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Structural and biochemical characteristics of chitin-binding protein *Se*CBP66 from *Spodoptera exigua* (Hübner)

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ABSTRACT. Peritrophic membrane proteins are important components of the insect peritrophic membrane. A novel cDNA gene encoding a chitin-binding protein, named *secbp66*, was identified by immunization screening of the cDNA library of *Spodoptera exigua*. The full length of *secbp66* is 1806 bp, which encodes 602 amino acids. The predicted weight of the protein is 64.2 kDa. Bioinformatic analysis showed that a signal peptide composed of 17 amino acids located at the N-terminal of *Se*CBP66 contained seven tandem putative Type-II functional chitin-binding domains and five potential N-glycosylation sites, but no O-linked glycosylation sites. To study the properties of *Se*CBP66, recombinant SeCBP66 was successfully expressed in the insect cell line BTI-Tn-5B1-4 with a Bac-to-Bac expression system. A chitin binding experiment showed that the recombinant *Se*CBP66 protein could bind to chitin strongly. This study of the novel chitin-

binding protein *Se*CBP66 provides a basis for developing new control targets for *S. exigua*.

Key words: *Spodoptera exigua*; *Se*CBP66; Chitin binding activity

INTRODUCTION

In arthropods and invertebrates, the gut is normally covered by a semi-permeable extracellular matrix called the peritrophic matrix (PM), which acts as an effective mechanical barrier and often lines the midgut of insects, preventing the potential invasion of bacteria. The PM is composed of a network of chitin and proteins, with glycans filling the interspace (Peters, 1992; Shen and Jacobs-Lorena, 1999; Tellam et al., 1999; Wang and Granados, 2001). Chitin and proteins are usually associated in a complex via covalent interactions, and the proteins are often classified as different types based on their manner of release from the PM (Tellam et al., 1999).

To date, several PM proteins have been identified from various insect species, including the larvae of Lepidoptera, Diptera, and Coleoptera, and other agriculturally important insects (Wang et al., 2004; Guo et al., 2005; Zhang and Guo, 2011; Liu et al., 2014; Zhao et al., 2014). One class of the major PM proteins includes members with multiple chitin-binding domains (CBDs) that can only be removed by mild or strong denaturants, which cross-link with chitin fibrils to form the PM (Wang and Granados, 1997, 2001). Given that the PM plays an important role in gut physiology, this disruption would decrease the permeability of the PM, which has harmful effects to insects and can even cause death (Wang and Granados, 2000; Bolognesi et al., 2001). Therefore, detailed study of the structure, biochemical properties, and function of PM proteins will help to understand the formation mechanism of the PM, which would further enrich our understanding of the mechanism of pathogenic bacterial infections and the interaction between the PM and microorganisms.

Spodoptera exigua is a worldwide pest; however, identification of PM proteins from *S. exigua* remains limited compared to that from other species. Several intestinal mucin and carboxylesterase genes have been screened and identified by our research team (Li et al., 2010; Zhang and Guo, 2011). In this study, we cloned a novel cDNA gene, *secbp66*, encoding a chitin-binding protein (CBP) of the *S. exigua* PM by immunization screening of the cDNA library of *S. exigua*. We expressed *Se*CBP66 protein in the insect cell line BTI-Tn-5B1-4 and analyzed its molecular structure and chitin binding activity. The discovery and characterization of the PM protein *Se*CBP66 provides a basis for developing new control targets for *S. exigua*.

MATERIAL AND METHODS

Insect larvae and tissue preparation

A laboratory colony of *S. exigua* was maintained on an artificial diet. The fifth-instar larvae were used to isolate the PMs, midgut, and various tissues, as described by Wang and Granados (1997), and were stored at -70°C before use.

Construction of a midgut cDNA expression library of *S. exigua*

The midgut was used for the preparation of total mRNA, and the mRNA was purified

according to the manufacturer instructions (Qiagen Inc., Chatsworth, CA, USA). mRNA was reverse transcribed and the cDNA was unidirectionally ligated into the Uni-ZAP XR vector by T4 DNA ligase. The library was constructed and amplified according to the manufacturer instructions (Stratagene, La Jolla, CA, USA).

Cloning of cDNA encoding *Se*CBP66 and bioinformatic analysis

The cDNA expression library of the midgut was screened with antiserum against the *Trichoplusia ni* PM according to the the picoblue™ immunoscreening Kit (Stratagene). Plasmid DNA was isolated and sequenced using T3 and T7 primers, which covered the entire cDNA length. Sequence features of the cDNA were analyzed using DNAMAN software. The signal peptide, and O-glycosylation and N-glycosylation sites were predicted with CBS Prediction Servers, respectively (http://www.cbs.dtu.dk/services/), and the trypsin and chymotrypsin cleavage sites were analyzed using the DNAStar software.

Construction of recombinant baculoviruses for expression of *Se*CBP66

The Bac-to-Bac baculovirus expression system was used to produce the recombinant *Se*CBP66 protein. Two primers (forward primer: CGGGATCCATGAAAGGAATAGCGCTG TTGC, reverse primer: CCCAAGCTT*ATGGTGATGGTGATGGTG*AGGTTTAATTCTGTTG CTGC; the restriction sites are underlined and His tag sequences are in italics) were used to amplify the full length of *secbp66*. *Eco*RI and *Hin*dIII restriction sites were designed at the end of the primer, respectively. A 6xHis tag was also designed at the end of the gene for protein detection and purification. The polymerase chain reaction fragment amplified from the cDNA of *secbp66* was digested with *Eco*RI and *Hin*dIII and cloned into pFastBac1, generating recombinant pFastBac-*secbp66*. The plasmid pFastBac-*secbp66* was transformed into *Escherichia coli* DH10Bac following the instructions provided by the Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA). *secbp66* was transposed into Bacmid, and Bacmid-*secbp66* was constructed. The procedure to generate the recombinant baculoviruses in the sF9 cell line was the same as that provided by the manufacturer. The first and second generations of the recombinant baculoviruses were named vSeCBP66-P1 and vSeCBP66-P2, respectively.

Determination of the viral titer of vSeCBP66-P2

The viral titer of vSeCBP66-P2 was determined according to the 50% tissue culture infective dose as follows. A 20 mL cell suspension was prepared at 1 x 10^5 cells/mL in complete growth medium. The cell suspension was serial-diluted to 10^{-10}, and 100 µL cells were seeded into each well of 96-well plates. Every dilution contained 8 copies per plate. One hundred microliters vSeCBP66-P2 was added, and a control was designed with no vSeCBP66-P2 but only growth medium. The cells were observed about 10 days later to detect the cytopathic effect. It is required that the cells in the negative control must have no cytopathic effect and show good growth, and that the lowest dilution must be 100% positive and that the maximum dilution is 100% negative. The number of plaque-forming units was calculated by the KARBER statistical method. This procedure was repeated twice.

Expression of recombinant *Se*CBP66 and chitin binding analysis

To produce the recombinant *Se*CBP66, the insect cell line BTI-Tn-5B1 (HighFive) was infected with vSeCBP66-P2, and the cell culture media were collected at 24, 48, 72, and 96 h post-infection to measure the level of protein production. The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting. To obtain a large protein amount, we infected the cells again and harvested the cell culture media at the best time after centrifugation at 500 *g* for 5 min, which were stored at -70°C.

The regenerated chitin was prepared as described by Molano et al. (1977), and the chitin binding activity assay of *Se*CBP66 was conducted as described by Wang and Granados (2001). Briefly, 40 mg (wet weight) regenerated chitin was incubated with recombinant *Se*CBP66 to allow the protein to bind to the chitin, and phenylmethanesulfonyl fluoride was added to inhibit protein degradation. Then, aliquots of the chitin-protein complex were incubated with phosphate-buffered saline, 1% Calcoflour; 6 M urea; 50 mM acetic acid; 2% SDS + 5% β-mercaptoethanol; and 2% SDS and 1 M NaCl, respectively. The proteins released from the chitin were analyzed by SDS-PAGE and silver staining, and were colorimetrically analyzed by western blotting.

RESULTS

cDNA cloning and structural analysis of SeCBP66

To obtain positive clones, two rounds of screening were performed. The clone was excised and sequenced, and the full length of the inserted cDNA fragment was 2196 bp. The gene was named *secbp66* (accession No. EU139126), with an open reading frame of 1806 bp encoding a 17-amino acid residue signal peptide and a 602- amino acid protein with a predicted molecular weight of 64.2 kDa. The result of BlastP revealed that the *Se*CBP66 protein had a similarity of 77% with *Spodoptera litura* CBP (Figure 1).

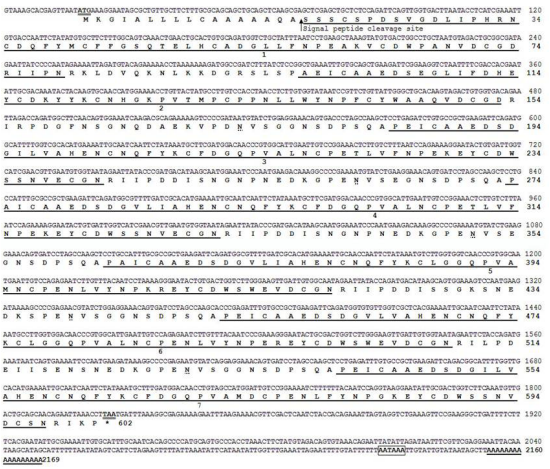

Figure 1. cDNA of secbp66 and its deduced amino acid sequences. The translation initiation codon ATG and stop codon TAA are double underlined. The predicted signal peptide cleavage site is indicated by a vertical arrow. PloyA is is underlined, the potential polyadenylaiton signal sequence marked in box. Senven underlined CBD are numbered.

DNAMAN analysis showed that *Se*CBP66 is rich in asparagine, aspartic acid, glutamate, and proline, accounting for 10.17, 9.55, 9.41, and 7.36% of the total amino acid residues, respectively. *Se*CBP66 contains 7 CBDs with conserved sequence motifs C-x(13,20)-C-x(5,6)-C-x(9,19)-C-x(10,14)-C-x(4,14)-C (Figures 2 and 3). There was no O-glycosylation site in *Se*CBP66, which indicated that SeCBP66 is not a mucin-type PM protein. Otherwise, it contained five potential N-glycosylation sites located in the gap between CBD2 and CBD7 (Figure 4). To confer the stability of the protein, there are three pairs of disulfide bonds between two adjacent cysteine residues. Collectively, these analyses indicated that the protein belongs to the peritrophin-A PM protein type.

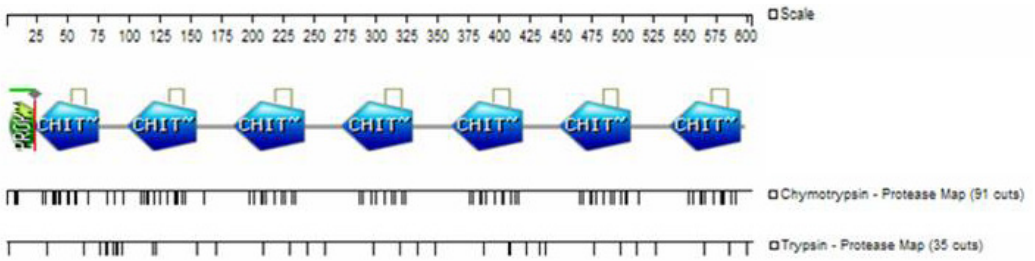

Figure 2. Structures and prediction of Trypsin and Chymotrypsin cleavage sites of *Se*CBP66. Seven type 2 chitin binding domains lines in *Se*CBP66 and disulfide bonds are present in each domain.

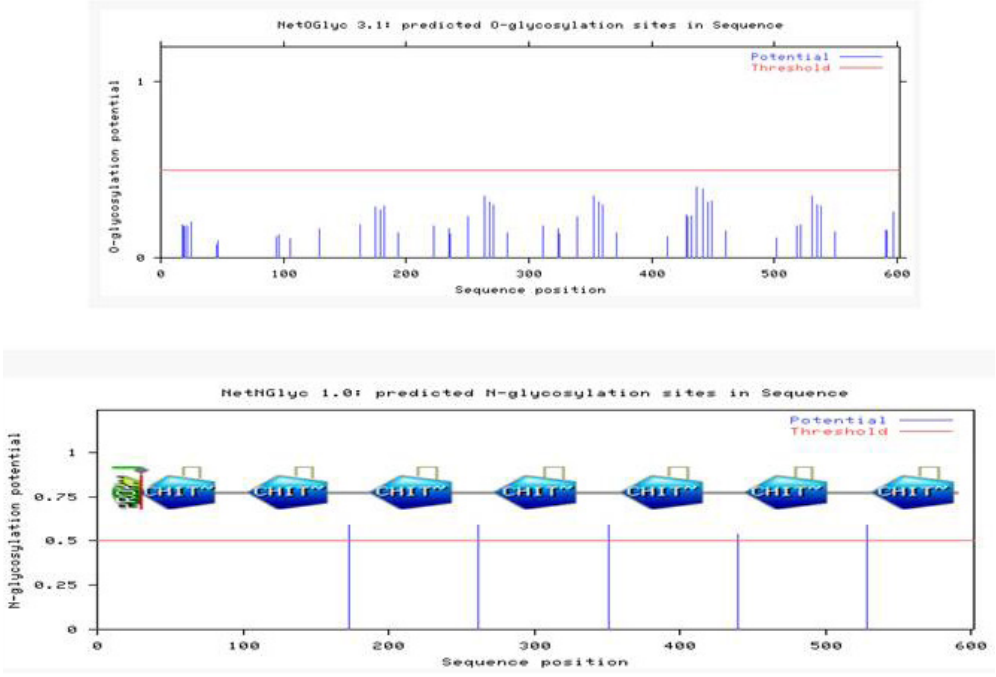

Figure 3. Prediction analysis of O-glycosylation site and N-glycosylation site.

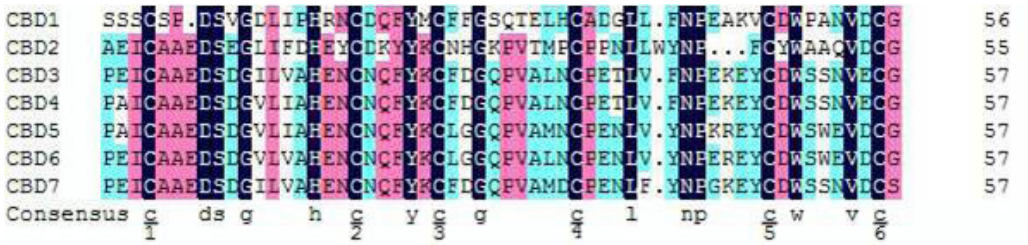

Figure 4. Analysis of CBDs of *Se*CBP66. Seven CBDs were aligned using the DNAMAN software. Every CBD has the conversed cysteine C-x(13,20)-C-x(5,6)-C-x(9,19)-C-x(10,14)-C-x(4,14)-C which belongs to the peritrophin-A domains.

The trypsin and chymotrypsin cleavage sites of *Se*CBP66 are primarily located within or in the vicinity of the CBD2 to CBD7 region, as identified using the DNAStar software. Some sites were located between CBD1 and CBD2, and almost no sites were found at the end of *Se*CBP66, which differs from the CBPs of *T. ni*, which have relatively more sites located between the last two CBDs and at the end of *Tn*CBP1 and *Tn*CBP2. A schematic diagram of the structure of *Se*CBP66 is shown in Figure 2.

Expression and detection of recombinant *Se*CBP66 in BTI-TN-5B1-4 (HighFive)

To produce the recombinant *Se*CBP66, the insect cell line BTI-Tn-5B1-4 (HighFive) was infected with vSeCBP66-P2, and the recombinant protein was secreted in the media successfully. The result showed that the molecular weight of recombinant *Se*CBP66 was almost 116 kDa, which is higher than the predicted molecular weight of 64 kDa, and is similar to the *Tn*CBP and other PM proteins that were previously identified (Figure 5).

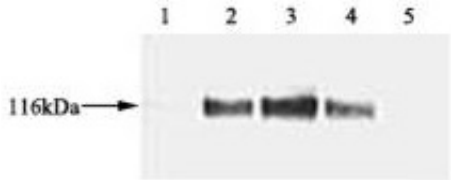

Figure 5. Western blot analysis of the recombinant *Se*CBP66 expressed in BTI- Tn-5B1-4 (HighFive). *Lanes 1*, *2*, *3*, and *4* show the culture medium harvested at 24, 48, 72, 96 h post-infection. *Lane 5* shows a negative control that the culture medium harvested at 96 h from the normal cell media.

Analysis of the chitin binding ability of recombinant *Se*CBP66

The recombinant *Se*CBP66 protein exhibited strong binding activity to chitin. The chitin-bound *Se*CBP66 could be released by treatment with the competitive chitin-binding reagent Calcofluor or 2% SDS+β-Me, and was partially dissociated with the strong denaturing reagent 6 M urea and 2% SDS, but did not dissociate following treatment with phosphate-buffered saline, 0.5 M NaCl, and 50 mM acetic acid (Figure 6A and B).

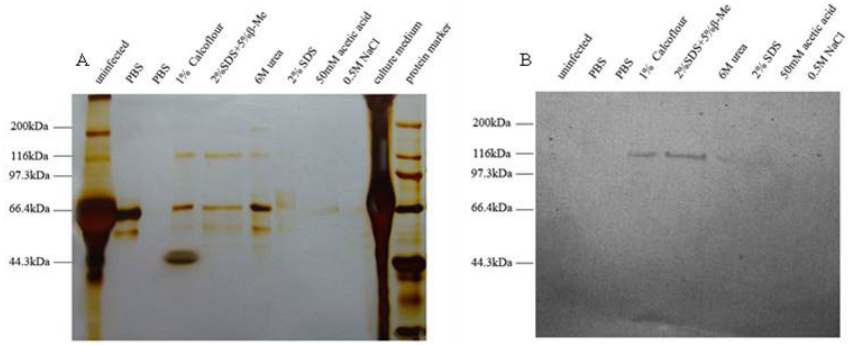

Figure 6. SDS-PAGE and western blot analysis of chitin binding activity of *Se*CBP66. **A.** Sliver staining. **B.** Western blot analysis of *Se*CBP66 bind to chitin. The molecular weight of *Se*CBP66 is almost 116 kDa.

Phylogenetic analysis of *Se*CBP66

Phylogenetic analysis was performed with the known insect PM proteins using the MEGA5.0 software. The phylogenetic analysis showed the highest degree of homology with *S. litura* CBP, but low similarity to the CBP of Coleoptera, which corresponds with evolutionary relationships (Figure 7).

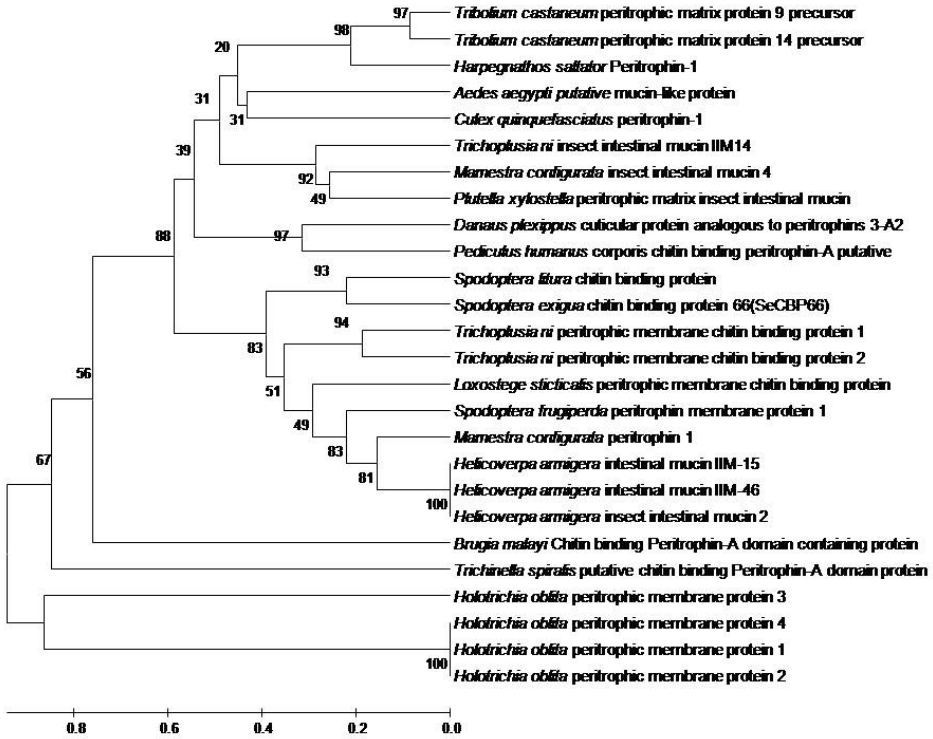

Figure 7. Clustering analysis of *Se*CBP66 with other PM proteins.

DISCUSSION

In this study, a new PM protein, *Se*CBP66, was isolated and identified from *S. exigua*, which belongs to the peritrophin-A protein type. *Se*CBP66 contains 7 tandem CBDs and is rich in asparagine, aspartic acid, glutamate, and proline. The deduced amino acid sequence was highly similar to that of the *S. litura* CBP. The *Se*CBP66 protein could be protected against midgut digestive proteinases in the midgut because of the trypsin and chymotrypsin cleavage sites, which is critically important, since PM proteins must function in an environment very rich in proteinases. The intradomain disulfide bonds within the structure could further stabilize the CBD. Chitin binding analysis of recombinant *Se*CBP66 showed that it tightly bound to chitin and could be released from bound chitin by treatment with 2% SDS + 5% β-Me and by 1% Calcofluor. This property is similar to the characteristics of other PMs.

Recombinant SeCBP66 was successfully expressed in BTI-Tn5B1-4. SDS-PAGE showed that its molecular weight was 116 kDa, which was higher than predicted. This is similar with most other PM proteins, including *Tn*CBP, whose predicted molecular weight is 120 kDa but SDS-PAGE shows its actual weight to be almost 200 kDa (Wang et al., 2004). This phenomenon might be associated with protein modification mechanisms in eukaryotic cells, such as glycosylation, given that *Se*CBP66 was predicted to have five potential N-glycosylation sites located at the gap between CBD2 and CBD7.

The strategy of using the PM as a target for pest control has been evaluated in many insects, including disruption of the disulfide bond, degradation of PM proteins using proteinase, and interference of the chitin bond of proteins (Elvin et al., 1996; Wang and Granados, 1997). This study of the biochemical properties and function of *Se*CBP66 indicates new suitable targets for pest-control product discovery, which could provide a theoretical reference for the biocontrol of *S. exigua*.

In conclusion, in this study, a novel cDNA gene encoding a CBP, named *secbp66*, was identified by immunization screening of the midgut cDNA library of *S. exigua*. *Se*CBP66 was blasted against the National Center of Biotechnology Information database, and the result showed that it is a PM protein, and contains the typical structural features of CBPs. Recombinant *Se*CBP66 was successfully expressed in the insect cell line HighFive using a Bac-to-Bac expression system. The chitin binding experiment showed that the recombinant *Se*CBP66 protein could strongly bind to chitin. This study of *Se*CBP66 provides a basis for developing new control targets for *S. exigua*.

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REFERENCES

Bolognesi R, Ribeiro AF, Terra WR and Ferreira C (2001). The peritrophic membrane of *Spodoptera frugiperda*: secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Arch. Insect Biochem. Physiol.* 47: 62-75. http://dx.doi.org/10.1002/arch.1037

Elvin CM, Vuocolo T, Pearson RD, East IJ, et al. (1996). Characterization of a major peritrophic membrane protein, peritrophin-44, from the larvae of *Lucilia cuprina*. cDNA and deduced amino acid sequences. *J. Biol. Chem.* 271: 8925-8935. http://dx.doi.org/10.1074/jbc.271.15.8925

Guo W, Li G, Pang Y and Wang P (2005). A novel chitin-binding protein identified from the peritrophic membrane of the cabbage looper, *Trichoplusia ni. Insect Biochem. Mol. Biol.* 35: 1224-1234. http://dx.doi.org/10.1016/j.ibmb.2005.06.003

Li W, Guo W, Zhang X, Li J, et al. (2010). Cloning, sequence analysis and detection of tissue-specific expression of the intestinal protein gene SeM-8 from *Spodoptera exigua* (Lepidoptera:Noctuidae) peritrophic membrane. *Acta Entomol. Sin* 53: 727-733.

Liu X, Li J, Guo W, Li R, et al. (2014). A new type I peritrophic membrane protein from larval *Holotrichia oblita* (Coleoptera: Melolonthidae) binds to chitin. *Int. J. Mol. Sci.* 15: 6831-6842. http://dx.doi.org/10.3390/ijms15046831

Molano J, Durán A and Cabib E (1977). A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* 83: 648-656. http://dx.doi.org/10.1016/0003-2697(77)90069-0

Peters W (1992). Peritrophic membranes. Springer.

Shen Z and Jacobs-Lorena M (1999). Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol.* 48: 341-347. http://dx.doi.org/10.1007/PL00006478

Tellam RL, Wijffels G and Willadsen P (1999). Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.* 29: 87-101. http://dx.doi.org/10.1016/S0965-1748(98)00123-4

Wang P and Granados RR (1997). An intestinal mucin is the target substrate for a baculovirus enhancin. *Proc. Natl. Acad. Sci. USA* 94: 6977-6982. http://dx.doi.org/10.1073/pnas.94.13.6977

Wang P and Granados RR (2000). Calcofluor disrupts the midgut defense system in insects. *Insect Biochem. Mol. Biol.* 30: 135-143. http://dx.doi.org/10.1016/S0965-1748(99)00108-3

Wang P and Granados RR (2001). Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol.* 47: 110-118. http://dx.doi.org/10.1002/arch.1041

Wang P, Li G and Granados RR (2004). Identification of two new peritrophic membrane proteins from larval *Trichoplusia ni*: structural characteristics and their functions in the protease rich insect gut. *Insect Biochem. Mol. Biol.* 34: 215-227. http://dx.doi.org/10.1016/j.ibmb.2003.10.001

Zhang X and Guo W (2011). Isolation and identification of insect intestinal mucin HaIIM86--the new target for Helicoverpa armigera biocontrol. *Int. J. Biol. Sci.* 7: 286-296. http://dx.doi.org/10.7150/ijbs.7.286

Zhao D, Guo W, Li S, Li R, et al. (2014). Identification of a new peritrophic membrane protein from larval *Holotrichia parallela* (Coleoptera: Motschulsky). *Molecules* 19: 17799-17809. http://dx.doi.org/10.3390/molecules191117799