

Identification and Molecular Characterization of a New Member of the Peritrophic Membrane Proteins from the Meadow Moth, *Loxostege Sticticalis*

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Abstract

The peritrophic membrane (PM) plays an important role in protecting insects. The PM proteins are important to determinate the formation and function of the PM. A new PM protein, named Lsti99, was identified from the PM of *Loxostege sticticalis* larvae by cDNA library screening. The full cDNA of Lsti99 is 1392 bp in length, contains an open reading frame (ORF) of 1245 bp that encodes a preprotein of 415 amino acid residues with a 17-amino acid signal peptide. The sequence of Lsti99 showed no homology to other known PM proteins. The recombinant Lsti99 was successfully expressed in insect cells (Sf9) using recombinant baculoviruses and was used to isolate the antibodies to Lsti99 from the polyclonal antiserum. Lsti99 was expressed mainly in the PM, but weaker bands could be detected in the head and integument as well. The Lsti99 protein could be separated from the PM complex by chitinase in vitro, but M2R did not show effect in vitro confirming the chitin-binding activity of Lsti99. The biochemical and physiological functions of Lsti99 in *L. sticticalis* require further investigation.

Key words: Peritrophic membrane protein; Chitin-binding activity; Recombinant protein; *Loxostege sticticalis*; Lsti99.

Introduction

In insects, the midgut is the main organ for digestion and nutrient absorption. In some insects the peritrophic membrane (PM) encircles the gut as a layer and is believed to protect insects from invasion by viruses, bacteria, protozoa, helminthes, and prevents damage to midgut cells by abrasive food particles [1-3]. The PM also appears to protect the midgut epithelium from toxic heme derivatives in mosquitoes [4] associated with digestion of blood meals, and the assimilation and elimination of toxic ammonium ions [5]. The PM is a potential target for insect control [6-7].

Insect PM is mainly composed of proteins and chitin. Four classes of PM proteins have been suggested based on the solubility of those proteins under different extraction conditions [2].

Using molecular methods, 20 PM proteins or putative PM proteins have been identified from several insects, including *Lucilia cuprina* [8-11], *Trichoplusia ni* [12-13], *Anopheles gambiae* [14-15], *Chrysomya bezziana* [10, 16], *Glossina morsitans morsitans* [17], *Plutella xylostella* [18], *Mamestra configurata* [19], *Spodoptera exigua* and *Helicoverpa armigera* [20]. These pro-

teins have chitin-binding activities as conferred by their characteristic chitin-binding domains or the peritrophin domains [2]. These domains contain 60-75 amino acid residues and are characterized by a conserved registry of cysteine residues and a number of aromatic amino acid residues [2]. It has been suggested that the conserved cysteine residues form intra-domain disulfide bonds contribute to protein stability in the protease-rich gut environment. In the case of TnPM-P42, a chitin deacetylase-like domain is reported instead of a peritrophin domain [2, 12-14]. Identification and characterization of PM from a wide variety of insects will help to develop pest management targets as well as a better understanding of the PM function and development.

In present study, we identified a new gene encoding a PM protein from *Loxostege sticticalis* larvae by cDNA library screening, which was named as Lsti99. The protein sequence features are different from known PM proteins, but can be liberated from the PM after chitinase treatment.

Materials and methods

Insect

A laboratory colony of *L. sticticalis* established by our laboratory was used in the present research. The larvae of *L. sticticalis* were maintained at 22±1 °C, 70-80% RH and L:D 16:8 photoperiod on LB Agar [21]. Tissues for analysis were isolated from fifth instar larval and were stored at -70°C until use.

Cloning and sequencing of the PM protein Lsti99 from *L. sticticalis*

A cDNA expression library of *L. sticticalis* midgut was screened by subtractive immunoscreening using antibodies made against a collection of *Spodoptera exigua* midgut PM proteins [20]. The screening procedure was as described by Wang et al (2004) [22-23]. The clones that positive to the antibodies of PM chitin-binding proteins were selected as candidate clones coding for unidentified PM proteins. These clones were cut and inserted into plasmids using Uni-ZAP XR Gigapack Cloning Kits (Stratagene, La Jolla, CA). The positive clones were sequenced using the dideoxynucleotide chain termination method (Takara Co., Dalian, China).

Recombinant Lsti99 expression

A recombinant baculovirus was constructed using the pENTR™ TOPO vector and BaculoDirect™ Linear DNA system (Invitrogen, USA) to over-express Lsti99 in insect cells. Two primers (99-ZFP: 5'-CACCGAGAACTATTCGCTTCTTAT-3' and 99-ZRP: 5'-TGGGATCCATTTCAGTATC-3') were

designed to amplify the Lsti99ORF. The PCR conditions were as follows: after 5 min 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 90 sec at 72 °C, then 10 min at 72 °C. The expected PCR products were excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan). Then, the obtained PCR products were cloned into pENTR™ TOPO vector. The LR recombination reaction was performed to transfer the Lsti99 gene into the BaculoDirect™ linear DNA following the protocols provided by the manufacturer. Recombinant Lsti99 was produced as a secreted protein by infecting Sf9 cells with the recombinant baculovirus and the infected culture was maintained in TNM-FH medium supplemented with 10% fetal bovine serum (FBS). The cell culture medium containing the secreted recombinant Lsti99 was collected at 72 h post-infection [24].

Preparation of antibodies reacting to Lsti99

Antibody reacting to the Lsti99 was prepared from an antiserum made against the whole collection of *S. exigua* midgut PM proteins. The recombinant Lsti99 protein was immobilized onto a piece of supported nitrocellulose membrane (Optitran BA-S85, Schleicher & Schuell, Keene, NH, USA) by incubation of the membrane with the recombinant protein at room temperature for 1 h, followed by extensive washing (5 times) with 0.1 M phosphate-buffered saline (PBS, pH7.4) and incubation in 3% bovine serum albumin (BSA) for 3 h. The nitrocellulose membrane was then incubated with a 100-fold dilution of the *S. exigua* PM proteins polyclonal antiserum in PBS (0.1M, pH7.4) with 3% BSA at room temperature for 3 h or at 4°C overnight to allow the antibodies in the antiserum to bind to the blotted membrane. Then, the membrane was thoroughly washed five times with 0.1 M PBS (pH7.4) and finally the bound antibodies specific to the Lsti99 were eluted from the membrane by incubation in 5ml of 0.1M glycine buffer (pH 2.5) at room temperature for 10 min, followed by addition of 0.5 ml of 1M Tris-HCl buffer (pH 8.0) to neutralize the pH of the antibody preparation [25].

Treatment of M2R and chitinase to *L. sticticalis* PM protein Lsti99

The dissected PM were treated with Calcofluor White M2R (M2R, Sigma, Beijing, China) or chitinase (Sigma, Beijing, China). 1.5 g (wet weight) of *L. sticticalis* PMs were incubated with 2.5 ml of 1% M2R (Sigma, Beijing, China) for 1.5 h at 37°C, followed by centrifugation at 1000 g for 5 min. The supernatant and the remaining PM pellet were stored for further protein extraction. The treatment of *L. sticticalis* PMs with 0.05 M chitinase (Sigma, Beijing, China) was

same as for M2R. The supernatant after M2R or chitinase were directly dried and stored for later use. The pellet were homogenized in 0.1M PBS (pH7.4) and centrifuged at 12,000g for 20 min at 4°C. The supernatants were dried and stored at -70 °C for western blot analysis [26].

Western blot analysis

Proteins for use in Western blotting were extracted from tissues by homogenization in 0.1 M PBS (pH7.4) and centrifugation at 12,000g for 20 min at 4°C. The supernatants were dried and stored at -70 °C. Protein extracts mixed with SDS-PAGE sample buffer were boiled for 10 min, and immediately loaded on the 12% SDS-PAGE gel. After SDS-PAGE, the proteins were blotted on a PVDF membrane (Hybond-P, Amersham), and the membrane was then incubated with Lsti99 antibodies for 2 h at 37 °C. After washing in PBS-Tween (PBST, 0.05% Tween-20 in PBS, pH 7.2), the membrane was incubated in secondary antibodies (HRP-conjugated goat anti-rabbit IgG, dilution 1/2000) for 2 h at 37 °C, and thoroughly washed in PBST (pH7.2). The binding was detected using a DAB stock stain kit (Sino-American Biotechnology Co., China) [27].

Result

Identification and sequence of the cDNA coding for PM protein of *L. sticticalis*, Lsti99

By screening of the cDNA expression library of the *L. sticticalis* midgut, 282 positive cDNA clones were obtained and sequenced. Homologous sequences were then identified in the GenBank database using blastp and tblastn programs [28]. The number 99 clone, named Lsti99, showed no similar homologous sequence in GenBank and may encode a previous unidentified PM protein.

The full cDNA of Lsti99 was 1392 bp, containing an ORF of 1245 bp, followed by an AT-rich untranslated region and a putative polyadenylation signal (AAATAA) located 49 bp upstream of the polyA tail (Fig. 1). The deduced protein sequence showed that Lsti99 being synthesized as a preprotein of 415 amino acid residues with a 17-amino acid signal peptide as predicted by the software SignalP [29]. The predicted molecular weight of mature Lsti99 protein was 45.06 kDa. Prediction of potential O-glycosylation sites us-

ing the NetOglyc 3.1 server [30] showed that the proteins had only two potential O-glycosylation sites (Fig. 1) with threonine at positions 200 and 410. Prosite protein pattern search predicted five potential N-glycosylation sites (Fig. 1) [31]. Interestingly, the distribution patterns of these characters did not resemble any of the peritrophin domains from either type I or II PM [2]. The hydropathic nature of Lsti99 was calculated and plotted for each residue in the sequence revealing that most of the residue regions were highly hydrophilic (Fig. 2).

Recombinant protein of Lsti99 and prepare the antibody

The recombinant Lsti99 were successfully expressed in insect cells (Sf9) as secreted proteins using baculoviruses system (Fig. 3). The apparent molecular weight of the recombinant Lsti99 was ~50 kDa, more than the predicted 45.06 kDa. This can be attributed to the modification to the recombinant protein in the eukaryotic expression system. We isolated the antibodies to the Lsti99 from the polyclonal antiserum using the Lsti99 recombinant protein, according to the methods of Wang and Granados (2003) [25].

Localization of Lsti99 in *L. sticticalis* larvae

The tissue distribution of Lsti99 in *L. sticticalis* larvae was detected by western blot using the antibody to the recombinant protein. The expected band of ~45.06 kDa was strongest in the PM, but weak band were detected in head and integument also. No bands were found in the hemolymph, midgut, fat body or frass (Fig. 4). Interestingly, there were unexpected clear bands that were co-detected in the hemolymph and midgut, head and integument (Fig. 4).

In vitro effect of M2R and chitinase on Listi99 protein

The PM were dissected from larvae and treated with M2R or chitinase. The western blot analysis revealed no band in the PM after the chitinase treatment, but a distinct band in the soluble solutions (Fig. 5). This indicated that the chitinase can dissolve the Lsti99 protein from the PM structure in vitro. When the dissected PMs were treated with M2R or no treatment, the clear band can be detected in PM, not in the soluble solutions (Fig. 5), indicating that M2R has no effect on the Lsti99 in vitro.

CTTAATATTGCGGCCGAT**ATG**GAGAACTATTCGCTTCTTATTTATTGTTGATGTGC 60
 M E N Y I R F L F I L L M C 14
 TCAAAATAGCTACTGTGATGAGTCATAACGTATAATTGGAGAAGGCTTGATGAAATG 120
 S N S Y C D E C I T Y N F E E G F D E M 34
 TTCTCATATAAATCTGGTGTCTGTAAAGACTTTGAACCCATGGACTAAAATAATTAC 180
 F S Y K S G V C K D F L N P W T K N N Y 54
 TCATCACTGGGTTGGAGAGTCCGCACCCATCAAGCAGCACATTATCACCTTCAGAT 240
 S S L G L E S P H P S S T T F I S P S D 74
 CAATTGAGCTGCGTATCATCCTTATGTTAATGCGACAAAGGAATTAGATATAAAA 300
 Q L S C V S S F M F N A T K G I L D I K 94
 GTTTACACGAAGAGTTGGGAAACAGTGGATTATATTAAAATATTAGTTATGAGTCTCA 360
 V Y T K S W G N S D Y I K I L V Y E S S 114
 GGC GG ATTAATTGGGTACACATACTTTCGTATTAATGGAAGGCTGGCATAATTGAGG 420
 G G L I G S H T F R D L M E G W H N L R 134
 CTTCCCATATCAAACGACTTAGAATTAGATGCTATATCGTGTGTTGGTACTGGACCA 480
 L P I S N D L E L D V Y I V F L G T G P 154
 GGCTCAACAGTATTAAATTGACTCATTTCGATATATTCCCTTGACGATGGACGAAGAAT 540
 G S T V L I D S F R Y I P L T M D E E D 174
 TGTTACATATTTATTCAACTCCTTCGTATCAACTGCTGATTCAATTACTGAAATG 600
 C Y I F Y Y S T P S S S T A D S I T E M 194
 AATACCATTGATGGAACTGATTTGATGAAAACAGTAACTGAAATCCATATTGAA 660
 N T I D G T D F D E N S V T E N P Y F E 214
 AGATGCATCAAATTCAATTGAAAATCACTCAACACACATTTCAGGGCTGG 720
 R C I K F N F E N H F N T T F S S G S G 234
 TTTTGCACTGGAGAACGCTGGACTTAAGTAACCTTACTTTGTTGGAGATGAATTGT 780
 F C T G A N A W T L S N F T L L E M N C 254
 TTTCATCCTTAAGTACTTCTTAATAGCACCTAACGTTAGTGCATTACATCT 840
 F H P L S T S L I A P K S T F S C I T S 274
 CCTAATTTCCTATGGGTGATGGAAAATTAGACTTAATGTTACATGGGAATGTGTCC 900
 P N F P M G D G K L E F N V Y M G N V S 294
 CGATCAAATTTCCTTTGGATGATCAACATATTGGTCCAAGTGAAGTCTGACGAC 960
 R S N F P L F W M I N I L V Q V K S D D 314
 GGCACCGATAAGACTATTGCAACTACGTACACTCACATTCTGCAATTGTGCCA 1020
 G T D K T I A T T S Y N S H S V N F V P 334
 GGCTGGAATACATTGAAAATAAACGTACAAACCTATGGCAAGAGGGACATGCGTGTG 1080
 G W N T L K I N V Q N L W Q E G H V V L 354
 ATGGGTATAAGACAGAAGGTTGATAGTATTGACTCATTCGCTTCATTCCATCG 1140
 M G I K T E G S I V F M D S F R F I P S 374
 ACTATGAATGATGATATGTTCTTACGAAAATCAAATAACAACGTGAAGATTCTGAA 1200
 T M N D D M C I L Y E N Q I T T E D S E 394
 ATAACAAAATTACTGAAACAGAAGCTGGAAACAGAACATCGGATACTGAAAAATGGATC 1260
 I T K F T V T E A W E T E S D T E K W I 414
 CCA**TAA**TTTCCTAATACCGATATGCTACACAAATGAACGTGTTAATTATTAAACAAAC 1320
 P . 415
 AAACATTGTTTCTTTCAATTAAACATTAATGCGTGTAAAAAAAAAAAAAAA 1380
 AAAAAAAAAAAA 1392

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the Lsti99 cDNA in *L. sticticalis*. The suggested start codon ATG and stop codon TAG are indicated in boxes. A putative polyadenylation signal is shaded with grey. The predicted signal peptide is underlined. Arrows under the nucleotide sequences represent the position of the different synthetic primers used in recombinant expression. The N-glycosylation sites in Lsti99 were predicted using Expert Protein Analysis System, and shaded in black. The O-glycosylation sites in Lsti99 were predicted using NetOglyc 3.1 server and showed with dotted line. The nucleotide sequence reported here is deposited in the GenBank database under the accession number FJ798745.

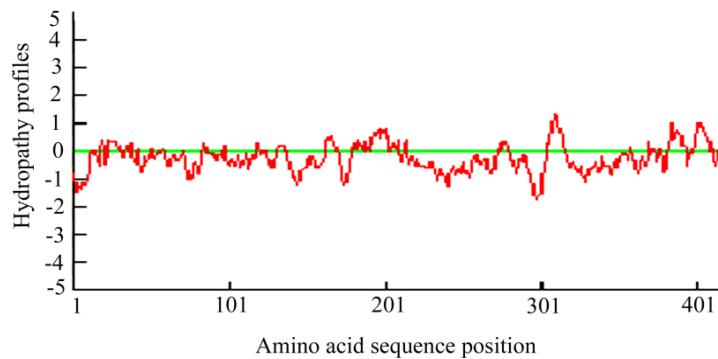


Fig. 2. The predicted hydropathy profiles of the deduced amino acid sequences of the Lsti99. The hydropathy profiles was predicted using DNAman 6.3.0.99.

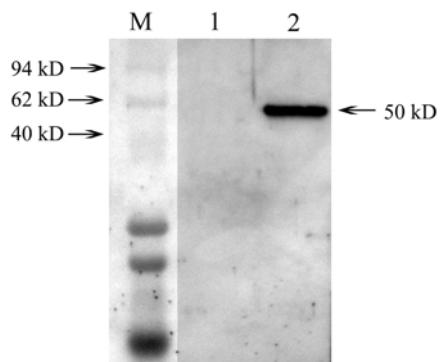


Fig.3. Western blotting analysis of expressed Lsti99 in Sf9 cells. M, represent the protein marker; lane 1, Western-blotting of the normal Sf9; lane 2, Western-blotting of the Sf9 being infected by recombinant virus.

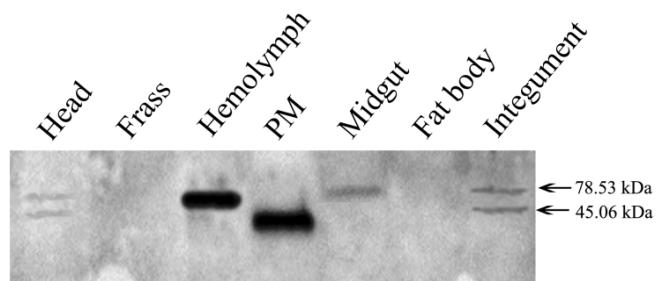


Fig. 4. Tissue distribution of the Lsti99 based on the western blot. 20 µg of protein samples extracted from several different larval tissues were loaded on each lane for immunoblot analysis.

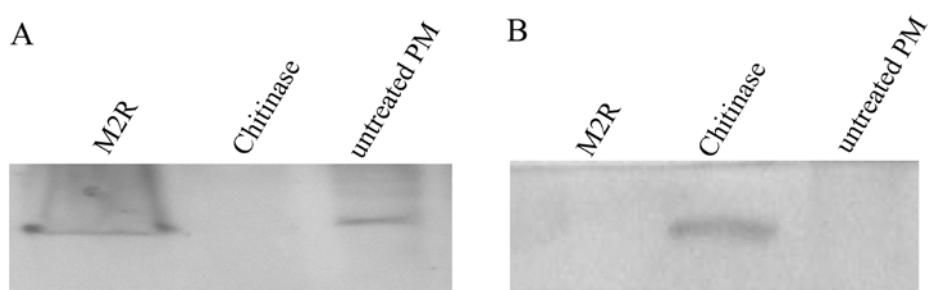


Fig. 5 Western blot analysis of the PM proteins treated with M2R and chitinase. A, the proteins from the pellet after treatment were used for analysis; B, the soluble solutions were used for analysis.

Discussion

To date, about 20 PM proteins have been identified and characterized, which possess invariably chitin-binding activities as conferred by their characteristic peritrophin domains [2]. 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 [32]. In this study, we identified a novel PM chitin-binding protein from *L. sticticalis*, Lsti99, distinctly different from the peritrophin-type chitin-binding proteins. Lsti99 lacks the peritrophin domain, but could be liberated from the PM after chitinase treatment (Fig. 5). The mature protein of Lsti99 was predicted to be 45.06 kDa. The baculovirus system was used to express the recombinant Lsti99 protein and the expressed protein was measured to be 50 kDa. The higher molecular weight can be attributed to the post-translational modifications in the eukaryotic expression system.

The antibodies to Lsti99 were used to characterize the tissue localization of the protein. The Lsti99

was mainly expressed in the PM, and also could be detected in the head and integument. The expression showed some differences to that of TnPM-P42 protein, another PM protein without peritrophin domains in *Trichoplusia ni* [23]. The TnPM-P42 protein was only expressed in the PM of midgut, not in hemolymph, malpighian tubules, salivary glands, fat body, or integument [23]. It is interesting that another bigger band also appeared in the head, hemolymph and integument (Fig. 4), which can be attributed to the cross activity of the Lsti99 antibodies to other proteins. Recently, another clone, named Lsti201 (GenBank FJ798746) from the *L. sticticalis* midgut cDNA library was sequenced and compared with Lsti99. The ORF of Lsti201 encodes a preprotein of 715aa, which is longer than Lsti99 (415aa). The two genes showed higher than 70% similarity at the N terminal and middle regions (Fig. S1). The antibody to Lsti99 showed cross activity to the recombinant protein of Lsti201 with *E.coli* expression system. However, the Lsti201 did not express in the PM (Fig 4), and showed no chitin binding activities.

Lsti99.txt	MENYIRFLFILLMCSNSYCYDECITYNFEEGFDEMFSYKSG..VCKDFLN..PWTKNNYSSLGLESPHPSSSTTFISPSDQLS	77
Lsti201.txt	MEHYTGFFFILLMFSSSYCYDECITYNFEEGFDEIFTPESSYICREMRISTPWTKNNYSSIGLESPHPSSSTIFIPSPNLSS	80
Consensus	me y f fillm s syc ecitynfeegfde f s c pwtknnyss glesphpsst fi ps s	
Lsti99.txtCVSSSFMFNATRGILDIKVYTKSWGNSDYIKILIVYESSCGCLIGSHTFRDLMEGWNLRLPISNDLFLDVYIVFLGTG	153
Lsti201.txt	GLTSCVSSPFVFVTRGILELTVYQKNLGPNDFIMILIS..LDGAITSY.FPDIVVEGMNNLRLTISNDIPEKETSIGIFGMW	157
Consensus	cvssf f gil vy k g d i ll g is f d egw nlrl isnd e i g	
Lsti99.txt	PGSTWLIDSFRYIPTMDEEDCYIFY..YSTPSSSTADSITEMNTIDGTFDENSVTEN.....PYFERCIKFNFENHFN	226
Lsti201.txt	EQGTLWLIDSFRYIPTMNEEDCVIYDSKYTTPSISTVWSTTEMDTINGTDFDENSVTELSTKSPSYFERQVKFNFENHFD	237
Consensus	t lidsfryip tm eedc i y tps st s tem ti gtdfdensvte yferc kfnnfenhf	
Lsti99.txt	TTFSSGSGFCTGANPWTLSNFTLLEINCFHPLSTSIAPKSTFSCITSPNFPMDGRLEFNVYMGNVSRSNFPLFWMINI	306
Lsti201.txt	STFSSGSGFCTGANPWTLSNFTLLEINCFHPLSTSIAPTSTFSCITSPNFIISDGTLLEFNVYMGNVSRSNFPLFWMIKI	317
Consensus	tfsssgsgfctgan wtlsnftlle ncfhplsts iap stfscitspnf m dg lefnvymgnvsrsnfplfwmi i	
Lsti99.txt	LVQVKSDDGTDKTIATTYSNHSWNVFPGWNTLKINVNLWQEGHIVLMLGIKTEGSIVFMDSFRIPIPSTMNDDMCILYEN	386
Lsti201.txt	LVQVKSHDGTDNTIATTYSNHSWDNFVPGWNTLKINVNLWQEGHIVLMLGMKTEGSIVFIDSFRFIPSTMNDDMQLYRN	397
Consensus	lvqvks dgtd tiattsynhs nfvgpgwntlkinv nlwqegh vlmg ktegsivf dsfrfipstmnddmcl y n	
Lsti99.txt	QITTE.....DSETTRFTVTEAWETESD.....	409
Lsti201.txt	QITTPSLTTMDSESTKTRITVTEAWETESDNSGHETENNQETNEQQTDATVSESFRSTDYDKETKTTVRDQWTTADTI	477
Consensus	qitt dse tk tvawew tes	
Lsti99.txtTEKWIP.....	415
Lsti201.txt	TDSTVTESWIKTTINNNQETTMAGIWTLDPTSSTDTETESTDLNEDATVTTKEMWTTTSKPNSTEICKYDFEKDFDK	557
Consensus	te wi	
Lsti99.txt	415
Lsti201.txt	LFSSRSGNCRGMTPWTLNNLTSVELESNLHPSSRLCISPCKTSLSCVTSFSFLMGSGTLEFSVYIGDSQFSNAINILVMES	637
Consensus		
Lsti99.txt	415
Lsti201.txt	SEGGFDYFRNYMYTPMSPNFVLGVNVLRITLDSPRDFEGIVLQGALAELNCSVVLVDSFQYIPSTIHEELCKIYQGQ	715
Consensus		

Fig. S1. Alignment of the amino acid sequences of Lsti99 and Lsti201. The conserved amino acids were shaded. Lsti99, FJ798745; Lsti201 FJ798746.

When the dissected PMs were treated with chitinase, no band was detected after western blot, but the soluble solutions showed a distinct band. When incubated with M2R, the Lsti99 band showed no difference to the control, which is in accordance to the results of M2R to *S. exigua* in vitro [26]. The above result indicated that the Lsti99 protein is one of the PM proteins, which can bind to chitin and be separated from the complex by chitinase. However, the biochemical and physiological functions of Lsti99 need to be further elucidated.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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