-binding proteins (Wang and Granados, 2003) and antibodies reacting to the known *T. ni* PM proteins, IIM (Wang and Granados, 1997b), CBP1 and CBP2 (Wang et al., 2004). The library screening procedure was similar to that previously described by Wang et al. (2004). Library phage plates were blotted with two nitrocellulose filters and the filters were probed with antibodies against all PM chitin-binding proteins and antibodies reacting to the known *T. ni* PM proteins (IIM, CBP1 and CBP2), respectively. The cDNA clones positive to the antibodies to all PM chitin-binding proteins but negative to the antibodies to the known PM proteins were selected as candidate clones coding for unidentified PM proteins. These phage clones were subsequently purified by additional rounds of plating and screening. Finally, these cDNA clones were processed to rescue the pBluescript SK(−) phagemids by in vivo excision following the procedures provided by the manufacturer (Stratagene, La Jolla, CA, USA). Selected positive cDNA clones were subjected to sequencing after restriction enzyme digestion analysis to exclude identical clones. DNA sequencing was performed by primer walking of both strands.

2.3. Preparation of antibodies reacting to TnPM-p42

Antibodies reacting to the novel *T. ni* PM protein, TnPM-P42, were prepared from an antiserum made against the whole collection of *T. ni* PM chitin-binding proteins as described by Wang and Granados (2003). Briefly, *E. coli* strain XL1-Blue with the full-length cDNA for TnPM-P42 in pBluescript was cultured in 12 ml LB medium and the expression of TnPM-P42 was induced by addition of 100 µl of 0.5 M isopropyl thiogalactopyranoside (IPTG). The *E. coli* cells were harvested by centrifugation and lysed by boiling in 0.5 ml of lysis buffer (2% SDS, 5% β-mercaptoethanol and 50 mM Tris–HCl, pH 8.0) for 5 min. The cell lysate was clarified by centrifugation at 16,000*g* for 10 min, and the supernatant was collected and diluted with 10 ml of deionized water. The solubilized proteins were immobilized onto a piece of supported nitrocellulose membrane (Optitran BA-S85, Schleicher & Schuell, Keene, NH, USA) by incubation of the membrane in the lysate at room temperature for 1 h, followed by extensive washing with PBS 5 times and incubation in 3% bovine serum albumin (BSA) for 3 h to block non-specific binding sites. The nitrocellulose membrane was then incubated with a 100-fold dilution of the antiserum (Wang and Granados, 2003) in PBS with 3% BSA at room temperature for 3 h or at 4 °C overnight to allow the anti-TnPM-P42 antibodies in the antiserum to bind to the blotted membrane. The membrane was then thoroughly washed five times with PBS and finally the bound antibodies specific to the TnPM-P42 were eluted from the membrane by incubation in 5 ml of 0.1 M glycine buffer (pH 2.5) at room temperature for 10 min, followed by addition of 0.5 ml of 1 M Tris–HCl buffer (pH 8.0) to neutralize the pH of the antibody preparation.

2.4. Identification and localization of TnPM-P42 in T. ni larvae

To identify and localize the TnPM-P42 in *T.ni* larvae, mid-fifth instar larvae were dissected to isolate various tissues/structures, including hemolymph, tissue from the anterior part of the midgut (2 mm segment of the most anterior part), tissue from the entire midgut, Malpighian tubules, salivary glands, fat bodies, integument and the PM. Exuviae from the larvae molting from fourth instar to fifth instar and fecal pellets from fifth instar larvae were also collected. Midgut digestive fluid was collected from mid-fifth instar larvae by stimulating the larval mouth parts in a test tube to induce regurgitation of the midgut fluid. The samples were analyzed for the presence of TnPM-P42 by Western blot analysis using the antibodies reacting to TnPM-P42. Briefly, proteins from the samples were solubilized by boiling in SDS-PAGE sample buffer (Laemmli, 1970). Similar amounts of proteins from each tissue sample, which were estimated visually by SDS-PAGE analysis of the samples, and proteins from 1 PM, 1 µl midgut fluid and 1 exuvia were resolved by SDS-PAGE analysis, followed by transfer of the proteins onto Immobilon-P membrane (Millipore, Bedford, MA, USA). Then, the membrane was probed with antibodies reacting to TnPM-P42 after treatment of the membrane with 3% BSA in PBS to block non-specific bindings. The positive antibody reaction was detected with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase and visualized by the colorimetric reaction with bromo-chloroindolyl phosphate/nitro blue tetrazolium as the substrate.

2.5. Recombinant TnPM-P42 expression and chitin-binding analysis

To over-express TnPM-P42 in insect cells, a recombinant baculovirus was constructed using the Bac-to-Bac system from Invitrogen (Carlsbad, CA, USA). The cDNA for the TnPM-P42 was excised from the cDNA clone in pBluescript by digestion with XbaI and XhoI and cloned into the vector pFASTBac1 between the cloning sites Xba1 and XhoI. The recombinant baculovirus with the cDNA for TnPM-P42 was generated by following the protocols provided by the manufacturer. Recombinant TnPM-P42 was produced as a secreted protein by infecting BTI-Tn-5B1-4 (HighFive) cells (Granados et al., 1994) with the recombinant baculovirus and the infected culture was maintained in TNM-FH medium (Hink and Strauss, 1976) supplemented with 10% fetal bovine serum (FBS). The cell culture medium containing the secreted recombinant TnPM-P42 was collected at 72 h post-infection.

The chitin-binding activity of TnPM-P42 was analyzed using the chitin-binding assaying method described by Wang et al. (2004). Recombinant TnPM-P42 was isolated by incubation of 1 ml TnPM-P42-containing cell culture medium with 40 mg (wet weight) of regenerated chitin to allow the TnPM-P42 protein to bind to chitin at 4 °C in suspension for 1 h in the presence of a cocktail of protease inhibitors (0.5 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). The regenerated chitin bound with TnPM-P42 was washed thoroughly with PBS followed by centrifugations. Aliquots of the resulting chitin bound with TnPM-P42 were incubated with PBS, 2% SDS, 6 M Urea, 0.2% Calcofluor, 50 mM *N*-acetylglucosamine (GluNAc), 0.5 M NaCl, 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.5) or 20 mM acetic acid. After 15 min incubation at room temperature, the supernatants containing the TnPM-P42 protein released from the chitin were collected after centrifugation and analyzed by SDS-PAGE analysis.

2.6. Detection of TnPM-P42-like proteins in the PMs from H. virescens and H. zea larvae

Fifth instar *H. virescens* and *H. zea* larvae were used to dissect the PMs and collect midgut fluids as described above. The PMs and midgut fluids were analyzed by Western blot analysis using the antibodies reacting to TnPM-P42 as described above to detect the presence of TnPM-P42-like proteins from the PMs and midgut fluids in these two insect species.

2.7. Analysis of TnPM-P42 from the PM treated with a baculovirus enhancin

To examine the susceptibility of TnPM-P42 to degradation by the baculovirus enhancin in the PM, *T. ni* PMs were incubated in 40 µl of 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 10.5) with 50 µg/ml baculovirus enhancin from *T. ni* granulovirus (Wang et al., 1994) at room temperature for 45 min, followed by washing thoroughly with deionized water. The treated PMs were boiled in SDS-PAGE sample buffer for 5 min to solubilize the remaining PM proteins and the proteins were separated by SDS-PAGE gel. Western blot analysis was performed to examine the presence and integrity of TnPM-P42 in the enhancin-treated PMs.

3. Results

3.1. Identification of the cDNA coding for a novel T. ni PM protein, TnPM-P42

By subtractive screening of the midgut cDNA library, two cDNA clones were identified to contain partial cDNA sequences coding for a polysaccharide deacetylase-like protein. Using these cDNA sequences, a full-length cDNA clone was identified from the *T. ni* midgut expressed sequence tag (EST) library that we have generated. The cDNA is 1238 bp in length, containing an ORF of 1155 bp, followed by an AT-rich untranslated region and a putative polyadenylation signal (AATATA) located at 15 bp upstream of the polyA tail (Fig. 1). The protein encoded by this cDNA is named TnPM-P42. The deduced protein sequence showed that TnPM-P42 is synthesized as a preprotein of 384 amino acid residues with a 17-amino acid signal peptide predicted by the software SignalP (Bendtsen et al., 2004). After removal of the signal peptide, the secreted TnPM-P42 protein is predicted to have a molecular weight of 41.6 kDa. The secreted TnPM-P42 has a highly acidic N-terminus (Glu^{18}–Glu^{19}–Asp^{20}–Glu^{21}–Glu^{22}) and contains one putative *N*-glycosylation site at Asn^{358} based on the presence of the sequence pattern Asn–Xaa–Thr/Ser (Blom et al., 2004) at residue positions from 358 to 360. TnPM-P42 contains 14 cysteine

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  GATGAAGTTGCTAGGATTACTGGCGTTACTTCTTGTGGCATCAGTGTACGCTGATGATGAAGATGATGGTCTTGCAAAGGATTGTGACCCTGAAGTCTGTGTTCTACCCAACTGCAGA  0118
    M  K  L  L  G  L  L  A  L  L  L  V  A  S  V  Y  A  D  D  E  D  D  G  L  A  K  D  C  D  P  E  V  C  V  L  P  N  C  R

TGTTCGAGCACAAACATTCCCGGAGGATTAAGTCCAAGAGATACGCCTCAGTTCGTCTCCGTGACTTTCGATGACGCCGTCAACGTCGTTAACATCTTGGACTACCGTGAGCTTCTCTAC  0238
 C  S  S  T  N  I  P  G  G  L  S  P  R  D  T  P  Q  F  V  S  V  T  F  D  D  A  V  N  V  V  N  I  L  D  Y  R  E  L  L  Y

AACCGTAAGAACAAGAACGGCTGTCCCGCTGGAGCCACCTTCTTCGTCAGCCACGAGTACACCAACTACCAACACGTCAACGAGTTGTACAACAATGGATTTGAGATCGCTCTTCACTCA  0358
 N  R  K  N  K  N  G  C  P  A  G  A  T  F  F  V  S  H  E  Y  T  N  Y  Q  H  V  N  E  L  Y  N  N  G  F  E  I  A  L  H  S

ATCTCTCATCAAACACCTCCTGCTTACTGGGCGGAGGCCACCGAAGAAATCTTAGAAAAGGAAATTGGAGAGCAAAGAATTCTTATGTCTCATTTCGCGAATATTCCTTTTACTTCTATC  0478
 I  S  H  Q  T  P  P  A  Y  W  A  E  A  T  E  E  I  L  E  K  E  I  G  E  Q  R  I  L  M  S  H  F  A  N  I  P  F  T  S  I

AAAGGTGTCCGTATGCCTTTCCTTCAGCTGGCTGGCGACAATAGCTTCAAAGTAATGGCCAAAAACAACTTGCTGTATGACCTGTCTTGGCCCACTGTTGCCCACACCAACCCTGGTCTG  0598
 K  G  V  R  M  P  F  L  Q  L  A  G  D  N  S  F  K  V  M  A  K  N  N  L  L  Y  D  L  S  W  P  T  V  A  H  T  N  P  G  L

TGGCCTTACTCCCTGGATTATAAATCAACCCACGACTGCATCATTGGACCCTGTCCTACTGCCTCTATTCCTAACGTGTGGGTCTTCCCCATGGTGTCTTGGACTGACTTGGCTGGATTC  0718
 W  P  Y  S  L  D  Y  K  S  T  H  D  C  I  I  G  P  C  P  T  A  S  I  P  N  V  W  V  F  P  M  V  S  W  T  D  L  A  G  F

CCCTGTTCTATGGTTGACGCTTGCTTCCAACCTCCTGCTGATGATGATGAAGAAGGATGGTTGCAGTTCATCCTGACCAACTTTGAGAGGCATTACTTCGGTAACAGAGCTCCATTCGGT  0838
 P  C  S  M  V  D  A  C  F  Q  P  P  A  D  D  D  E  E  G  W  L  Q  F  I  L  T  N  F  E  R  H  Y  F  G  N  R  A  P  F  G

TTCTACGCTCACCAGCCCTTGATCAGCCAAGAAAAGCCGGCTATCCGCAGGGCCTTCAGTAGGTTCCTTGACATTATCAACAATTTGGATGATGTTTTCATGGTCAACGCTGAACAAGTC  0958
 F  Y  A  H  Q  P  L  I  S  Q  E  K  P  A  I  R  R  A  F  S  R  F  L  D  I  I  N  N  L  D  D  V  F  M  V  N  A  E  Q  V

ATCGACTGGGTAAAGAACCCAGTACCTGTCGATGAGTACAAGAAACAGAGCTGCTACCATTTCGTTCCAAGCCTTTGCCCTCAATTCAGCTGTGGGAACCTCCAGTCAAGTCATAATCCC  1078
 I  D  W  V  K  N  P  V  P  V  D  E  Y  K  K  Q  S  C  Y  H  F  V  P  S  L  C  P  Q  F  S  C  G  N  L  Q  S  S  H  N  P
                                                                                                                    ↑
ACTAACGCCTACTGGATGCAGACCTGCAATGTTTGCCCGGCCACGTACCCATGGGTCAACAACCCTCTTGGACAGTAATTAGGAACGAACTTGTTACGATTGTGTTAAAGATAATATATT  1198
 T  N  A  Y  W  M  Q  T  C  N  V  C  P  A  T  Y  P  W  V  N  N  P  L  G  Q

CTTATGTTTAATTAAAAAAAAAAAAAAAAAAAAAAAAAAA  1238
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Fig. 1. Nucleotide sequence of the cDNA for TnPM-P42 and its deduced amino acid sequence (GenBank™ accession number: AY966402). The translation initiation codon ATG and stop codon TAA are indicated in boxes and the predicted signal peptide sequence is underlined. The potential polyadenylation signal sequence is double underlined. A putative *N*-glycosylation site is indicated by an arrow.

residues localized in the N-terminal (5 cysteines between amino acid residue 28 and 87), middle (4 cysteine residues between residue 212 and 247) and C-terminal (5 cysteine residues between residue 337 and 371) regions of the protein. However, the distribution patterns of these cysteine residues in TnPM-42 do not resemble any of the peritrophin domains from both types I and II PMs (Tellam et al., 1999).

Search of the GenBank database using both blastp and tblastn programs (Altschul et al., 1990) for homologous sequences to the TnPM-P42 only resulted in identification of similar sequences for hypothetical proteins, but no sequences from known proteins, indicating that the TnPM-P42 is a novel protein. However, conserved domain database search using CD-Search (Marchler-Bauer and Bryant, 2004) identified a putative polysaccharide deacetylase-like domain in the TnPM-P42 sequence from amino acid residues 54–186. This polysaccharide deacetylase-like domain showed sequence similarities to the chitin deacetylase domain sequences from the nodulation protein B (NodB) from *Sinorhizobium meliloti* (Torok et al., 1984), a chitin deacetylase from *Amylomyces rouxii* (Kafetzopoulos et al., 1993) and the chitin deacetylase 1 and chitin deacetylase 2 from *Saccharomyces cerevisiae* (Mishra et al., 1997) (Fig. 2).

Although no known proteins with a significant overall sequence similarity to TnPM-P42 were identified from the GenBank, ESTs from various arthropods, including insect, acari and crustacean species, were identified from the GenBank EST database to code for proteins with high sequence similarities to the *T. ni* TnPM-P42. Deduced protein sequences of the ESTs from several selected insect and acari species are highly similar to the *T. ni* TnPM-P42 with amino acid residue identities ranging from 40% to 70% (Fig. 3), suggesting the expression of a *tnpm-p42* homolog in other arthropods.

3.2. Identification of TnPM-P42 from the T. ni PM

Antibodies reacting to TnPM-P42 recognized a single protein from the *T. ni* PM proteins by Western blot analysis (Fig. 4). This protein band on the Western blot correlated with a distinct PM protein band on the SDS-PAGE gel with an apparent molecular weight of 42 kDa, which is in agreement with the predicted molecular weight of 41.6 kDa for TnPM-P42 (Fig. 4). Furthermore, both SDS-PAGE and Western blot analyses of the PM proteins confirmed that the abundance of TnPM-P42 in the anterior, middle and posterior regions of the PM was similar (Fig. 4), indicating that TnPM-P42 is evenly distributed throughout the entire length of the PM.

3.3. Localization of TnPM-P42 in T. ni larvae

Western blot analysis of proteins from various tissues isolated from fifth instar *T. ni* larvae showed that in addition to the PM, TnPM-P42 was detected in midgut tissue but was not detectable from the larval hemolymph, Malpighian tubules, salivary glands, fat bodies and integument (Fig. 5). In addition, a positive 42 kDa protein was also detected in the midgut fluid, exuvia and fecal pellets (Fig. 6). In proteins from the midgut fluid, a major 42 kDa protein band and a number of minor bands with lower molecular weights were detected by Western blot analysis. The minor bands could be degradation products of TnPM-P42.

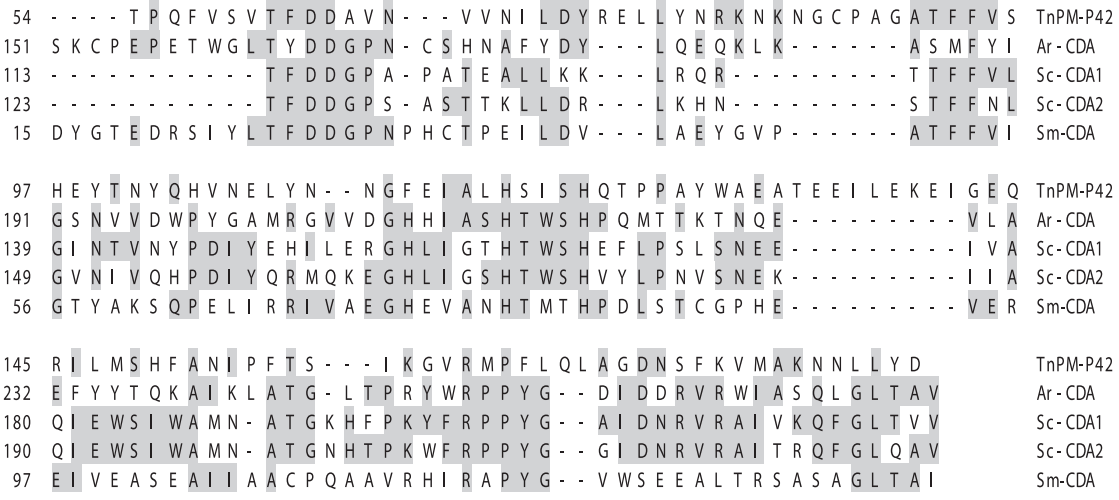

Fig. 2. Alignment of the predicted chitin deacetylase domain sequence from TnPM-P42 with chitin deacetylase domain sequences from *Amylomyces rouxii* chitin deacetylase (Ar-CDA) (GenBank accession number: P50325), *Saccharomyces cerevisiae* chitin deacetylase 1 (Sc-CDA1) (GenBank accession number: Q06702) and chitin deacetylase 2 (Sc-CDA2) (GenBank accession number: Q06703), and *Sinorhizobium meliloti* chitooligosaccharide deacetylase (Nodulation protein B) (Sm-CDA) (GenBank accession number: P02963). Residues identical to the consensus sequence are shaded.

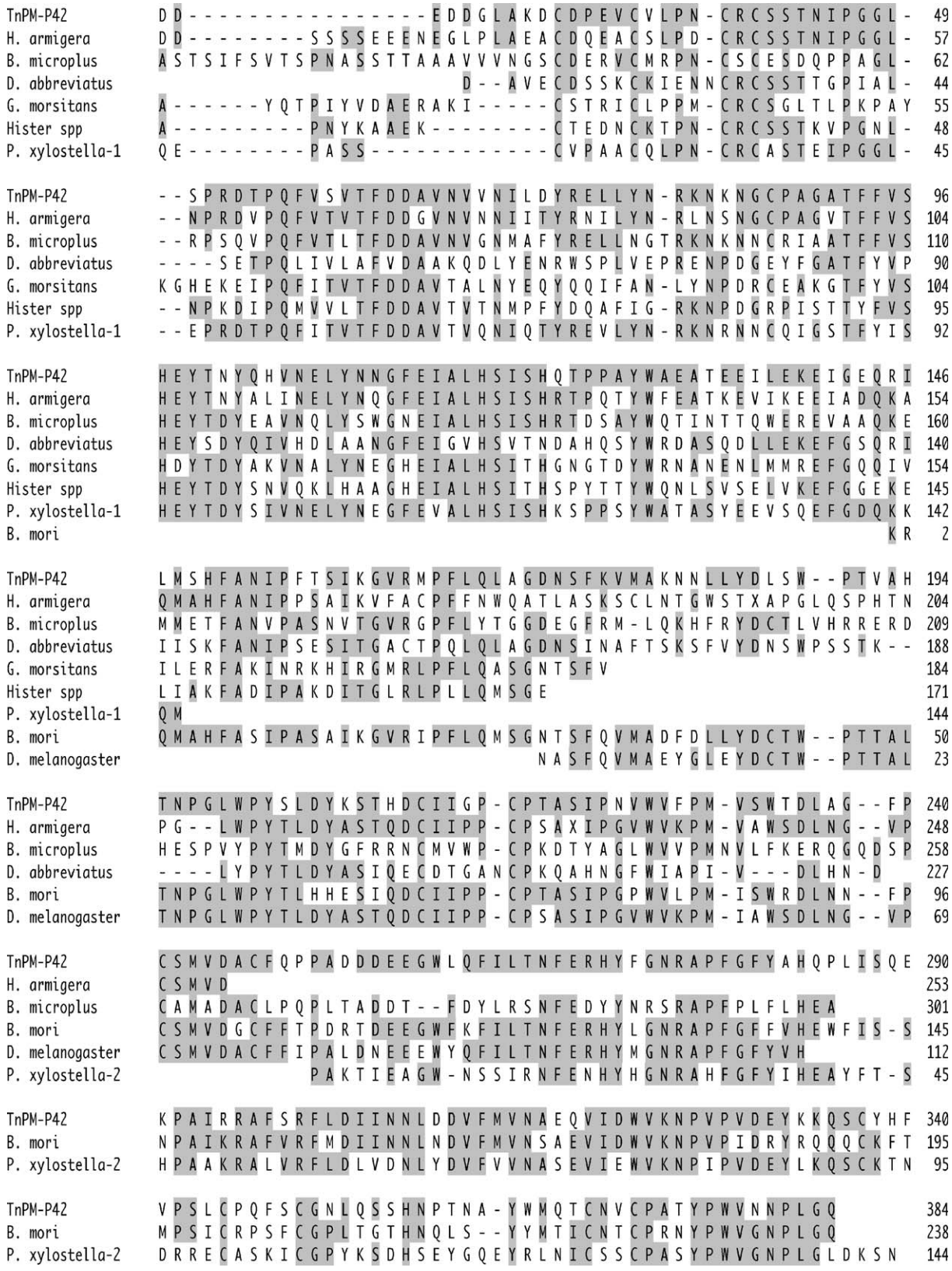

Fig. 3. Alignment of TnPM-P42 sequence with the deduced protein sequences from the ESTs from *Helicoverpa armigera* (GenBank accession numbers: BU038484, BU038562, BU038607, BU038695 and BU038804), *Bombyx mori* (GenBank accession numbers: AU002310, CK494771, CK496072, CK496401, CK496460, CK495488 and CK497999), *P. xylostella* (GenBank accession numbers: BP937146 and BP937383), a *Hister* species (GenBank accession number: CV158446), *Diaprepes abbreviatus* (GenBank accession number: DN200131), *Drosophila melanogaster* (GenBank accession number: CO326302), *G. morsitans morsitans* (GenBank accession numbers: BX560614, BX551086 and BX561657) and *Boophilus microplus* (GenBank accession number: CK182189). Predicted signal peptide sequences are not included for the sequence alignment. Residues identical to the consensus sequence are shaded.

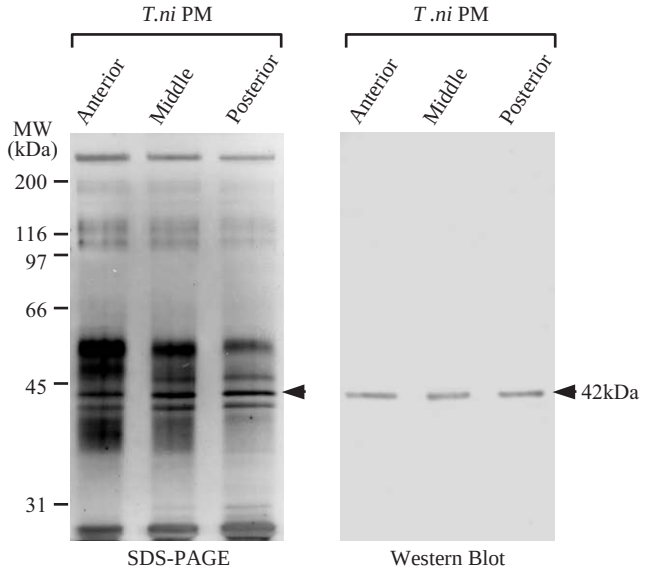

Fig. 4. Identification of TnPM-P42 from *T. ni* larval PM proteins by SDS-PAGE analysis and Western blot analysis with antibodies specific to TnPM-P42. Proteins were from the anterior, middle and posterior regions of the *T. ni* PMs. Arrow indicates the 42 kDa TnPM-P42 protein band recognized by the antibodies on the Western blot and the corresponding protein band shown in the SDS-PAGE gel visualized by silver staining.

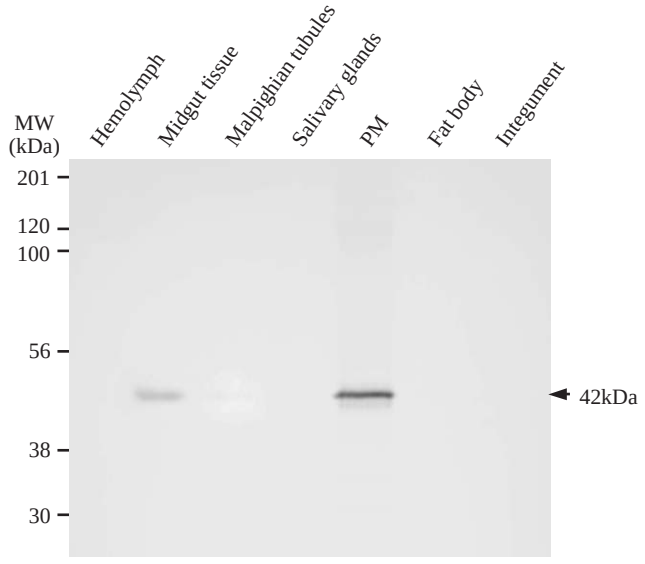

Fig. 5. Detection of TnPM-P42 in various *T. ni* tissue and structure samples by Western blot analysis.

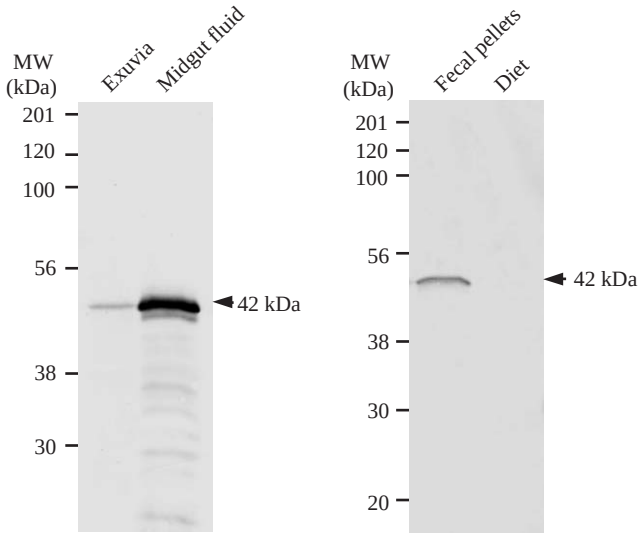

Fig. 6. Detection of TnPM-P42 in larval exuvia, midgut fluid and fecal pellets by Western blot analysis using antibodies reacting to TnPM-P42. Artificial diet was used as a negative control. Arrow indicates the TnPM-P42.

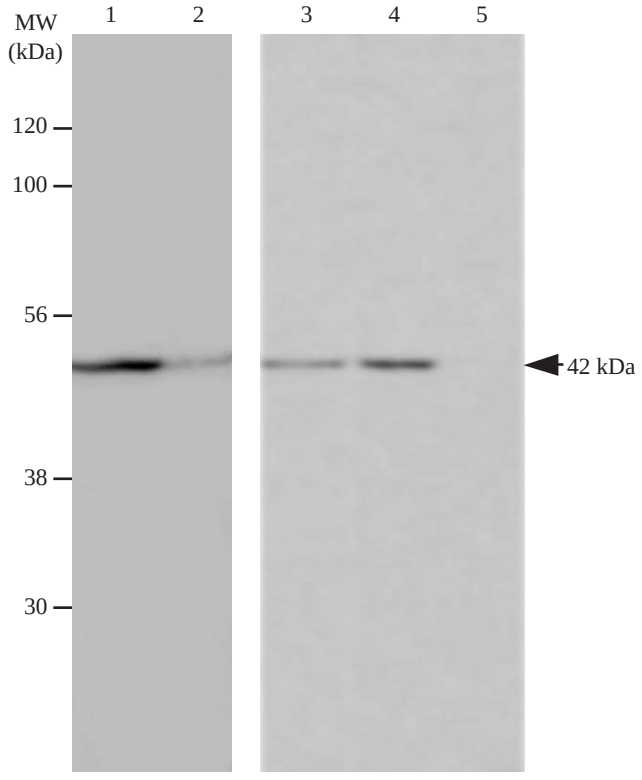

Fig. 7. Western blot analyses of TnPM-P42 from (1) the most anterior part of the midgut epithelium and (2) the whole midgut epithelium isolated from mid-fifth instar *T. ni* larvae, and TnPM-P42 from whole midgut epithelium isolated from (3) early, (4) middle and (5) late stages of fifth instar larvae. Equal amount of proteins from each sample were used for the analysis.

Western blot analysis of proteins of midgut tissues from different regions using antibodies reacting to the TnPM-P42 showed that the TnPM-P42 protein was more abundant in the most anterior region of the midgut (Fig. 7), indicating that TnPM-P42 was primarily synthesized in the anterior part of the midgut. The presence of TnPM-P42 in the midgut tissue of fifth instar larvae was only detected during the early

and middle stages of the instar, or the feeding stages (Fig. 7). When the larvae had stopped feeding in the late stage of the fifth instar and in a pre-pupae stage, TnPM-P42 became absent in the midgut tissue, which is consistent with the PM production in the midgut.

3.4. Chitin-binding activity of recombinant TnPM-P42

TnPM-P42 was expressed successfully in insect cells (Tn-5B1-4) with the recombinant baculovirus. The recombinant protein, which was secreted into the cell culture medium, exhibited its activity to bind chitin (Fig. 8A). The chitin-bound TnPM-P42 could only be released from the chitin by the competitive chitin-binding reagent Calcofluor (Sigma, St Louis, MO, USA) or the denaturing reagent urea (6 M). Treatments of the TnPM-P42/chitin complex with PBS, 0.5 M NaCl, 50 mM GluNAc, 2% SDS, 20 mM acetic acid and 0.1 M sodium carbonate buffer (pH 10.5) did not result in detectable dissociation of TnPM-P42 from chitin (Fig. 8B).

3.5. Presence of TnPM-P42-like protein in other Lepidopteran larvae

Western blot analysis of samples from *H. virescens* and *H. zea* larvae positively detected the presence of a TnPM-P42-like protein from the PMs of *H. virescens* and *H. zea* larvae (Fig. 9). In contrast to the observation in *T. ni* larvae, no positive reactions were detected in the proteins from the midgut fluids from *H. virescens* and *H. zea* larvae.

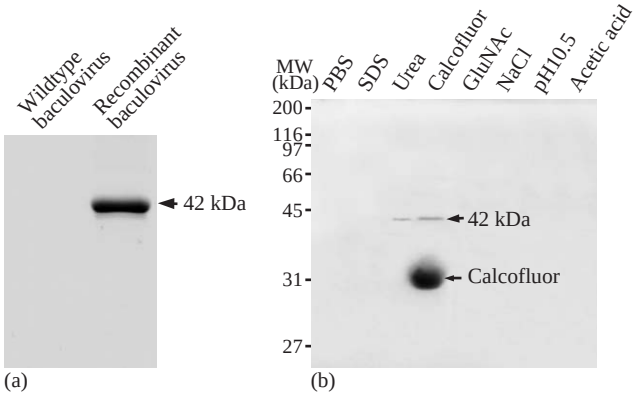

Fig. 8. Analysis of chitin-binding activity of recombinant TnPM-P42. Panel A: SDS-PAGE analysis of chitin-bound proteins isolated from the culture media of insect cells infected with wild-type baculovirus and the TnPM-P42-expressing recombinant baculovirus, showing the binding of the recombinant TnPM-P42 to chitin. Panel B: SDS-PAGE analysis of recombinant TnPM-P42 dissociated from the TnPM-P42/chitin complex by incubation with PBS, 2% SDS, 6 M urea, 0.2% Calcofluor, 50 mM GluNAc, 0.5 M NaCl, 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.5) and 20 mM acetic acid.

3.6. Presence of TnPM-P42 in PMs treated with the baculovirus enhancin

Treatment of *T. ni* PMs with the baculovirus enhancin to degrade the IIM in the PM (Wang and Granados, 1997a) neither led to degradation of the TnPM-P42 protein nor dissociation of the protein from the PM (Fig. 10). Similar amounts of TnPM-P42 were detected

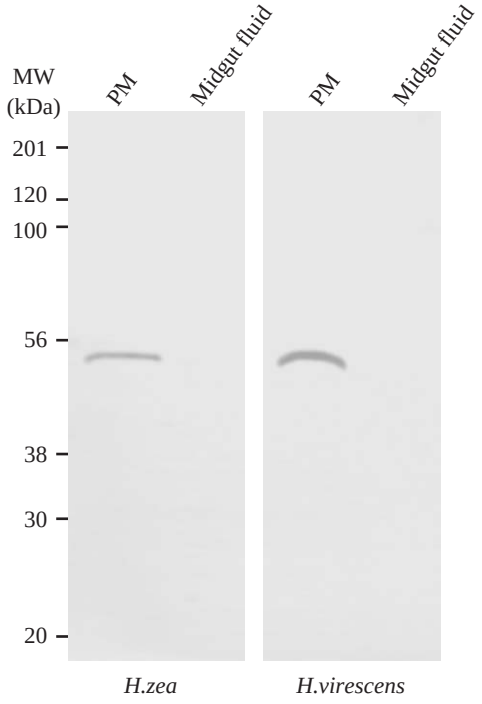

Fig. 9. Western blot analysis of PM and midgut fluid proteins from *H. zea* and *H. virescens* larvae with anti-TnPM-P42 antibodies.

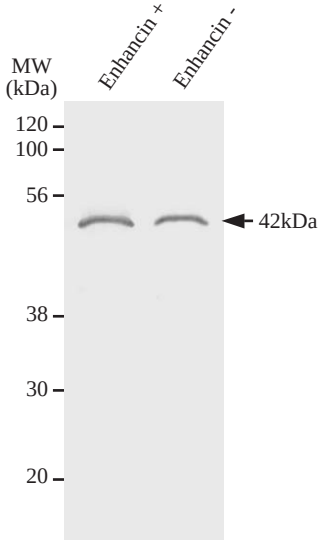

Fig. 10. Western blot analysis to detect the TnPM-P42 in *T. ni* PMs that were treated with the baculovirus enhancin (left lane) and mock-treated with buffer only (right lane).

from both the enhancin-treated and non-enhancin-treated PMs. Therefore, TnPM-P42 in the PM is not susceptible to degradation by the baculovirus enhancin.

4. Discussion

PMs are a gut lining in the complex digestive tract. Therefore, it is difficult to define the integral protein constituents of the PM structure without a good understanding of the PM proteins and mechanisms involved in the PM formation. At present, over a dozen PM proteins intrinsic to the PM structure have been identified from several insect species. All these identified PM proteins are tightly associated with the PM and are common in their activities to bind chitin. The chitin-binding activities of these identified PM proteins are attributed to their peritrophin domains (Tellam et al., 1999; Wang et al., 2004). In this study, we identified a novel PM chitin-binding protein, TnPM-P42, distinctly different from the peritrophin-type chitin-binding proteins. The TnPM-P42 extracted from the *T. ni* PM appeared to be 42 kDa in molecular weight (Fig. 4), identical to its predicted molecular weight based on its protein sequence without significant post-translational modifications (Fig. 1). In vitro chitin-binding assays clearly demonstrated the strong chitin-binding affinity of TnPM-P42 (Fig. 8), which is an important mechanism for the protein–chitin association in the PM formation (Wang and Granados, 2001).

The baculovirus enhancin from *T. ni* granulovirus, a viral pathogen of *T. ni* larvae, disrupts the *T. ni* PM structure in the midgut by degrading the IIM of the PM (Wang and Granados, 1997a). However, treatment of the PM with the baculovirus enhancin neither resulted in degradation of the TnPM-P42 nor in dissociation of the protein from the PM (Fig. 10). Therefore, in the larval midgut, the TnPM-P42 in the PM is not susceptible to degradation by the baculovirus enhancin and its integrity and association with the PM is not dependent on the IIM. TnPM-P42 from the anterior, middle and posterior regions of the PM similarly appeared as a distinct protein band without significant difference in abundance among all the PM proteins and with no detectable proteolytic degradation (Fig. 4). These observations indicate that TnPM-P42 is evenly distributed throughout the entire PM and is fairly resistant to the midgut digestive proteases in the PM structure. Consequently, TnPM-P42 can be detected in the excreted fecal pellets (Fig. 6).

The TnPM-P42 belongs to the Class 3 PM proteins and is similar to the peritrophin type PM proteins in its activity to bind chitin. However, TnPM-P42 represents a uniquely different family of proteins from the peritrophin-type PM proteins in its sequence features. Although there are no sequences of known proteins from GenBank with significant overall similarities to TnPM-P42, TnPM-P42 has a putative polysaccharide deacetylase domain with sequence similarities to the chitin deacetylase domains from fungi and a bacterium (Fig. 2). This finding suggests that the chitin-binding activity of TnPM-P42 may be attributed to its chitin deacetylase-like domain, which is uniquely different from the previously reported peritrophin-type PM proteins from various insects.

TnPM-P42 is a novel insect protein with no significant overall sequence similarity to known proteins. However, blast search of ESTs from a number of arthropods clearly suggested that TnPM-P42-like proteins are widely present in arthropods (Fig. 3). Particularly, several ESTs from the larval midgut of two lepidopteran species, *H. armigera* and *B. mori*, code for proteins with remarkable sequence similarity to the TnPM-P42 from *T. ni*. Consistent with the finding from the EST sequences, Western blot analysis of PM proteins from *H. virescens* and *H. zea*, two species in the same family as *T. ni*, detected a PM protein similar to the *T. ni* TnPM-P42 (Fig. 9), suggesting that TnPM-P42 represents a family of PM proteins in insects.

In *T. ni* larvae, the midgut epithelium is the only tissue where TnPM-P42 was localized, suggesting that TnPM-P42 is synthesized in the midgut cells. Notably, TnPM-P42 appeared to be more abundant in the most anterior part of the midgut epithelium (Fig. 7), which is similar to the observations made in other insects that the syntheses of PM proteins and PM chitin synthesizing enzymes are more abundantly localized in the anterior part of the midgut (Bolognesi et al., 2001; Hogenkamp et al., 2005). Western blot analysis weakly detected a 42 kDa protein from the exuvia of a *T. ni* larva molting from fourth to fifth instar (Fig. 6), but not from the larval integument (Fig. 5). Further analysis of proteins from the integument from various larval stages, including the time point immediately prior to ecdysis, still did not show a visible positive reaction by Western blot analysis (data not shown). To detect the presence of TnPM-P42 from the exuvia required one entire exuvia. Therefore, the presence of TnPM-P42 in the integument appears to be minute and is associated with the cuticle which becomes an exuvia after ecdysis. TnPM-P42 was also detected in the midgut digestive fluid (collected as regurgitant) from *T. ni* larvae (Fig. 6). Whether the TnPM-P42 in the midgut fluid is associated with PM precursors and degraded PMs, or is present as soluble molecules has yet to be investigated.

Our results from this study showed that TnPM-P42 is a novel insect protein associated with the PM and proteins similar to TnPM-P42 appear to be present in other insects and acari. TnPM-P42 has chitin-binding activity and shows sequence similarity to chitin deacetylase domains. However, analysis of the recombinant TnPM-P42 for chitin deacetylase activity using an in-gel

assaying method (Trudel and Asselin, 1990) did not show the enzyme activity (data not shown). Therefore, the biochemical and physiological functions of this new family of proteins need to be elucidated. The identification of TnPM-P42 in this study has provided the foundation for further investigation on the biochemical and physiological function of this new family of proteins in insects.

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