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A chymotrypsin-like serine protease cDNA involved in food protein digestion in the common cutworm, *Spodoptera litura*: Cloning, characterization, developmental and induced expression patterns, and localization

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ABSTRACT

A full-length cDNA (*Slctlp2*) encoding a chymotrypsin-like serine protease was cloned from *Spodoptera litura*. This cDNA encoded a putative serine protease with a predicted molecular mass of 30.6 kDa, which contained a serine protease catalytic motif GDSGGPL. Temporal and spatial expression of *Slctlp2* mRNA and protein detected by Northern blotting, RT-PCR, qPCR and Western blotting analyses revealed that both *Slctlp2* mRNA and protein were mainly present in the foregut and midgut of the 5th and 6th instar larvae during the feeding stages. *In situ* hybridization and immunohistochemistry confirmed that both *Slctlp2* mRNA and protein were predominately present in the midgut. Expression of the gene was not induced by bacterial infection. Juvenile hormone III induced the gene expression, while 20-hydroxyecdysone had no impact on the expression. The expression of *Slctlp2* mRNA and protein was down-regulated by starvation but up-regulated by re-feeding. The SlCTLP2 protein was detected in the lumen residues of the anterior, middle and posterior midgut and feces of the feeding 6th instar larvae, suggesting that it was secreted from the epithelium into the lumen of the gut. The results suggest that this *Slctlp2* gene may be involved in digestive process of food proteins during the feeding stages of the larval development.

1. Introduction

Serine proteases have been reported to be involved in immune response (Jiang and Kanost, 2000; Jiang et al., 2003a, 2003b; Kanost et al., 2004), food protein digestion (Mazumdar-Leighton and Broadway, 2001; Herrero et al., 2005; Broehan et al., 2008), and molting process (Samuels and Reynolds, 1993; Chamankhah et al., 2003; Wei et al., 2007; He et al., 2009) in insects. As the digestive enzyme system in the midgut of polyphagous lepidopteran insects can be targeted in development of new insecticidal technologies, it has been the subject of many studies for insect control (Gatehouse et al., 1997; Brito et al., 2001; Srinivasan et al., 2006; Chougule et al., 2008). Generally, serine proteases such as trypsin and chymotrypsin, cysteine proteases, carboxypeptidases and amino-peptidases are the major digestive enzymes in the midgut of lepidopteran larvae (Ortego et al., 1998; Terra and Ferreira, 1994; Broehan et al., 2008). Many protease inhibitors (PIs) have been tested for their possible pesticide activities by using either diet bioassays (Jongsma et al., 1995; Hilder et al., 1987; Ortego et al., 1998; Bown et al., 1997; Srinivasan et al., 2006; Chougule et al., 2008; George et al., 2008) or transgenic plants (Johnson et al., 1989; McManus et al., 1994; Thomas et al., 1995; Gatehouse, 2008). Identification and characterization of insect digestive proteases would improve the use of PIs for plant protection strategies (Srinivasan et al., 2005, 2006).

Several cDNA and genes for chymotrypsin-like serine proteases have been cloned and characterized in different insect species such as *Manduca sexta* (Peterson et al., 1995; Broehan et al., 2007, 2008), *Helicoverpa armigera* (Srinivasan et al., 2005; Chougule et al., 2008), *Aedes aegypti* (Bian et al., 2008), *Ostrinia nubilalis* (Coates et al., 2006) and *Drosophila melanogaster* (Muharsini et al., 2001). Many studies have suggested that midgut chymotrypsin-like serine proteases may be involved in food protein digestion. However, direct experimental evidence for dietary protein digestion in the midgut and characterization of insect midgut chymotrypsins needs to be further documented to better understand the protein digestion process and action mechanisms of midgut chymotrypsins.

Spodoptera litura is a major pest of economically important crops in subtropical and tropical regions around the world. The larval stage is highly polyphagous and is known to feed on more than 290 species of host plants from 99 families, including cole, cabbage, radish, potato, tomato, spinach, bean, etc. (Qin et al.,

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2006). In a previous study we identified a midgut-specific chymotrypsin-like serine protein (*Slctlp1*) in *S. litura* (Zhan et al., 2010, in press). In the present study, another chymotrypsin-like serine protease cDNA (*Slctlp2*) was cloned from the *S. litura* midgut. Developmental and induced expression of the *Slctlp2* gene was analyzed using RT-PCR, qPCR, northern blot and western blot analyses. Localization of the mRNA and protein of the *Slctlp2* gene was investigated using *in situ* hybridization and immunohistochemistry. The results suggest that this gene may be involved in food protein digestion in the midgut of the insect.

2. Materials and methods

2.1. Experimental insects and sample treatments

S. litura (Lepidoptera: Noctuidae) was obtained from the Entomology Institute of SUN YAT-SEN University, Guangzhou, China. Larvae were reared on artificial diet (soybean powder: 100 g, wheat bran: 80 g, yeast: 26 g, casein: 8 g, Vitamin C: 8 g, choline chloride: 1 g, sorbate: 2 g, cholesterol: 0.2 g, inositol: 0.2 g, agar: 26 g and formaldehyde: 2 ml in 1 l) at 26 °C, 70–80% humidity and a photoperiod of 12 h light and 12 h dark until they reached adult moths.

Larvae that were molting into 6th instar stage with a white head capsule were used for hormone treatments. The larvae were injected with 4 μl (1 μg) of juvenile hormone III (JH III) or 20-hydroxyecdysone (20E) (Sigma–Aldrich Co., Guangzhou, Guangdong, China) at 0.25 μg/μl in DMSO in the penultimate abdominal seqment using a microinjector and the injection site was sealed with Vaseline. The injected larvae were then reared on artificial diet at 26 °C until sample collection. Larvae were collected at 8, 16 and 24 h post-treatment for RNA isolation from the midgut. The larvae injected with the same volume of PBS were used as controls. Twenty larvae were treated and used for analysis in each of three replicates. For *in vitro* hormone treatment experiments, the midguts were isolated from larvae that were molting into 6th instar stage with white head capsule and incubated at 26 °C in the Grace's medium (Invitrogen, USA) containing 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and JH III or 20E at a final concentration of 1 μM. The *in vitro* incubated midguts were collected for RNA extraction after 12 and 24 h culture.

For the study of dose effects of JH III on *Slctlp2* expression, larvae that were molting into 6th instar stage with a white head capsule were injected with different concentrations (0, 0.5, 1, 2 and 3 μg per larva) of JH III or 20E and RNA was extracted and analyzed at 12 h after injection. For the midgut *in vitro* incubated, different final concentrations (0, 1, 2, 3 and 4 μM) of JH III or 20E were added into the medium and the midgut was incubated for 12 h before RNA extraction. The other conditions were the same as described above.

For the experiments of pathogen-induced expression, larvae that were molting into 6th instar stage with a white head capsule were used for pathogen infection. The larvae were injected respectively with three Gram-positive bacteria *Bacillus thuringiensis*, *B. subtilis*, *Staphylococcus aureus*, and two Gram-negative bacteria *Escherichia coli* strains DH5α and $K_{12}D_{31}$ at 1×10^5 cells in 5 μl of phosphate buffered saline (PBS) (pH 7.4) into the hemolymph in the penultimate abdominal seqment. Larvae were collected at 6 and 24 h post-treatment for RNA isolation from the midgut, and larvae injected with the same volume of PBS were used as controls. Thirty larvae were treated and used for analysis in each of three replicates.

For the starvation experiments, larvae that were molting into 6th instar stage with a white head capsule were transferred from the artificial diet to the culture dishes without diet. The larvae were collected at 6, 12, 24, 48 and 72 h post-starvation treatment for RNA isolation. For the re-feeding assay, the larvae that had been starved for 12, 24 and 36 h were fed again for additional 12, 24 and 36 h, respectively. All the treated larvae were dissected for midgut isolation on ice and immediately frozen in liquid nitrogen. The samples were stored at −80 °C until they were used for RNA isolation. Sixty-five larvae were treated and used for analysis in each of three replicates.

For collection of food residues from the gut and feces, individual 6th instar larvae were reared on either the artificial diet or leaves of *Arabidopsis thaliana*, which was obtained from the Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, South China Normal University, Guangzhou, China. At the day 3 of 6th instar stage, larvae, as well as the feces, were collected. Different portions of the gut epithelium and lumen food residues were carefully separated and collected with the aid of a microscope for protein extraction. Total soluble proteins were extracted from the gut epithelium, lumen food residues, feces and diets using homogenization buffer (50 mM Tris, 10 mM EDTA, 15% glycerol, pH 7.8) as previously described (Feng et al., 2001). Eight larvae were treated and used for analysis in each of three replicates.

2.2. Cloning and sequence analysis

Sequences of serine protease cDNAs were identified from an expressed sequence tag (EST) dataset from a cDNA library that was constructed in the Uni-ZAP XR vector by using the ZAP cDNA Gigapack II Gold Cloning Kit (Stratagene, La Jolla, CA) and using mRNA isolated from the midgut of 6th instar larvae of *S. litura*. DNA sequencing was conducted using an ABI377 automatic sequencer. Annotation and comparison of sequences were performed using the National Center for Biotechnology Information BLAST search services (http://www.ncbi.nlm.nih.gov; Altschul et al., 1990). Multi-sequence alignment and phylogenetic tree analysis were performed using the CLUSTAL W method in MegAlign 5.01 of DNASTAR software (DNASTAR, Inc., Madison, WI) at a gap penalty of 10, a gap length penalty of 0.2, Gonnet series matrix of 250 and a bootstrap value of 10,000 times. A 95% bootstrap confidence interval was used in the program.

2.3. RNA isolation and northern blot analysis

Total RNA was isolated from larval tissues using Trizol-reagent (Invitrogen, Guangzhou, Guangdong, China). The midgut was dissected from larvae of the 5th and 6th instar and pupal stages. Ten micrograms of total RNA from each of the samples was separated on 1.0% formaldehyde agarose gels and then transferred onto nylon membranes after electrophoresis. A fragment of *Slctlp2* cDNA was labelled with ^{32}P-dCTP using the Random Primer DNA Labelling Kit (TaKaRa BIO Inc., Otsu, Japan) and used as a probe for northern blot analysis. Pre-hybridization, hybridization and post-hybridization washes were carried out according to the protocol by Béliveau et al. (2000). Membranes were scanned and photographed using Typhoon TRIO Variable Mode Imager (Typhoon 9400, GE Healthcare Life Sciences, USA).

2.4. Reverse-transcription PCR (RT-PCR) and quantitative real-time reverse-transcription PCR (qPCR) analysis

Total RNA was extracted as described above. For RT-PCR, 1.5 μg of each of the RNA samples was treated with 2 units of DNase I to remove trace amount of genomic DNA. Reverse-transcription was preformed using the Reverse Transcriptase M-MLV Kit according to the manufacturer's instruction (TaKaRa BIO Inc., Otsu, Japan), followed by PCR amplification at the following conditions: 95 °C 2 min, 27 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and final extension at 72 °C for 10 min. The *Slctlp2*-specific primers were 5′-CCGTCAACATGAAGGTCTTGGC-3′

(forward) and 5′-TTAAAGACGTTGGTTGATCCAGC-3′ (reverse). The size of the expected PCR product of *Slctlp2* was 0.9 kb. The primers for the cDNA amplification of *β-actin* as internal control were 5′-CTCCCTCGAGAAGTCCTACGAACT-3′ (forward) and 5′-GGATGCCGCACGATTCCATAC-3′ (reverse). The size of the expected PCR product of *β-actin* was 340 bp. The PCR products were analyzed using 0.8% agarose gel electrophoresis.

For quantitative real-time RT-PCR (qPCR), SYBR Green Kit was used according to the manufacturer's instruction (TaKaRa BIO Inc., Otsu, Japan). For each of qPCR reaction, 1.5 μg of template RNA was used. The *Slctlp2* ORF-specific primers used for qPCR analysis were 5′-CTTCATCGCCCTGCCCTCTG-3′ (forward) and 5′-GCTCCGCTAACACTTCCACC-3′ (reverse). The primers for *β-actin* as internal control were 5′-CTCCCTCGAGAAGTCCTACGAACT-3′ (forward) and 5′-GGATGCCGCACGATTCCATAC-3′ (reverse). The expected PCR products are 108 and 122 bp for *Slctlp2* and *β-actin*, respectively. The qPCR was performed at the following conditions: SYBR® Premix Ex Taq™ (2×): 10 μl in 20 μl of reaction volume; the primer concentrations: 0.4 μl (10 μM); 95 °C 10 s, then 40 cycles (C_q) at 95 °C 5 s, 60 °C 31 s according to SYBR Green1 fluorescent relative quantitative approaches (TaKaRa BIO Inc., Otsu, Japan) using ABI7300 fluorescence quantitative PCR system. The slopes of the cDNA template dilution standard curves were −2.93 and −2.85, and the r^2 values were 0.98 and 0.93 for *Slctlp2* and *β-actin*, respectively. Tm was 60 °C for both the genes and the specificity of each reaction was tested by melting curve analyses, which showed only one PCR product for both the genes. Relative expression levels were calculated based on the equation of $2^{-\Delta Ct}$, where ΔCt equals the difference between the *Slctlp2* Ct-values and *β-actin* Ct-values according to the manufacturer's instruction. Three independent duplicates for each of the data were conducted.

2.5. Production of recombinant protein and antibody

For *in vitro* protein expression in a bacterial system, the open reading frame (ORF) of the *Slctlp2* cDNA was amplified by PCR using a paired primer (forward: 5′-GAATTCATGAAGGTCTTGGCTGTGACT-3′ and reverse: 5′-CTCGAGGCTCTAGAAGTACTCTCGAG-3′). The amplified PCR product was inserted into the pPROEXTM HTa expression vector (Life Technologies, Burlington, Canada) with a 6xHis-tag on the C-terminal end of the target sequence. *E. coli* DH5α cells were transformed with the recombinant plasmid DNA (pPROEXTM HTa-*Slctlp2*). Expression of recombinant SlCTLP2 protein in the *E. coli* DH5α was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.6 mM. The recombinant protein was purified using His-tag affinity column (Novagen, Darmstadt, Germany).

The purified recombinant SlCTLP2 was mixed with the Freund's adjuvant. The protein was then injected into a New Zealand White rabbit. Antiserum was collected after three-booster injections, each with 0.5 mg of the recombinant protein. Pre-immune serum collected from the same rabbit prior to immunization was used as a control.

2.6. Localization of mRNA by in situ hybridization

The ORF sequence of *Slctlp2* cDNA was inserted into the plasmid pBluescript II SK$^+$ to produce a recombinant plasmid DNA, pBluecript II SK$^+$-*Slctlp2*. The recombinant plasmid was then linearized with *Xho* I or *EcoR* I for 4 h and then separated by agarose gel electrophoresis. The target DNA was extracted using DNA Gel Extraction Kit (AXYGEN, Hangzhou, China). Messenger RNAs were synthesized using DIG RNA Labelling Kit (Roche, Basel, Switzerland) at 37 °C for 2 h. The reaction mixture contained 1 μg of the linearized DNA, 2 μl 10× DIG RNA labelling mix, 4 μl 5× transcription buffer, 2 μl RNA polymerase T3 for sense probe or RNA polymerase T7 for antisense probe. After reaction, 2 μl 0.2 M EDTA (pH 8.0) was added to stop the reaction and 1 μl RNase inhibitor was added to protect the synthesized mRNA from degradation. The quality and quantity of sense and antisense mRNA products were examined and estimated by agarose gel electrophoresis.

Two-day-old 6th instar larvae were fixed in 4% paraformaldehyde and processed for paraffin sectioning. The 5 μm-thick sections were mounted on glass slides. After deparaffinization, the sections were washed with 1× PBS, followed by treatment with proteinase K (10 μg/ml in PBS) for 20 min at 37 °C. After rinsing with cold glycine-PBS buffer (2 mg/ml), the sections were fixed for 10 min with 4% paraformaldehyde in 1× PBS before acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0). After a 10-min washing with 4× SSC, the sections were pre-hybridized at 58 °C for 1 h in pre-hybridization buffer (25% 4× SSC, 50% formamide and 25% DEPC water) and then hybridized at 58 °C for overnight with the DIG-labelled antisense or sense probe in the hybridization buffer (50% formamide, 10% dextran sulphate, 1× Denhard's solution, 10 mM DTT, 1 mg/ml tRNA and 1 mg/ml salmon sperm DNA). After two washes with 2× SSC and one wash with formamide: 2× SSC (1:1) at 58 °C, the sections were incubated at 37 °C in NTE buffer (0.5 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 5 min, followed by a treatment with RNase A (20 μg/ml) in NTE buffer at 37 °C for 30 min. The sections were further washed once with the NTE buffer and 0.1× SSC at 58 °C, equilibrated in TBS buffer (100 mM Tris, 150 mM NaCl, pH 7.5) for 10 min and the blocking solution containing 10% sheep serum and 1% Blocking Reagent (Roche, Basel, Switzerland) for 30 min. The probes were then detected with anti-DIG-alkaline phosphatase according to the manufacturer's protocol (Roche, Basel, Switzerland). The color development was stopped by carefully rinsing the slides with distilled water and the sections were observed and photographed with a fluorescence microscopy (DMI4000B, Leica, Germany).

2.7. Localization of protein by immunohistochemistry

Immunohistochemistry localization of SlCTLP2 protein was performed as described by Feng et al. (2001). Two-day-old 6th instar larvae were fixed in 4% formaldehyde at 4 °C overnight. The larvae were then embedded in paraffin and 5 μm-thick cross-sections were made for immunostaining. The sections were hybridized with the anti-SlCTLP2 antibody at a dilution of 1:200. After washing the sections with 1× PBS, the secondary antibody, goat anti-rabbit IgG conjugated with SABC-FITC (StreptAvidin-Biotin Complex-fluorescein isothiocyanate) (Boster, Wuhan, China), was applied at a dilution of 1:1000. The sections were counter-stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) at a dilution of 1:1000 for 30 min and examined under a fluorescence microscopy (DMI4000B, Leica, Germany). The photographs were taken as double-exposures using fluorescein and DAPI filters.

2.8. Statistical analysis of data

All of the data are presented as mean ± standard deviation (SD) with $n = 3$. Significance analysis of the experimental data was performed using ANOVA method followed by Duncan's Multiple Comparison Test at $p < 0.01$ and $p < 0.05$.

3. Results

3.1. Cloning and sequence analysis of S. litura Slctlp2 cDNA

After assembly and Blast search in the NCBI protein database, 84 sequences out of 4656 ESTs from the *S. litura* midgut cDNA

library were found to putatively encode chymotrypsin-like serine proteases. Two full-length cDNAs that encoded a chymotrypsin-like serine protease ORF were assembled and are referred as *Slctlp1* and *Slctlp2*, respectively. *Slctlp1* (GenBank accession number: GQ354838) has been described previously by Zhan et al. (2010, in press). *Slctlp2* (GenBank accession number: GQ891130) is characterized and described in this report. This cDNA was 1003 base pairs in length and encoded a 295-amino acid protein with a predicted molecular mass of 30.6 kDa and a p*I* of 8.74 (Fig. 1). Recombinant SlCTLP2 protein was expressed in an *E. coli* expression system and the apparent molecular mass of the recombinant protein was approximately 31.6 kDa (Fig. 2), closely similar to the predicted size based on the deduced amino acid sequence, indicating that the *Slctlp2* cDNA did encode a full-length protein. There was a 17-amino acid signal peptide at *N*-terminal end of the deduced amino acid sequence. A typical serine protease catalytic motif, GDSGGPL, which is found in most of the members of the serine protease gene family, is present in the C-terminal region. A catalytic triad (His105, Asp152 and Ser247) for serine protease activity was also found in the sequence; therefore it belongs to the peptidase S1 family (Barrett and Rawlings, 1995). Six conserved cysteine residues that putatively form three paired internal disulfide bonds as seen in invertebrate serine proteinase were found in the SlCTLP2 sequence. The conserved amino acids, Ser191, Gly268 and Ala279, which form a specific S1 hydrophobic reaction pocket and are believed to determine substrate specificity, were found and this is consistent with those in both vertebrate and invertebrate chymotrypsins (Peterson et al., 1995).

Multi-sequence alignment of the two SlCTLPs and 16 most homologous serine proteases from other lepidopteran species is shown in Fig. 3A. The catalytic motif (GDSGGPL) is identical in all of the proteins, except *M. sexta* elastase (AAA67842) and *H. virescens* chymotrypsin-like protease C3 (ABR88233), in which the Ser residue at the third place is substituted by Tyr. The cleavage site (RIVGG) for removal of the prepeptide from the mature protein is also highly conserved among the sequences. There are 40 amino acid residues

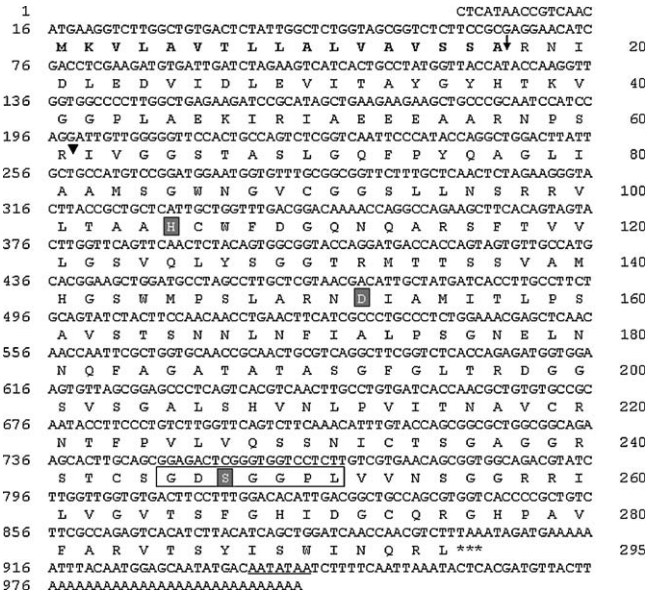

Fig. 1. Nucleotide and deduced amino acid sequences of *Slctlp2* cDNA cloned from *S. litura*. The numbers on the left refer the nucleotide sequence and the numbers on the right refer the amino acid sequence. The putative signal peptide of 17 amino acids is bolded and the cleavage site is indicated by an arrow. The cleavage site for the putative prepropeptide removal is indicated by an arrowhead. The stop codon TAA is indicated by asterisks. A putative polyadenylation signal (AATATAA) is underlined. A putative serine protease conserved motif (GDSGGPL) is boxed. The three amino acid residues (His105, Asp152 and Ser247) of the catalytic triad for serine protease activity are indicated in white letters with grey background. The GenBank accession number for this cDNA sequence is GQ891130.

that are identical in all of the aligned sequences, particularly in the catalytic motif region and the AAHC region, implying that these amino acids might be essential for the secretory and/or functional activity of these chymotrysin-like serine proteases. The three conserved His105, Asp152 and Ser247 residues of a catalytic triad

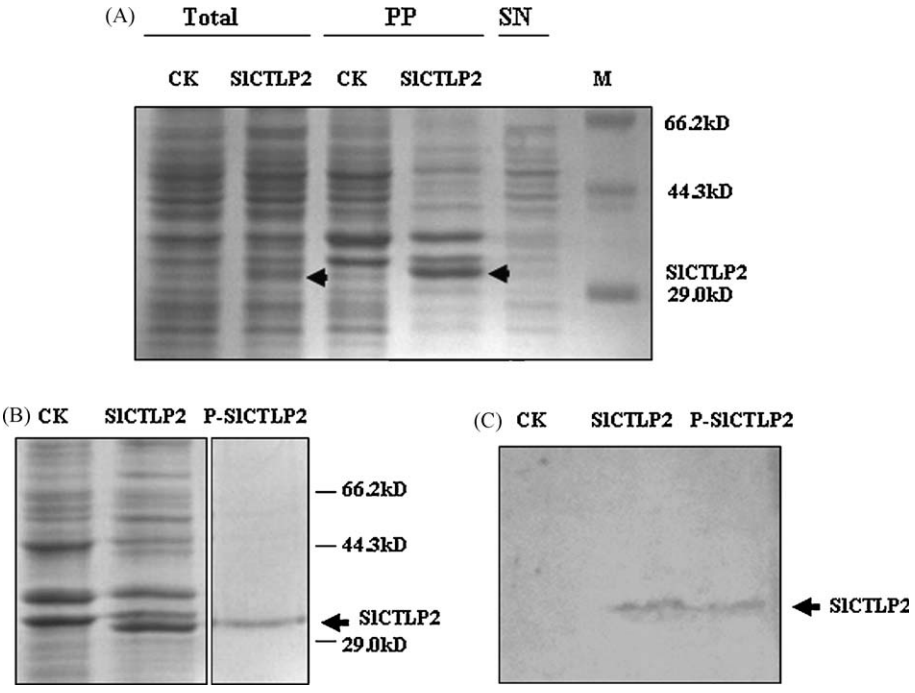

Fig. 2. Production of recombinant SlCTLP2 protein and anti-SlCTLP2 antibody. (A) Expression of the recombinant protein with pPROEXTM HTa expression vector in *E. coli* strain DH5α; (B) purification of the recombinant protein by His-tag affinity column; (C) production and specificity analysis of the anti-SlCTLP2 antibody by western blot. PP: precipitation fraction; SN: supernant fraction; CK: control, the pPROEXTM HTa expression vector alone; SlCTLP2: recombinant protein in *E. coli* cells or precipitation fraction; P-SlCTLP2: His-tag purified SlCTLP2; M: molecular mass marker.

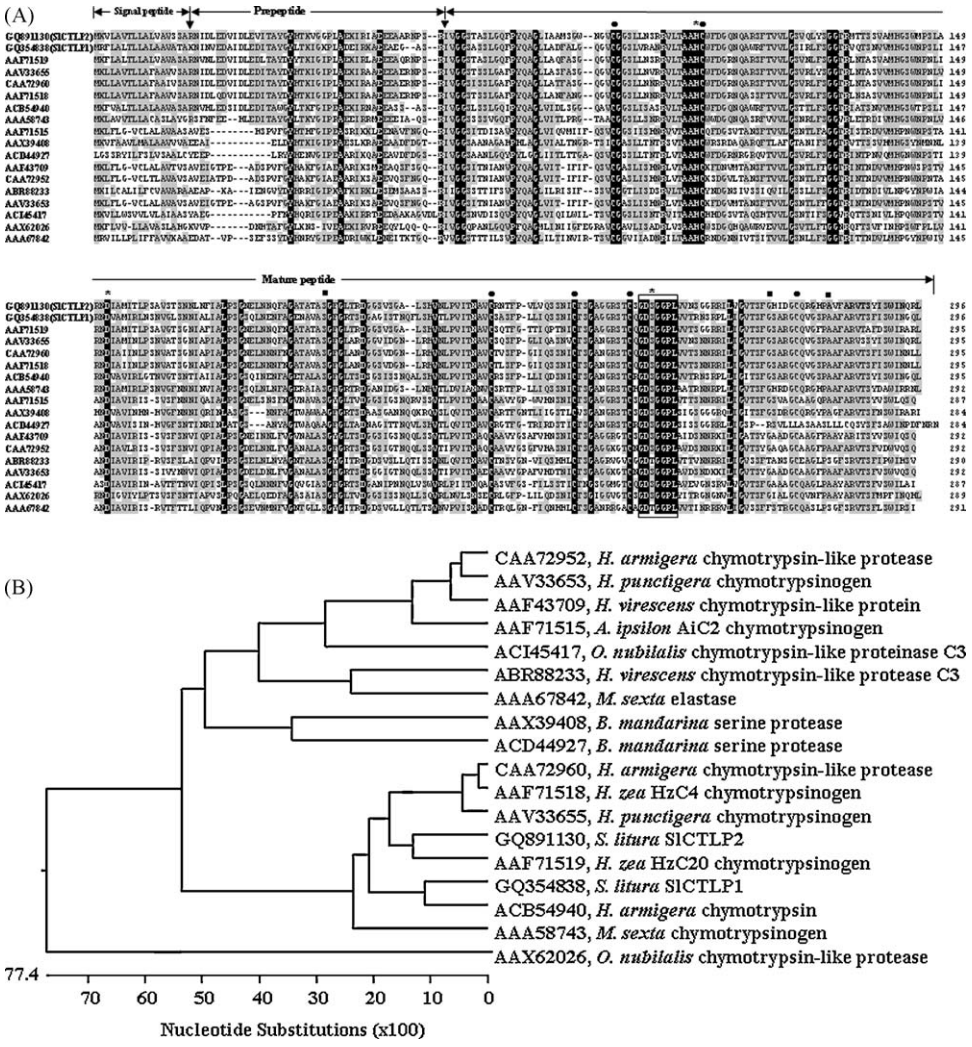

Fig. 3. (A) Multi-sequence alignment of SlCTLP2 (GQ891130) and SlCTLP1 (GQ354838) with homologues of 16 lepidopteran chymotrypsin-like serine proteases. The species names and gene description of the sequences are listed in the phylogenetic tree in (B). The amino acid residues that are identical in all of the sequences are indicated in white letters in black background. The amino acid residues that are conserved in at least 9 out of 18 sequences are shown in grey background. The putative cleavage site for the signal peptide is indicated by an arrow. The conserved cleavage site for removal of the prepropeptide from the mature protein is indicated by an arrowhead. The six conserved cysteine residues that putatively form three paired internal disulfide bonds are indicated with black circles. The three conserved residues His105, Asp152 and Ser247 of the catalytic triad of serine proteases are indicated with asterisks. The three conserved residues Ser191, Gly268 and Ala279 of the S1 specificity pocket are indicated with black squares. The putative serine protease catalytic motif, GDSGGPL, is boxed. (B) Phylogenetic tree analysis of SlCTLP2 and SlCTLP1 with 16 homologues of other lepidopteran chymotrypsin-like serine proteases. Multi-sequence alignment and phylogenetic tree analysis were performed using the CLUSTAL W method at a gap penalty of 10, a gap length penalty of 0.2, Gonnet series matrix of 250 and a bootstrap value of 10,000 times.

were highly conserved among the sequences (Fig. 3A). Six conserved cysteine residues that may contribute to the formation of three paired internal disulfide bonds were highly conserved in these lepidopteran chymotrypsin-like proteins. *S. litura* SlCTLP2 had about 50–81% identities at amino acid level to the homologues of the lepidopteran species, for example, 67% identity to SlCTLP1 (Zhan et al., 2010, in press) and 35–50% identities to the homologues of dipteran species. Phyologenetic tree analysis of the 18 lepidopteran chymotrypsin-like proteins indicated that there are two major groups (Fig. 3B). The first group, which include SlCTLP2 and SlCTLP1, has more conserved sequences of the signal peptides, prepeptides, catalytic motif region and the C-terminal ends than the members of the second group (Fig. 3A) SlCTLP2 was highly similar and close to *Helicoverpa zea* chymotrypsinogen HzC20 (AAF71519) (81% identity) (Fig. 3B). *H. zea* HzC20 was identified from the *H. zea* midgut and, unlike mammalian chymotrypsins, HzC20 was insensitive to the dietary plant protease inhibitor, soybean Kunitz trypsin inhibitor (SKTI) (Mazumdar-Leighton and Broadway, 2001).

3.2. Tissue and developmental expression of *Slctlp2* gene

To examine where the *Slctlp2* gene is expressed, total RNA and proteins were isolated from different tissues, including the head, epidermis, fat body, foregut, midgut, hindgut, Malpighian tubules and hemocytes of 3-day-old 6th instar larvae and the expression of the gene and protein was analyzed by RT-PCR and western blot, respectively (Fig. 4). The transcripts were detected only in the Malpighian tubules, foregut and midgut, and no transcripts were found in other tissues tested, such as head, epidermis, fat body, hindgut and hemocytes (Fig. 4A). The protein was also detected only in the Malpighian tubules, foregut and midgut (Fig. 4B). An unidentified protein with a molecular mass of approximate 34 kDa was immunologically recognized by the anti-SlCTLP2 antibody in the epidermis and fat body. Identity of this protein is not clarified yet.

Expression of the *Slctlp2* mRNA and protein during development through embryo to adult was analyzed with the whole

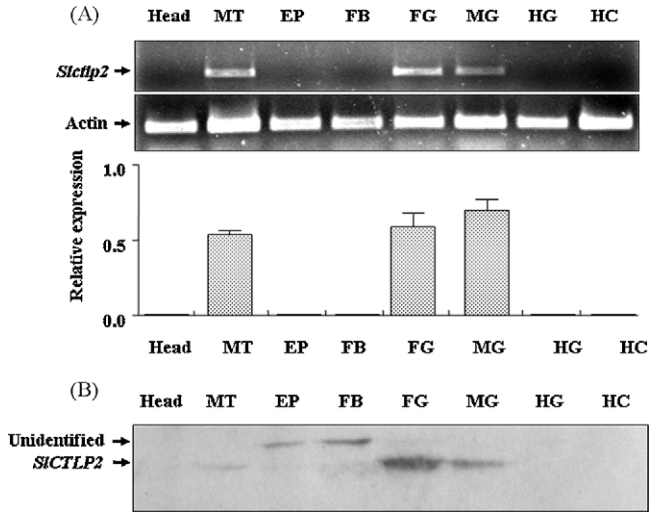

Fig. 4. RT-PCR (A) and western blot (B) analyses of the expression of *Slctlp2* mRNA and protein, respectively, in different tissues of 3-day-old 6th instar larvae of *S. litura*. Total RNA and protein were isolated from head, Malpighian tubules (MT), epidermis (EP), fat body (FB), foregut (FG), midgut (MG), hindgut (HG) and hemocytes (HC). In the RT-PCR analysis (A), actin expression was used as an indicator for equal use of RNA. The size of the PCR products is 0.9 kb. The lower panel shows the quantitative analysis of the RT-PCR results from three independent repeats of the experiment. In the western blot analysis (B), 30 μg proteins were used in each of the lanes. The size of SlCTLP2 protein is 30.6 kDa. The size of the unidentified protein is approximate 34 kDa.

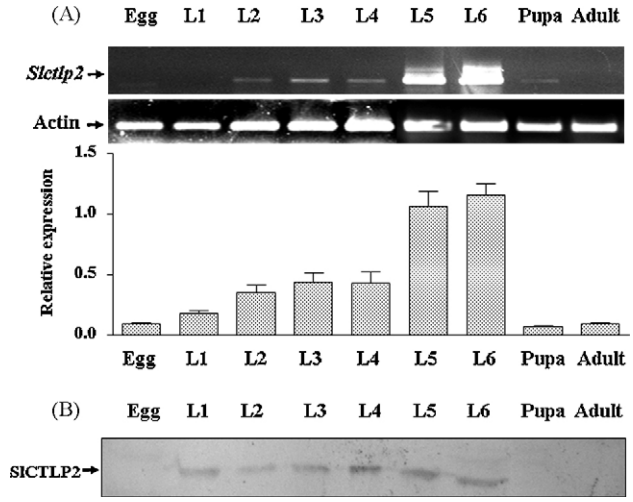

Fig. 5. RT-PCR (A) and western blot (B) analyses of the expression of *Slctlp2* mRNA and protein, respectively, at different developmental stages of *S. litura*. Total RNA and protein were isolated from the eggs (day 1), 1st, 2nd, 3rd, 4th and 5th instar larvae (day 1), 6th instar larvae (day 3), pupae and adults (day 1). In the RT-PCR analysis (A), actin expression was used as an indicator for equal use of RNA. The size of the PCR products is 0.9 kb. The lower panel shows the quantitative analysis of the RT-PCR results from three independent repeats of the experiment. In the western blot analysis (B), 40 μg proteins were used in each of the lanes. The size of SlCTLP2 protein is 30.6 kDa.

insects (Fig. 5). All of the samples were collected at the first day in the corresponding stages prior to 6th instar stage, for which the larvae were collected at day 3. The transcripts were first detected at the 2nd instar stage and reached the highest levels at the 6th instar stage (Fig. 5A). No transcripts were found at the adult stage. Similar results were observed for the protein profile and the protein was first detected at the first instar stage but not detected at the pupal and adult stages (Fig. 5B).

Detailed expression pattern of the *Slctlp2* transcripts and protein was examined with the midguts of 5th and 6th instar larvae using northern blot (Fig. 6A), RT-PCR (Fig. 6B), qPCR (Fig. 6C) and western blot (Fig. 6D). A major transcript of approximate 1000 bp was detected in the epithelium cells of the midgut by northern blot (Fig. 6A). The size of the detected transcript coincided with that of the *Slctlp2* cDNA, which was 1003 bp in length, indicating that the cloned *Slctlp2* cDNA was a full-length

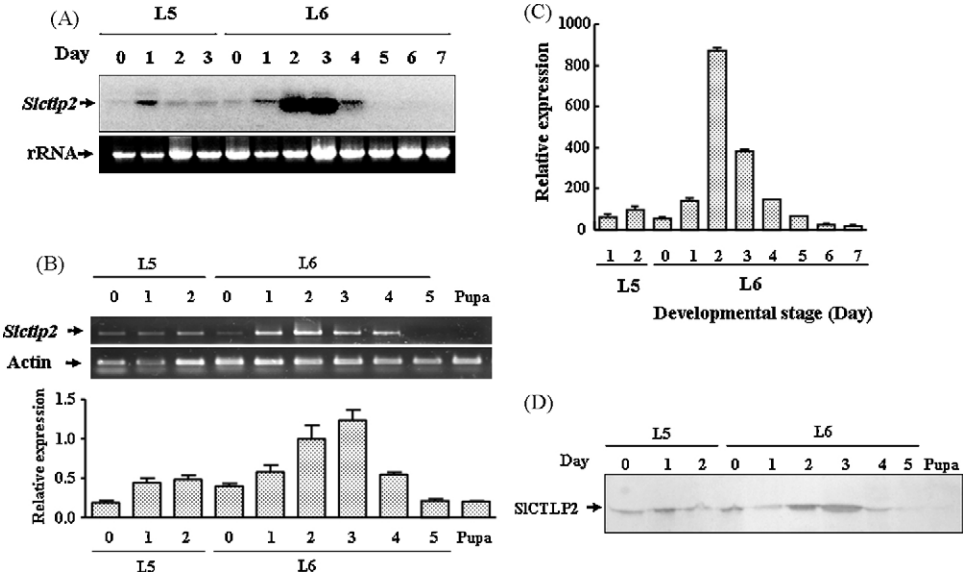

Fig. 6. Northern blot (A), RT-PCR (B), qPCR (C) and western blot (D) analyses of the expression of *Slctlp2* mRNA and protein at different stages of 5th and 6th instar larvae of *S. litura*. Total RNA and protein were isolated from the midguts of 5th instar larvae at days 0–3 and of 6th instar larvae at days 0–7, as well as pupae at day 1. Day 0 is referred as to the stage when the larvae just molted with a head capsule slippage. At days 6 and 7 of the 6th instar stage, the larvae stopped feeding and entered prepupal stage. In the northern blot analysis (A), 10 μg of total RNA was used in each of the lanes and hybridized with a ^{32}P-labelled *Slctlp2* cDNA probe. Ribosomal RNA was stained with ethidium bromide to show equal loading of RNA. The size of the *Slctlp2* mRNA is 1 kb. In the RT-PCR analysis (B), actin expression was used as an indicator for equal use of RNA. The size of the PCR products is 0.9 kb. The lower panel shows the quantitative analysis of the RT-PCR results from three independent repeats of the experiment. In the qPCR analysis (C), quantitative and relative expression of the *Slctlp2* transcripts was calculated and normalized with actin transcript expression as an internal control. Each of the data points represents independent triplicates of the experiment. In the western blot analysis (D), 30 μg proteins were used in each of the lanes. The size of SlCTLP2 protein is 30.6 kDa. L5: 5th instar stage; L6: 6th instar stage.

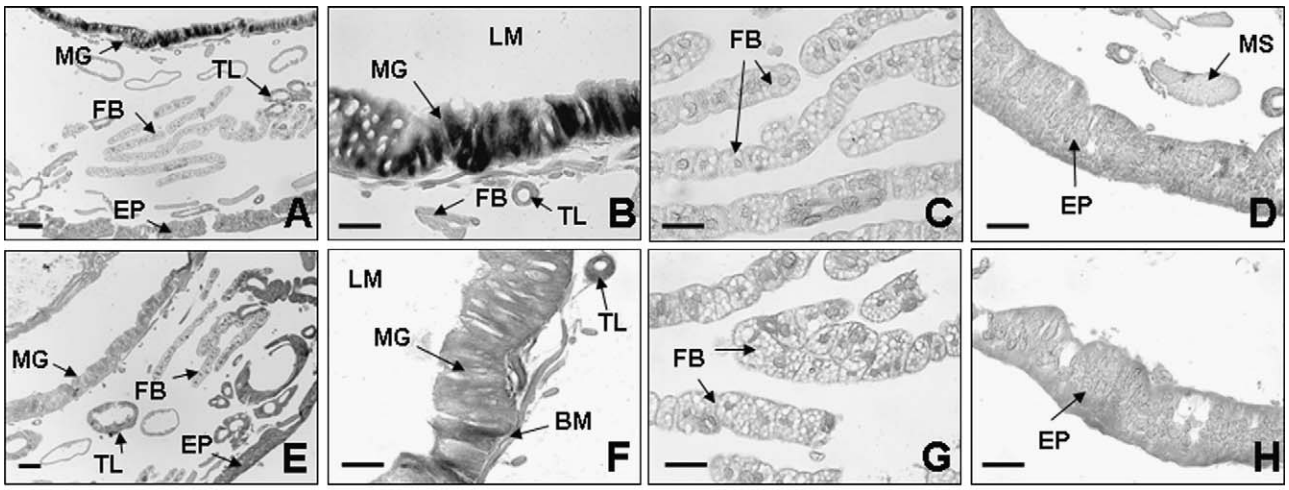

Fig. 7. *In situ* hybridization of *Slctlp2* mRNA in 2-day-old 6th instar larvae of *S. litura*. (A) and (E) show a portion of whole cross-section of the 6th instar larvae; (B) and (F) show the midgut; (C) and (G) show the fat body; (D) and (H) show the epidermis. In (A), (B), (C) and (D) the sections were hybridized with the DIG-labelled antisense *Slctlp2* mRNA probe; in (E), (F), (G) and (H) the sections were hybridized with the DIG-labelled sense *Slctlp2* mRNA probe as control. The black stain in (A) and (B) indicates the presence of *Slctlp2* mRNA in the midgut. MG: midgut; EP: epidermis; FB: fat body; LM: lumen: MS: muscle; TL: tracheal; BM: basal membrane. The bars represent 80 μm in (A) and (E), and 30 μm in (B), (C), (D), (E), (F) and (G).

sequence. High levels of the mRNA expression was found at day 1 of 5th instar stage and days 2 and 3 of 6th instar stage, when the larvae were actively feeding, followed by a decrease and it was not detectable at the prepupal stage (days 5–7). This expression pattern of the *Slctlp2* transcripts in the midgut was confirmed by the results of the RT-PCR (Fig. 6B) and qPCR analysis (Fig. 6C). The expression level rapidly increased at day 2 of 6th instar stage and then gradually decreased during the later stage (prepupal stage) of the 6th instar. The protein expression profile was similar to the transcript profile and the highest levels of the protein were detected at days 2 and 3 of the 6th instar stage when the larvae were actively feeding (Fig. 6D). All of these results indicated that during the feeding stages, the expression of mRNA and protein was much higher than non-feeding stages, such as molting, prepupal and pupal stages.

3.3. Localization of Slctlp2 mRNA and protein

To further investigate where the *Slctlp2* gene was expressed, *in situ* hybridization of *Slctlp*2 mRNA was conducted using *Slctlp2*-specific RNA probes (Fig. 7). In the 2-day-old 6th instar larvae, which had the highest level of the *Slctlp2* transcript expression (Figs. 5 and 6), the transcripts were detected by the antisense mRNA probe mainly in the epithelial cells of the midgut (Fig. 7A

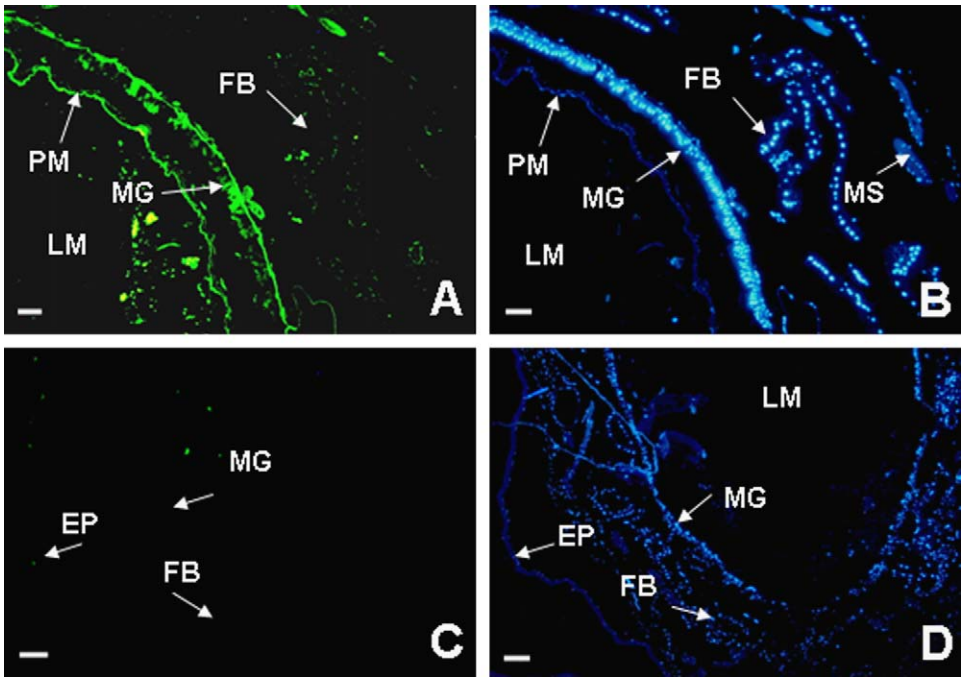

Fig. 8. Immunohistochemistry localization of SlCTLP2 in 2-day-old 6th instar larvae. Five-micron cross-sections were immunostained with anti-SlCTLP2 antibody at a dilution of 1:200 (A and B) or pre-immune serum as control (C and D), followed by goat anti-rabbit IgG conjugated with SABC-FITC at a dilution of 1:1000 (A and C) and counter-staining with DAPI (B and D). The sections were observed under a fluorescence microscopy and the photographs were taken in double-exposures with fluorescein (A and C) and DAPI filters (B and D). The green fluorescence indicates the presence of SlCTLP2 protein (A). MG: midgut; FB: fat body; EP: epidermis; LM: lumen: MS: muscle. The bars represent 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

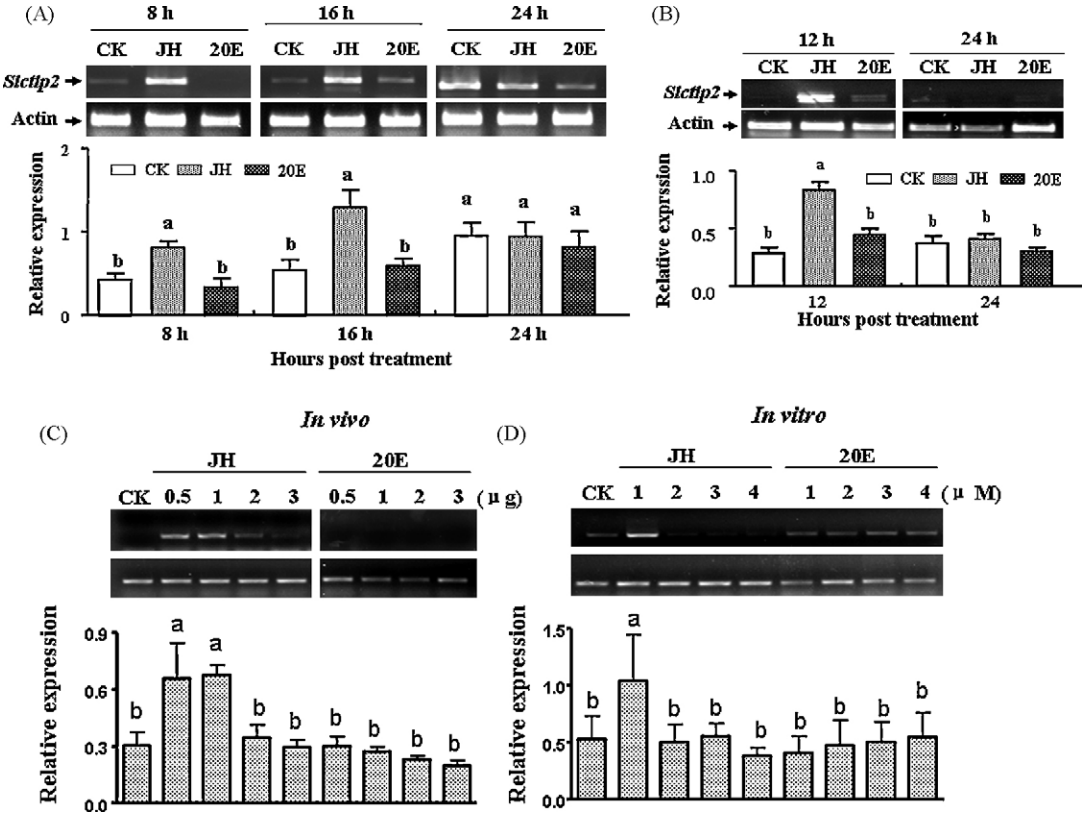

Fig. 9. Induced expression of *Slctlp2* mRNA by JH III and 20E in the midgut of 6th instar larvae of *S. litura*. (A) and (C): Larvae that just molted into the 6th instar with head capsule slippage were injected with either JH III or 20E at 1 μg (A) or at the indicated concentrations (C). The midguts were collected at 8, 16 and 24 h post-injection and the mRNA expression was detected using RT-PCR. The control (CK) was the midgut of the larvae injected with DMSO. Actin expression was used as an indicator for equal use of RNA. (B) and (D): The midguts of 6th instar larvae with head capsule slippage were isolated and *in vitro* incubated in the medium containing either JH III or 20E at 1 μM for 12 and 24 h (B) or the indicated concentrations (D) for 12 h before RNA isolation for RT-PCR analysis. The control (CK) was the *in vitro* cultured midgut treated with DMSO. Actin expression was used as an indicator for equal use of RNA. The lower panels in (A–D) show the quantitative analysis of the RT-PCR results from three independent repeats of the experiment. Significance of the differences were satisfied at $p < 0.05$ using ANOVA analysis followed by the Duncan's Multiple Comparison Test. The same letters indicate no significant differences between the treatments, while the different letters indicate significant differences at $p < 0.05$.

and B), and no transcripts were detected in the fat body (Fig. 7C) and epidermis (Fig. 7D). This result coincided with RT-PCR analysis of the transcripts (Fig. 4A).

To examine where SlCTLP2 protein was present in the 2-day-old 6th instar larvae, immunolocalization of SlCTLP2 protein was performed using the anti-SlCTLP2 antibody. The result revealed that SlCTLP2 protein was present mainly in the epithelial cells, peritrophic membrane and the lumen of the midgut (Fig. 8), while scatter positive signals were also found in the Malpighian tubules, epidermis and fat body. This result is consistent with the result of the western blot analysis (Fig. 4B). The analyses of *in situ* hybridization of *Slctlp2* mRNA and immunohistochemistry localization of *SlCTLP2* protein indicated that the *Slctlp2* mRNA was synthesized and its protein product was present mainly in the midgut of the 6th instar feeding larvae. The presence of the protein in the lumen of the midgut implied that the protein is secreted into the lumen from the midgut after synthesis.

3.4. Induced expression of Slctlp2 by insect hormones

Because the expression of *Slctlp2* gene appeared to be increased with the feeding stage and decreased during the molting and pupation stages, it was of interest to examine whether this gene was inducible by the insect hormones, juvenile hormone (JH) and 20-hydroxyecdysone (20E). Larvae at the first day of 6th instar stage, which had low expression levels of *Slctlp2* transcripts during normal larval development, were injected with JH III and 20E, respectively. JH III significantly ($p < 0.05$) induced the expression of *Slctlp2* mRNA starting from 8 h post-injection (Fig. 9A), whereas 20E had no impact on the expression. By 24 h post-injection, JH III induction was no longer evident because *Slctlp2* expression started to increase at this time of the normal development (Fig. 6A and B). Similar results were obtained with the midguts that were isolated from larvae just molting into the 6th instar stage and were *in vitro* incubated in the medium containing JH III or 20E at 1 μM (Fig. 9B). The expression of *Slctlp2* mRNA was induced by JH III at 12 h post-treatment. The effect of JH III no longer displayed at 24 h post-treatment. The expression of *Slctlp2* in response to JH III was dose-dependent and was up-regulated in larvae injected with JH III at the concentrations lower than 1 μg/larva; however, when the concentrations were higher than 1 μg/larva, the expression of the gene was not increased any more and there seemed no increase over the control levels at 2 and 3 μg/larva (Fig. 9C). In the midgut *in vitro* incubated, the expression of *Slctlp2* was induced by JH III only at a final concentration of 1 μM and higher concentrations of JH III appeared not effective (Fig. 9D). 20E had no impact on the expression of *Slctlp2* mRNA either in larvae or in the midgut *in vitro* incubated.

3.5. Induced expression of Slctlp2 by bacteria infection

Because the expression of *Slctlp2* mRNA and protein was found mainly in the gut and lumen during the feeding stages, its possible involvement in anti-microbial activity was suspected. To examine

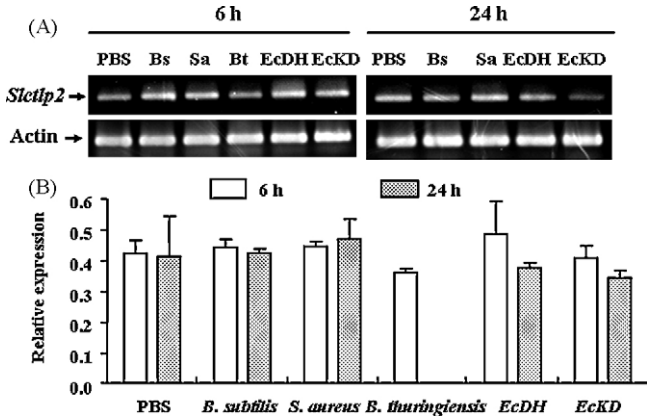

Fig. 10. RT-PCR analysis of induced expression of *Slctlp2* mRNA by bacterial treatments. Larvae that just molted into the 6th instar with head capsule slippage were injected with *B. thuringiensis* (Bt), *B. subtilis* (Bs), *S. aureus* (Sa), *E. coli* DHα5 (EcDH) and *E. coli* K12D31 (EcKD) at 1×10^5 cells in 5 μl of phosphate buffered saline (PBS) (pH 7.4). Total RNA was extracted from the midgut of three infected larvae at 6 and 24 h post-injection. The larvae injected with 1x PBS were used as a control. (A) The RT-PCR result. Actin expression was used as an indicator for equal use of RNA. (B) Quantitative RT-PCR analysis of relative expression of *Slctlp2* mRNA, which is normalized over actin expression. Each of the data points includes three independent experimental replicates. No significant differences were found by ANOVA analysis followed by the Duncan's Multiple Comparison Test at $p < 0.05$. The larvae injected with Bt died at 24 h post treatment.

whether or not the expression of the *Slctlp2* gene was regulated by bacterial infection, several bacteria, including the Gram-positive bacteria *B. thuringiensis*, *B. subtilis*, *S. aureus* and Gram-negative bacteria *E. coli*, were tested for their possible induction effects on the expression of *Slctlp2* mRNA in larvae that just molted into the 6th instar stage with head capsule slippage (HCS) (Fig. 10). After the larvae were injected with the bacteria, the expression of *Slctlp2* mRNA was examined using RT-PCR at 6 and 24 h post-injection. The results indicated that no significant ($p < 0.05$) changes in the expression level of mRNA were found at either 6 or 24 h post-treatment.

3.6. Induced expression of Slctlp2 by starvation and re-feeding

Another putative function of the *Slctlp2* gene is its involvement in food protein digestion. To determine whether or not the expression of the *Slctlp2* gene was regulated by feeding and starvation, larvae that just molted into the 6th instar stage were starved for 6, 12, 24, 48 and 72 h without feeding with artificial diet, or starved for 12, 24 and 36 h followed by re-feeding for 12, 24 and 36 h, respectively. The expression of the *Slctlp2* mRNA and protein in the midgut were determined by RT-PCR and western blot analyses. The results indicated that the expressions of both *Slctlp2* mRNA (Fig. 11A) and protein (Fig. 11B) were significantly ($p < 0.05$) down-regulated by starvation in all of the treatments and up-regulated again by re-feeding.

3.7. Secretion of SlCTLP2 into the lumen of the midgut

Sequence analysis indicated that the SlCTLP2 protein contains a signal peptide and a transmembrane prepropeptide that is putatively removed after synthesis and secretion into the target site (Figs. 1 and 3A). If the protein is involved in the food protein digestion, it has to be secreted into the interspaces between the epithelium and peritrophic membrane or the lumen of the gut, where it digests the ingested food proteins. To examine whether or not the enzyme is present in the diet inside the lumen and feces, western blot analysis was performed with the proteins isolated from the epithelium and the lumen food residues of the anterior, middle and posterior midgut, as well as feces, of the 6th instar larvae fed with artificial diet or *A. thaliana* leaves. The results showed that a protein (slightly larger than 29 kDa) was present not only in the epithelium of the anterior, middle and posterior midgut, but also in the lumen food residues of the anterior, middle and posterior midgut, as well as the feces of the larvae feeding on either the artificial diet (Fig. 12A) or the plant (Fig. 12B), suggesting that the protein was secreted into the lumen of the gut after removal of the signal peptide. It was noticed that the protein appeared to be partially cleaved into additional four smaller fragments (approximate 23, 17, 12 and 8 kDa) when the larvae fed

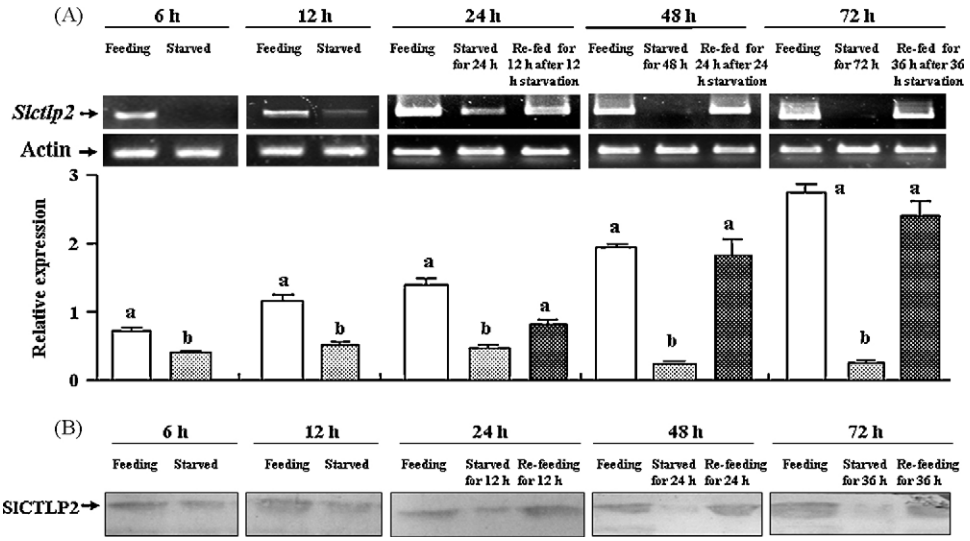

Fig. 11. RT-PCR (A) and western blot (B) analyses of induced expression of the *Slctlp2* mRNA by starvation and re-feeding. Larvae that just molted into the 6th instar stage were starved for 6, 12, 24, 48 and 72 h without feeding with artificial diet, or starved for 12, 24 and 36 h followed by re-feeding for 12, 24 and 36 h, respectively. The total RNA and proteins were extracted at 6, 12, 24, 48 and 72 h post 6th instat ecdysis and analyzed by RT-PCR (A) and western blot (B) analyses, respectively. Actin expression was used as control for equal use of RNA. The size of the PCR products is 0.9 kb. 30 μg proteins were used in each of the lanes. The size of SlCTLP2 protein is 30.6 kDa. The lower panel in (A) shows the quantitative analysis of the RT-PCR results from three independent repeats of the experiment. Significance of the differences were satisfied using ANOVA analysis followed by the Duncan's Multiple Comparison Test at $p < 0.05$. The same letters indicate no significant differences between the treatments, while the different letters indicate significant differences at $p < 0.05$.

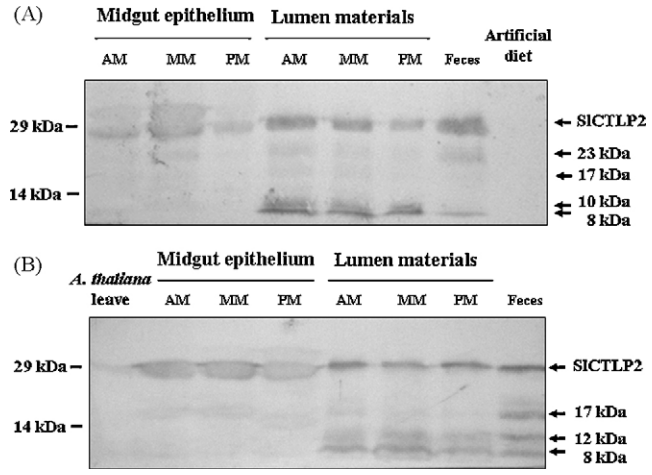

Fig. 12. Western blot analysis of SlCTLP2 protein in the epithelium of the anterior midgut (AM), middle midgut (MM) and posterior midgut (PM), lumen residues of the anterior, middle and posterior midgut, and the feces of the 6th instar larvae that fed on artificial diet (A) and *A. thaliana* leaves (B), respectively. Total proteins were extracted from the gut epithelium, lumen residues and feces, as well as the diets, after feeding on the diets for 72 h. Each of the lanes contained 30 μg proteins and hybridized with the anti-SlCTLP2 antibody at a dilution of 1:1000. The 29-kDa protein is proposed to be the mature enzymogen and those smaller protein bands detected in the lumen residues and feces are proposed to be the active forms or the degraded peptides of the SlCTLP2 protein.

on artificial diet (Fig. 12A) or three smaller fragments (approximate 17, 10 and 8 kDa) when the larvae fed on *A. thaliana* leaves (Fig. 12B), as immunologically detected by the antibody.

4. Discussion

In this study, cDNA (*Slctlp2*) of a chymotrypsin-like serine protein was cloned from *S. litura* and characterized in terms of cDNA structure, expression, localization and functional analysis. Sequence analysis revealed that this cDNA is a typical insect chymotrypsin and contained a highly conserved catalytic motif GDSGGPL (Figs. 1 and 3A). Identical sequence of this catalytic motif is also present in most of the lepidopteran chymotrypsin (Fig. 3A). However, this motif is changed to GDSGSAL in the *M. sexta* chymotrypsin-like serine protease (MsCTLP2-4) (CAM84317, CAM84318 and CAM84319, Broehan et al., 2008). Because of this GP/SA substitution, MsCTLP is proposed to play other roles in *M. sexta* rather than to be involved in digestion of dietary proteins (Herrero et al., 2005).

Several lines of evidence indicate that this gene is very likely associated with food protein digestion. First, RT-PCR, qPCR, northern blot and western blot analyses (Figs. 4–6) showed that the transcripts and protein of the gene was predominately expressed in the gut, particularly the anterior and middle midgut, during the larval feeding stages, whereas during the non-feeding molting and pupal stages, the expression of the transcripts and protein was decreased. This result indicates that expression of this gene coincided with the feeding function of the gut. Several studies reported that expression of the gut chymotrypsins that are involved in food protein digestion is highly parallel to feeding behaviour and gut-specific (Marshall et al., 2008; Broehan et al., 2008). The treatment with JH III, which can prolong the larval stage and feeding period (Riddiford, 2007), induced expression of the *Slctlp2* gene, whereas the treatment of 20E, which can initiate non-feeding, molting and metamorphosis, had no impact on the expression of the gene (Fig. 9). Up-regulation of a chymotrypsin gene, *JHA15*, by JH is also found in adults of the yellow fever mosquito *A. aegypti* (Bian et al., 2008). It would be interesting to know whether or not the treatment of JH induces the expression of the gene indirectly by prolonging the feeding stage or directly by acting at the regulation of the gene. Secondly, the results of *in situ* hybridization of *Slctlp2* mRNA (Fig. 7) and immunohistochemistry localization of SlCTLP2 protein (Fig. 8) indicated both mRNA and protein of the gene were specifically located in the epithelium of the foregut and midgut and the protein was also found in the lumen. This suggests that the mRNA was transcribed and the protein was translated in the midgut epithelium and secreted into the lumen. It has been suggested that food proteins are digested by chymotrypsin to peptides in the anterior midgut inside the endoperitrophic space and subsequently undergo further digestion in the ectoperitrophic space of the posterior midgut of *Lutzomyia longipalpis* (Fazito do Vale et al., 2007). In *Dermestes maculates*, it is suggested that protein digestion starts in anterior midgut and ends in the surface of posterior midgut cells (Caldeira et al., 2007). In *Stomoxys calcitrans* expression of two chymotrypsins occur predominantly in the opaque region responsible for secretion of digestive enzymes from the midgut (Lehane et al., 1998). The results of this study indicate that higher levels of the *Slctlp2* mRNA and protein were detected in the anterior and middle midgut than the posterior midgut (Figs. 4 and 12), although the lumen of the posterior midgut also contained the protein (Fig. 12). Thirdly, the expressions of both *Slctlp2* mRNA and protein were down-regulated by starvation but up-regulated again by re-feeding (Fig. 11), implying that it may be essential for food protein digestion. Similar results are also found in *M. sexta*, in which starvation suppressed the expression of a chymotrypsin-like serine protease MsCTLP2, which contains a GDSGGPL catalytic motif, whereas other chymotrypsins (MsCTLP1, MsCTLP3 and MsCTLP4) that contain an alterative GDSGSAL motif, were not significantly changed by starvation (Broehan et al., 2008). Fourthly, the protein sequence contains a signal peptide and a transmembrane prepropeptide, which is putatively cleaved after synthesis and secretion of the protein. Western blot analysis detected the presence of the protein in the food residues inside the lumen of the midgut and the feces of 6th instar larvae feeding on either artificial diet or the plant *A. thaliana* (Fig. 12), confirming that the protein was secreted into the gut lumen, where it may digest the dietary proteins. In the malaria mosquito *Anopheles gambiae*, after blood feeding, two chymotrypsin-like serine proteases, Anchym1 and Anchym2, are secreted into the midgut lumen where zymogens of the proteins are activated by partial tryptic digestion (Vizioli et al., 2001). In this study, in addition to the mature protein, several smaller peptides were also detected in the lumen residues and feces (Fig. 12), but not in the epithelial cells, although it is not clear yet whether or not these smaller peptides are the active forms of the enzyme or degraded products generated during the food digestion. It has been reported that a chymotrypsinogen can be cleaved by other trypsins and chymotrypsins through different catalytic processes, generating various active forms (Appel, 1986). Fifthly, numerous studies have reported that many insect serine proteases are involved in immune defense response (Jiang et al., 1998). In this study, three Gram-positive bacteria (*B. thuringiensis*, *B. subtilis*, *S. aureus*) and two Gram-negative bacteria (*E. coli* strains DH5α and K12D31) were used to inject 6th instar larvae at HCS stage. The expression of *Slctlp2* was not affected by injection of these tested bacterials (Fig. 10). This preliminary result indicates that this gene is unlikely associated with immune defence responses. In addition, no *Slctlp2* mRNA and protein were detected in the fat body and hemocytes (Figs. 4 and 7), where many of immune response-related serine proteases are synthesized (Zou et al., 2005).

In conclusion, the evidence derived from the experiments of temporal (for example, feeding-associated) and spatial (predominately in the anterior midgut and middle midgut) expression and regulated expression (up-regulated by feeding and re-feeding, as

well as JH, and down-regulated by starvation), localization and distribution of mRNA and protein (in the epithelial cells of the foregut and midgut, and secretion into the lumen residues and feces of the feeding larvae) suggests that this *S. litura Slctlp2* gene is most likely involved in food protein digestion in the gut of the insect during the larval feeding stage. Despite the evidence cited above, two other possible functions can not completely be ruled out in this study: first, it was proposed that a possible role for the *S. exigua* chymotrypsin protease SeCT34 is the proteolytic remodelling of the gut during the larval to pupal molt (Herrero et al., 2005). Secondly, a chymotrypsin-like serine protease (MsCTLP1) interacts with the chitin synthase in the midgut of *M. sexta* (Broehan et al., 2007). Further investigations will determine the potential protein substrates for SLCTLP2 and its proteolytic reaction mechanisms so that these possibilities can be clarified and application strategies for controlling this insect pest can be developed, for example, by blocking the activity of the gene and/or protein.

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