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Cloning, expression and characterization of four *serpin-1* cDNA variants from the spruce budworm, *Choristoneura fumiferana*

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ABSTRACT

Four cDNAs (*Cfserpin-1a*, *Cfserpin-1b*, *Cfserpin-1c* and *Cfserpin-1d*) of the *Choristoneura fumiferana* serpin-1 gene were cloned from an epidermis cDNA library. Analysis of the deduced amino acid sequences indicated that the cloned cDNAs encode four different proteins displaying identical *N*- but distinct *C*-termini, the latter region containing the inhibitory loop. The entire *CfSerpin-1* gene is transcribed while the variants are generated. Antibodies generated against the purified recombinant serpins cross-reacted with the other three. Each of the four *Cfserpin-1* cDNA variants was transcribed throughout larval development, from the 4th to the 6th instar, but transcript levels during the intermolt phases were generally higher than during the molting phase. The epidermis and fat body had higher levels of *Cfserpin-1* transcripts than the midgut. Cfserpin-1 proteins, detected with the Cfserpin-1a antibody, were found in the epidermis, midgut, fat body, plasma and molting fluid of 6th instar larvae and pre-pupae. Prepupal and pupal insects had higher levels of the proteins than the 6th instar feeding larvae, despite a drop in transcript levels. Cfserpin-1a could bind with the serine proteinase elastase and form a complex in vitro. We hypothesize that the cloned serpins could be involved in the regulation of cuticle degradation during the insect molting cycle.

1. Introduction

Serine protease inhibitors (serpins) form a superfamily of proteins that are capable of inhibiting the activity of serine proteinases (Potempa et al., 1994). Serpins are typically about 350–400 amino acid residues in length, with a reactive center loop near the carboxyl terminal end. Like mammalian serpins, members of the insect *serpin-1* gene family are mainly involved in the regulation of innate immune responses (Kanost et al., 2004; Gan et al., 2001; Christophides et al., 2002; Danielli et al., 2003). A well-studied example of serine protease-controlled immune response is the activation of prophenoloxidase (PPO) (Kanost et al., 2004; Jiang and Kanost, 2000). When insects suffer septic injury by pathogenic microorganisms, a series of hemolymph-borne serine proteinases is rapidly activated to ultimately convert the zymogen PPO into the active phenoloxidase (PO). PO catalyzes the formation of melanin at injury sites or around invading organisms, resulting in the melanization of invaders, as well as the production of toxic byproducts such as *O*-quinones. Serpins involved in the negative regulation of the PPO cascade during the innate immune response have been isolated from *Drosophila* (Scherfer et al., 2008) and *Manduca sexta* (Kanost et al., 2004; Jiang and Kanost, 2000).

Lepidopteran serpins, such as those of *Manduca sexta* and *Bombyx mori*, are highly similar in their *N*-terminal amino acid sequences, whereas their last 30–40 amino acid residues, which form the reactive center loop at the *C*-terminus, tend to differ (Jiang et al., 1994; Sasaki, 1991). The *Manduca sexta serpin-1* gene and its products have been well characterized in terms of structure, expression and physiological functions (Kanost et al., 1995; Jiang et al., 1996; Jiang and Kanost, 1997; Jiang et al., 2003). The *Manduca sexta* gene has 10 exons, among which exon 9 codes for the reactive center loop and has 12 alternate forms, generating 12 cDNA variants of the gene through alternative splicing (Jiang et al., 1996; Jiang and Kanost, 1997). In *Mamestra configurata*, the *serpin-1* gene also contains 10 exons but only 10 alternate splicing variants have been identified so far (Hegedus et al., 2008). The serpin reactive center loop binds to the active site of its serine proteinase substrate, forming a serpin–proteinase complex, which leads to a loss of proteinase activity. The specific amino acid residues of the reactive center loop usually determine the specificity towards substrates (Irving et al., 2000).

In the present study we report on the cloning and characterization of four cDNA variants (*Cfserpin-1a*, *Cfserpin-1b*, *Cfserpin-1c* and

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Cfserpin-1d) of the *serpin-1* gene from the spruce budworm, *Choristoneura fumiferana*. We compared their sequences and expression patterns and assessed their possible functions. Our data support earlier speculation by Hegedus et al. (2008) and Chamankhah et al. (2003) that insect serpin-1 isoforms may be involved in processes distinct from plasma coagulation and melanization during the innate immune response, namely the regulation of the activity of proteases in cuticle degradation during the molting cycle.

2. Materials and methods

2.1. Experimental animals

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) were reared at the Great Lake Forestry Centre (Sault Ste. Marie, Ontario, Canada). Third instar larvae were transferred onto a synthetic diet (McMorran, 1965) and reared at 22 °C, 70% relative humidity and under a photoperiodic cycle of 12-h light:12-h dark, until they reached the adult stage. To ensure accurate staging of later instars, 4th instar larvae were selected after the insects had undergone head capsule slippage for sampling.

To collect molting fluid samples, a small hole was made with a needle at the bottom of a 0.5 ml PCR tube, which was then inserted into a 1.5 ml microfuge tube containing 1.5–2.0 µl cold PBS (0.1 M, pH 8.0). Old integument that had just separated from prepupal larvae was collected and put in the 0.5 ml PCR tube, which was then centrifuged (12,000*g*) for 5 s at 4 °C. The resultant buffered molting fluid was collected in the 1.5 ml tube and kept at −20 °C until used for SDS-PAGE, western blotting analyses and antibody production.

2.2. Cloning and sequence analysis of Cfserpin-1 cDNAs

An expression cDNA library was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) using mRNA isolated from the epidermis of larvae molting from the 5th to the 6th instar. This library was screened with antibody produced against total proteins of the molting fluid as described below. The positive clones were isolated and the cDNA inserts were sequenced. Those cDNA clones whose partial sequence revealed high similarity to insect serpins were completely sequenced in both directions.

Annotation of sequences was performed using the National Center for Biotechnology Information BLAST search services (http://www.ncbi.nlm.nih.gov; Altschul et al., 1990). Multiple sequence alignment and phylogenetic tree analysis were performed using the Clustal W Alignment Program of DNASTAR (DNASTAR, Inc., Madison, WI) in MegAlign 5.01 version at a gap penalty of 10, a gap length penalty of 0.2, Gonnet series matrix of 250 and a bootstrap value at 10,000 times.

2.3. Production of recombinant Cfserpin-1 proteins

To produce protein in a bacterial expression system, the open reading frames (ORF) of the four *Cfserpin-1* cDNAs were amplified by PCR and inserted between the *Bam*H I and *Xho* I restriction sites of the pPROEXTM HT expression vector so as to place a hexahistidine tag on the *C*-terminal ends (Invitrogen, USA). The recombinant pPROEXTM HT-*Cfserpin-1* plasmids were used to transform DH10BTM *Escherichia coli* cells for protein expression *in vitro*. Protein expression was induced by adding IPTG at a final concentration of 1 mM. The cells containing the expressed recombinant proteins were homogenized by sonication and the protein inclusion bodies were isolated by centrifugation at 10,000×*g* for 20 min. The purified occlusion bodies were denatured with 8 M urea at 4 °C overnight, followed by refolding in a series of dialysis solutions of 4 M urea and 0.15 M NaCl for 2 h; 2 M urea and 0.15 M NaCl for 2 h; 0.15 M NaCl for 6 h using a D-Tube Dialyzer (Novagen, Canada).

2.4. Antibody production

Total proteins of the molting fluid from 6th instar larvae were used to produce a polyclonal antiserum by injecting a New Zealand rabbit (Cocalico Biologicals, Inc., Reamstown, PA, USA). The antiserum was collected after three booster injections, each with 0.3 mg of total molting fluid proteins.

To produce antibody against individual recombinant serpin proteins, the recombinant proteins were separated by SDS-PAGE and the target protein bands were excised from the gel. The protein was extracted and injected into New Zealand rabbits. The antiserum was collected after three booster injections, each containing 0.1 mg of the recombinant protein.

2.5. RNA isolation and northern blot analysis

Total RNA was isolated from larval tissues using the guanidinium isothiocyanate method (Chomyczynski and Sacchi, 1987). Total RNA (10 µg per lane) was separated on a 1.2% formaldehyde agarose gel and transferred onto nylon membrane. Variant-specific regions (nucleotides 1060–1411 for *Cfserpin-1a*; 1058–1394 for *Cfserpin-1b*; 1039–1334 for *Cfserpin-1c* and 1063–1496 for *Cfserpin-1d*) of *Cfserpin-1* cDNA were labeled with ^{32}P-dCTP using the RadPrime DNA Labeling System (Life Technologies, Burlington, Canada) and used as probes for northern blotting analysis. Pre-hybridization, hybridization and post-hybridization washes were carried out as described (Beliveau et al., 2000). The membranes were exposed to X-ray films at −75 °C.

2.6. Quantitative real-time PCR

Post-diapause 2nd instar larvae of the spruce budworm were reared on artificial diet and sampled at 26 different times during development, starting with 3rd instars at the time of head capsule slippage (L3HCS) and ending with day-7 pupae (PD7). Total RNA was extracted from a pool of 3–5 whole insects in three biological replicates, using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. The RNA was treated for 15 min with 1 U DNAse I (Invitrogen, USA) per µg of RNA. Reverse transcription was carried out for 50 min at 42 °C, in 20 µL 1X-RT buffer containing 0.5 µg of an oligo(dT)12-18 primer (Invitrogen, USA), 50 U SuperScript II

Table 1
Designations, oligonucleotide sequences, length, Tm and amplicon sizes for primers used in q-PCR quantification of four individual *Choristoneura fumiferana serpin-1* transcripts.

Specificity	Name	Sequence	Length (nt)	Tm (°C)	Amplicon size (bp)
Cfserpin-1a	Ser-1a F2	5′-CATCGCAGACCGTCAGTTTGTTTACCA-3′	27	70.2	92
	Ser-1a R4	5′-GCCTCAGCTTCAACAACTAATTTCGG-3′	26	68.0	
Cfserpin-1b	Ser-1b F3	5′-GACCACCGCCGCCTGTG-3′	17	72.5	189
	Ser-1b R3	5′-ACCCAATTACATTACTTGGTATGGACGTGTTGATGAA-3′	37	70.2	
Cfserpin-1c	Ser-1c F4	5′-GCAGCATTCGAGGAAACGCCA-3′	21	70.5	150
	Ser-1c R3	5′-CAAGCGAGTCGTCACATAAACTTTGCTTTATTGAATACT-3′	39	69.5	
Cfserpin-1d	Ser-1d F2	5′-GGACCGAAGCCGCCCAAA-3′	18	71.2	95
	Ser-1d R2	5′-GCAAAGACACCGCTGAATAACTTGTTGTCG-3′	30	70.8	

"F" and "R" in the primer names designate forward and reverse primers.

Table 2
Features of the four *Cfserpin-1* cDNA variants and of proteins they encode.

cDNA variants	Nucleotide length (bp)	No. of amino acids	Molecular mass (Da)	Isoelectric point (pI)
Cfserpin-1a	1410	394	44,261	5.23
Cfserpin-1b	1393	395	43,779	5.05
Cfserpin-1c	1333	394	43,854	4.92
Cfserpin-1d	1495	395	44,057	5.13

RNase reverse transcriptase and 2 μg total RNA. The cDNAs thus obtained were diluted in 10 mM, Tris–Hcl (pH 8.0) at a final concentration of 10 ng/μL.

qRT-PCR primer pairs for *Cfserpin-1a* to *Cfserpin*-1d cDNA were designed using the Oligo Explorer software (Version 1.2) and analyzed for dimer formation with Oligo Analyzer (Table 1). Alignments were done using Vector NTI to find primers specific for each serpin homolog. Specificity of the primers was verified by assessing the ability of each pair to amplify only one of the four *Cfserpin-1* cDNA variants. PCR amplifications were carried out on aliquots of individual RT reactions containing an amount of cDNA obtained from the conversion of 10 ng total RNA. Four replicate amplification reactions containing 500 nM of each primer were conducted for each biological replicate using a MX3000P spectrofluorometric thermal cycler (Stratagene, USA) and QuantiTectTM SYBR® Green PCR Kit (Qiagen, USA), initiated with a 15-min incubation at 95 °C, followed by a cycling regime of denatuation at 95 °C for 10 s; annealing and extension at 65 °C for 120 s. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Amplification efficiency was determined for each amplification reaction using linear regression of efficiency analysis. The number of target molecules was calculated using lambda genomic DNA as a quantitative standard (Rutledge and Stewart, 2008a,b).

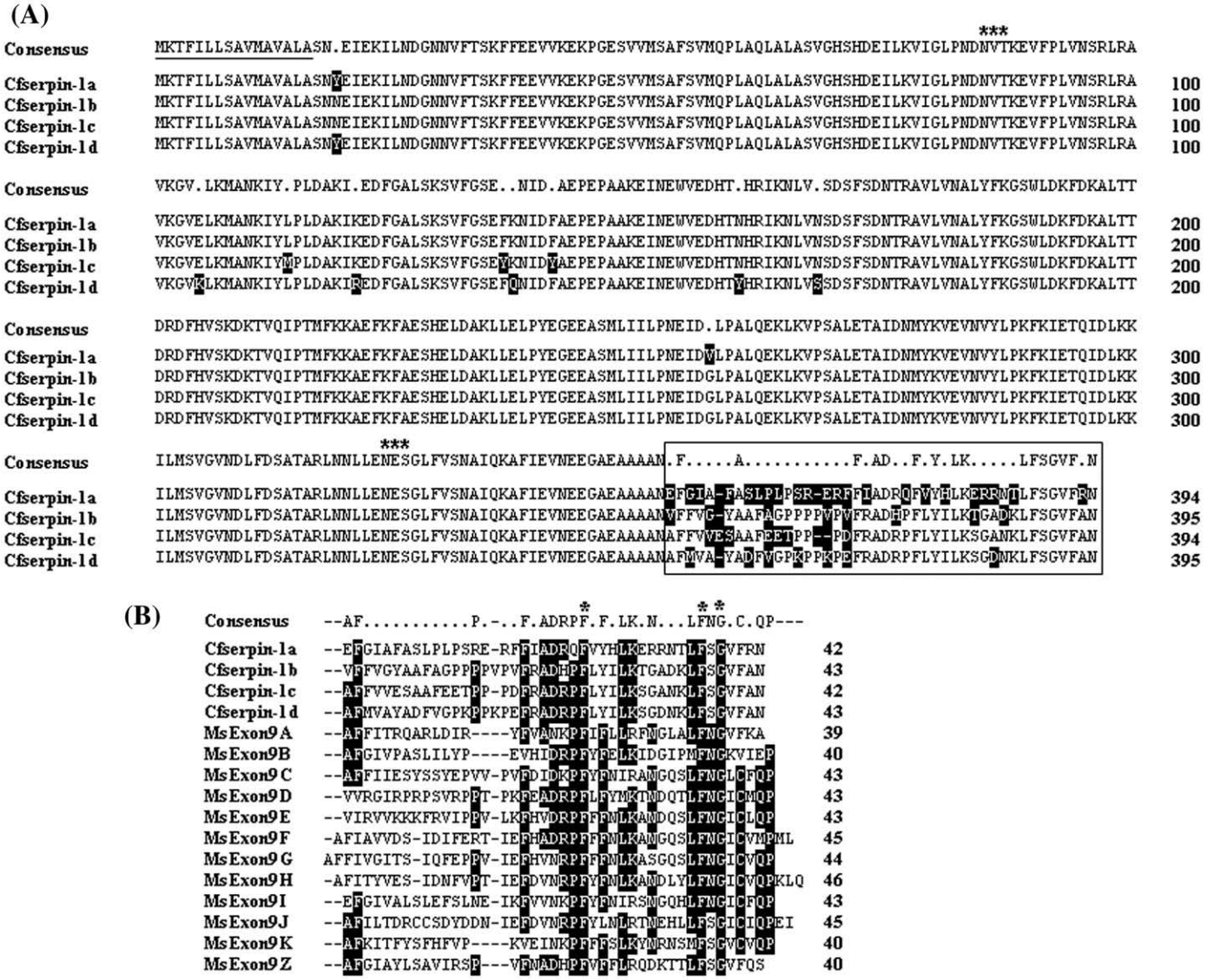

Fig. 1. (A) Alignment of the amino acid sequences of Cfserpin-1a, Cfserpin-1b, Cfserpin-1c and Cfserpin-1d, using Cluster W program in DNASTAR (DNASTAR, Inc., Madison, WI, USA). The signal peptide sequence is underlined. The reactive site loop region is boxed. Perfectly conserved residues that are the same in all of the four sequences are shown in the consensus at the top of the alignment, otherwise they are shown as ".". The amino acid residues that are different from those at the same positions of other sequences are shown in white letters over black background. GenBank accession numbers for these protein sequences are **ABW17155**, **ABW17156**, **ABW17157** and **ABW17158**, respectively. (B) Alignment of the *C*-terminal reactive site loops of Cfserpin-1 with those of the exon 9 isoforms of *Manduca sexta* (Jiang et al., 1996) and *Mamestra configurata serpin-1* genes (Hegedus et al., 2008). The amino acid residues that match the conserved residues (at least 13 out of 25 sequences) are shown in white letters over black background and shown in the consensus at the top of the alignment, otherwise shown as ".". The amino acid residues that match in all of the sequences are indicated by "*". (C) Phylogenetic tree analysis of the Cfserpin-1 proteins with 70 homologues, which are most identical to the Cfserpin-1 proteins, from other insect species. The tree was made by using the Clustal W method at a gap penalty of 10 and a gap length penalty of 0.2 and bootstrap value at 10,000 times.

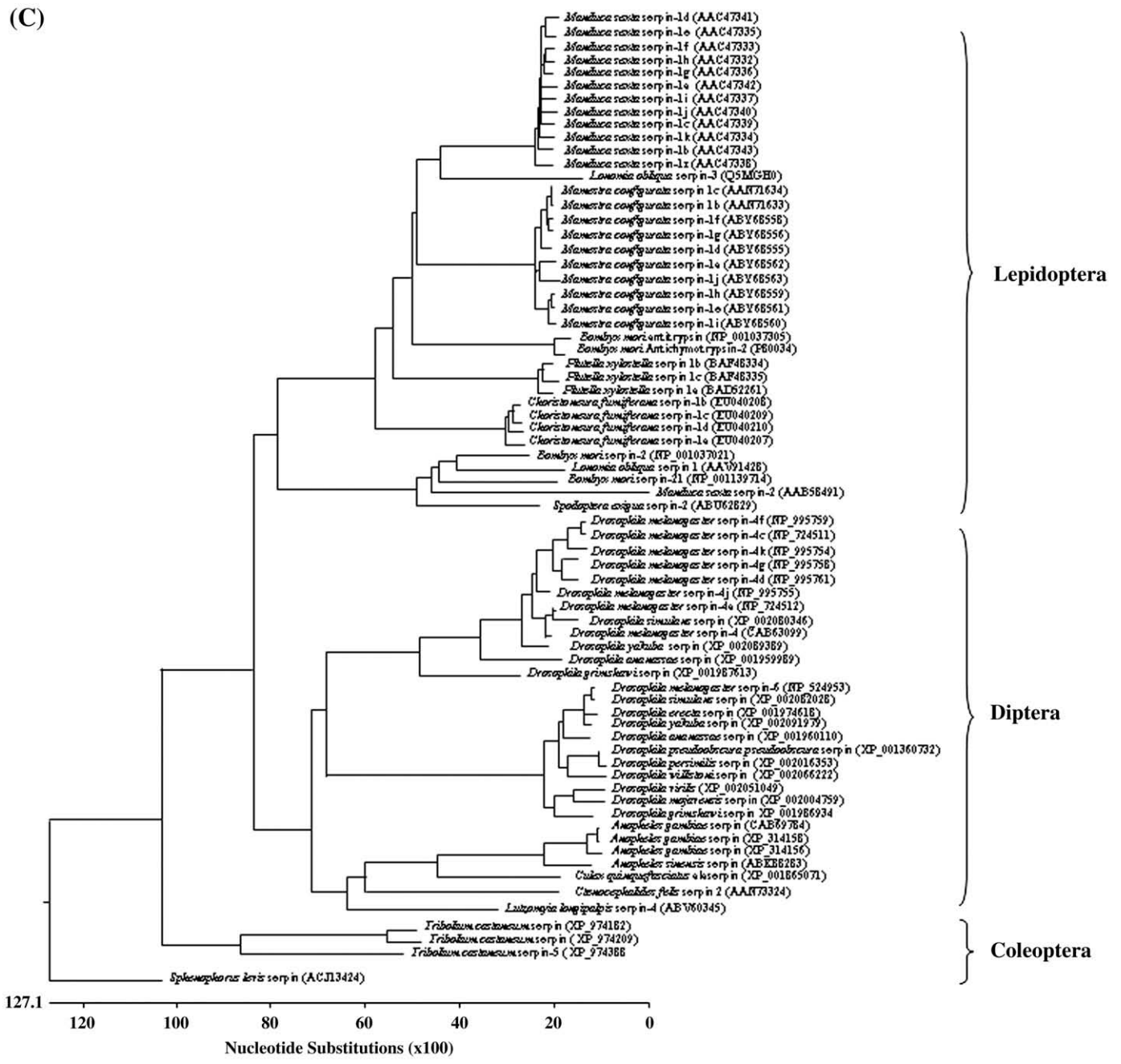

Fig. 1 (*continued*).

2.7. SDS-PAGE and western blot analysis

Protein samples were denatured at 100 °C for 3 min in an equal volume of 2× protein loading buffer (0.1 M Tris buffer, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 40% glycerol, and 0.002% bromophenol blue). SDS-PAGE was performed in 7.5% polyacrylamide gels, which were stained with Coomassie Brilliant Blue R-250 after electrophoresis. For western blotting analysis, proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane, which was then blocked with 3% bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS) for 30 min at room temperature, and incubated with the anti-Cfserpin-1 antibodies (1:2000) at room temperature for 1 h. Goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody at a dilution of 1:2000. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for color development.

2.8. Binding assay

Binding of the recombinant Cfserpin-1a protein, which was purified by a hexahistidine-tag affinity column, to elastase was assayed by incubating 2 μg of the Cfserpin-1a recombinant protein with 8 μg of porcine pancreatic elastase (Sigma, Canada) in TBS buffer (25 mM Tris, 50 mM NaCl, pH 8.0) at 22 °C for 5 min. The protein mixtures were then separated in 7.5% polyacrylamide native gel to determine whether protein complexes had formed.

3. Results

3.1. Cloning of Cfserpin-1 cDNAs and properties of the Cfserpin-1 proteins

An expression cDNA library constructed with mRNA isolated from the epidermis of larvae molting from the 5th to the 6th stadium was

screened using the antiserum raised against molting fluid proteins. Out of 9×10^4 colonies screened, 278 positive clones were identified. The inserts of these positive clones were partially sequenced. Thirteen clones (4.5% of the positive clones) were found to encode proteins displaying similarity to insect serpins. These clones were then completely sequenced. Four full-length cDNA variants closely related to the *Manduca sexta serpin-1* gene (Jiang et al., 1996), while the other nine clones encoded other serpins (data not shown). On the basis of their high levels of identity with the *Manduca sexta serpin-1* cDNAs, these four *serpin-1* cDNAs were named *Cfserpin-1a*, *Cfserpin-1b*, *Cfserpin-1c* and *Cfserpin-1d* (GenBank accession numbers: EU040207, EU040208, EU040209 and EU040210, respectively),. Table 2 summarizes the length of the nucleotide and amino acid sequences of the ORFs, as well as molecular mass and isoelectric points (pI) of these four *Cfserpin-1* cDNAs. A 16 amino acid signal peptide was predicted for all four deduced proteins using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004) (Fig. 1). Amino acid sequence alignment of the four *Cfserpin-1* variants revealed that they had almost identical *N*-termini and central regions, whereas they displayed different *C*-terminal ends, starting at residue 353 (Fig. 1A). The highly similar regions correspond to exons 1–8 of the *Manduca sexta* (Jiang et al., 1996) and *Mamestra configurata* (Hegedus et al., 2008) *serpin-1* genes. The *C*-terminal regions (i.e., the reactive loop region) of Cfserpin-1a and Cfserpin-1b proteins were most similar to that encoded by exon 9Z of the *Manduca sexta serpin-1* gene (ca 40% identity at the amino acid level; Fig. 1B). The *C*-terminal region of Cfserpin-1c protein was most similar to that encoded by exon 9G of the *Mamestra configurata* serpin-1 gene (46% identity) and the *C*-terminal region of Cfserpin-1d was most similar to that encoded by exon 9D of the *Mamestra configurata serpin-1* gene (37% identity). Only three amino acid residues (Phe, Phe and Gly) within the reactive center loop were identical among all *Choristoneura fumiferana*, *Manduca sexta* and *Mamestra configurata serpin-1* proteins, although a few others were common to a majority of serpins (Fig. 1B).

Phylogenetic tree analysis of the Cfserpin-1 proteins with 70 homologues from other insect species revealed three groups: the Lepidoptera group, including the proteins from *Choristoneura fumiferana* (this study), *Manduca sexta* (Jiang et al., 1996), *Mamestra configurata* (Hegedus et al., 2008), *B. mori* (Sasaki, 1991), *Plutella xylostella* (Song et al., 2008), *Lonomia oblique* (Veiga et al., 2005), and *Spodoptera exigua*, was separated from the Diptera group, which included *Drosophila*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Ctenocephalides felis* and *Lutzomyia longipalpis*, and the Coleoptera group, which included *Tribolium castaneum* and *Sphenophorus levis* (Fig. 1C). It appears that the different isoforms of lepidopteran serpin-1 proteins are paralogues diverged from gene duplication or alternative splicing within a species after speciation, because these isoforms were grouped into the separate species clusters. The selected serpins of Diptera and Coleoptera for phylogenetic tree analysis were the most homologous to the Cfserpin-1 proteins, but it is not clear yet if they can be classified into the *serpin-1* gene family, although they do have the similar conserved domains to the serpin-1 proteins and share about 30–40% identities to the lepidopteran serpin-1 proteins. Additionally, they appeared not to have as many as paralogues as the lepidopteran *serpin-1* genes.

3.2. In vitro production of recombinant Cfserpin-1

All four *Cfserpin-1* variant proteins were produced as hexahistidine-tagged fusion proteins in *E. coli* expression system (Fig. 2A). The recombinant proteins were produced as protein inclusion bodies (Fig. 2B). The apparent molecular mass of the recombinant proteins, as assessed by SDS-PAGE, was approximately 45 kDa, close to the calculated size based on the deduced amino acid sequences. The antibody generated against individual recombinant Cfserpin-1 proteins cross-reacted with each of the other three recombinant serpins (Fig. 2D). In addition, the anti-Cfserpin-1b antibody showed cross-reactivity with an unknown high molecular-mass protein of bacterial origin (Fig. 2Db).

3.3. Assessment of Cfserpin-1 transcript and protein abundance in vivo

Quantitative PCR assessment of transcript abundance was conducted for the four *Cfserpin-1* variants as a function of developmental stage (Fig. 3A). Absolute amounts of transcripts varied widely among the four *Cfserpin-1* cDNA variants, with *Cfserpin-1a* being the most

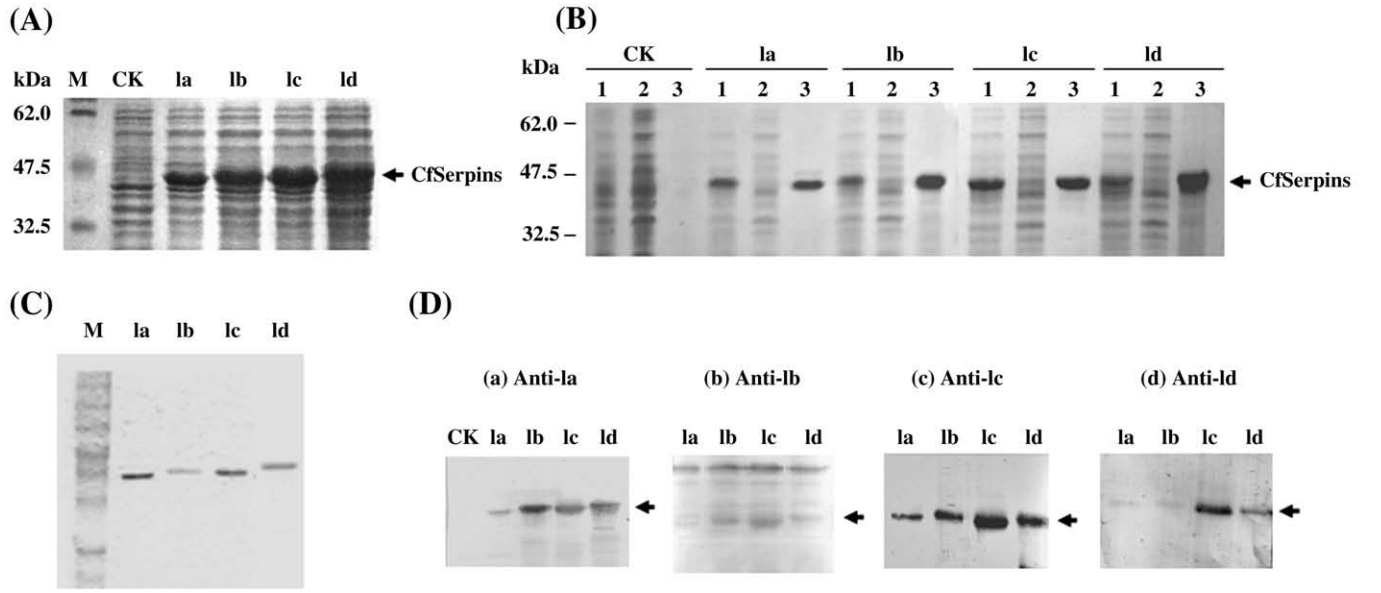

Fig. 2. SDS-PAGE analysis of Cfserpin-1a, Cfserpin-1b, Cfserpin-1c and Cfserpin-1d proteins produced *in vitro*. (A) Total proteins from the transformed *E. coli* cells; (B) protein separation after centrifugation to pellet the protein occlusion bodies. Lane 1: total proteins from the cell lysate; Lane 2: supernatant fraction of the cell lysate; Lane 3: the pellet fraction. (C) Purified and refolded proteins, which were used for the protein binding activity assays. (D) Western blot analysis of the specificity of anti-Cfserpin-1 antibodies. (a–d) are for anti-Cfserpin-1a, anti-Cfserpin-1b, anti-Cfserpin-1c and anti-Cfserpin-1d antibodies, respectively. M: protein mass marker; CK: control, the proteins isolated from the bacteria transformed with the pPROEXTM HT empty vector alone. 1a–1d: the proteins from the bacteria transformed with Cfserpin-1a, Cfserpin-1b, Cfserpin-1c and Cfserpin-1d, respectively.

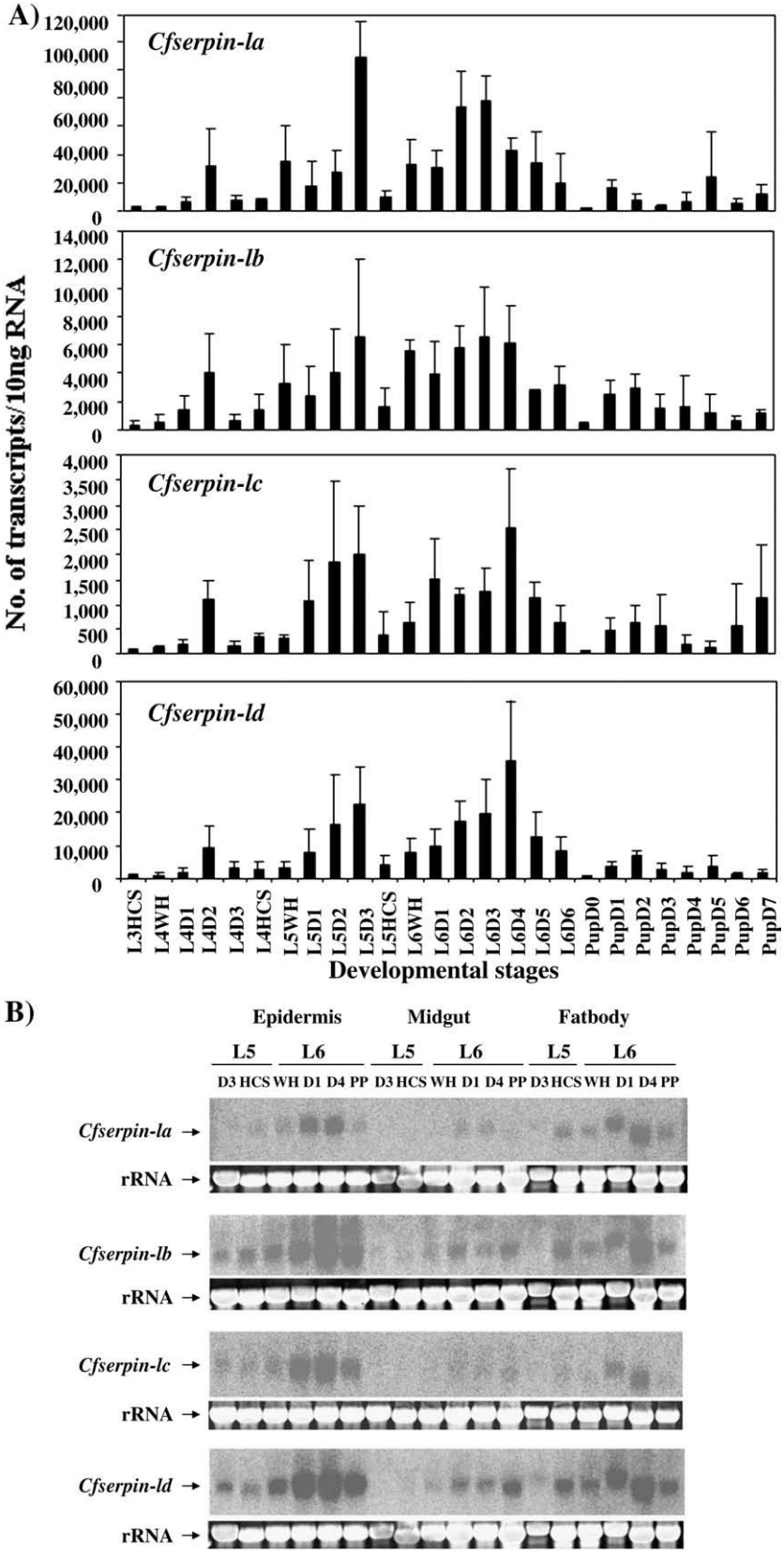

Fig. 3. (A) Quantitative RT-PCR analysis of the transcripts of *Cfserpin-1a*, *Cfserpin-1b*, *Cfserpin-1c* and *Cfserpin-1d* of *Choristoneura fumiferana*. Numbers of transcripts per 10 ng total RNA for each of the four *Cfserpin-1* variants were measured by q-PCR from whole-body extracts made at 26 different developmental stages, from 3rd instar (L3) at head capsule slippage (HCS) to the 7th day (D7) of the pupal stage (Pup). Each value is the mean of four technical replicates for each of three biological replicates. WH: white head stage. (B) Northern blotting analysis of the tissue-specific expression of *Cfserpin-1a*, *Cfserpin-1b*, *Cfserpin-1c* and *Cfserpin-1d* in the epidermis, midgut and fat body during 5th and 6th instar larval stages. L: larval stage; D: day; HCS: head capsule slippage. WH: white head. PP: Pre-pupae. Stained rRNA indicates equal loading of 10 μg of total RNA in each of lanes.

abundant, followed by *Cfserpin-1d*, *Cfserpin-1b* and *Cfserpin-1c*. The transcriptional profiles revealed a tendency for transcripts to reach a maximal level during the intermolt phase and to reach a minimum level just before the molt (head capsule slippage; HCS) and/or just after (white head or pupa D0) (Fig. 3A). The levels of the transcripts gradually increased after the molts and decreased again when the animals started larval molting (e.g. L5HCS) or entered prepupal stage (e.g. L6D5–L6D6). This trend was particularly striking for *Cfserpins-1b*, *-1c* and *-1d*, whose transcriptional profiles were more similar to one another than to *Cfserpins-1a*, which displayed an earlier increase in the transcript levels after the molts (e.g. L6D1–D2) and earlier decrease before the prepupal phase (e.g. L6D4) (Fig. 3A).

Tissue-specific abundance of the serpin transcripts during 5th and 6th instar stages was examined by northern blot analysis (Fig. 3B). All four transcript variants displayed higher abundance in the epidermis and fat body than in the midgut, particularly after ecdysis to the 6th instar, followed by a decrease at the prepupal stage. The results by northern blot analysis confirmed the tendency of the expression changes of the transcripts detected by qRT-PCR analysis, except that *Cfserpins-1a* abundance was higher than the other variants in qRT-PCR analysis.

When the anti-Cfserpin-1a antibody was used to examine protein expression during the 6th larval stadium and pupal stage, two protein bands (41 and 42 kDa) were detected and protein expression levels increased after ecdysis to the 6th instar, reaching its highest level in pre-pupae and pupae (Fig. 4A). Serpin proteins were detected in the epidermis, midgut and fat body of the 4-day-old 6th instar larvae (L6D4) and pre-pupae (PP) (Fig. 4B). In the hemolymph, the proteins were mainly present in the plasma, with only trace levels of protein detected in the hemocytes at both stages (Fig. 4C and D). In the plasma of the L6D4 larvae, which were actively feeding, the antibody cross-reacted with a high molecular mass protein (Fig. 4C). In the molting fluid collected from the 6th instars molting into to pupae, two major bands of 41 and 42 kDa, the sizes similar to those observed in whole larvae, were detected (Fig. 4A and E). They likely represent differentially glycosylated forms of the protein, as shown in *Mamestra configurata* by Chamankhah et al. (2003).

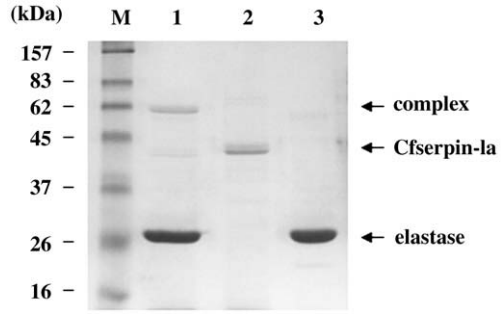

Fig. 5. Binding assay of Cfserpin-1a protein to porcine pancreatic elastase. The purified Cfserpin-1a protein was incubated with porcine pancreatic elastase in TBS buffer (25 mM Tris, 50 mM NaCl, pH8.0) at 22 °C for 5 min. The protein mixtures were then electrophoresed a 7.5 % of native gel.

3.4. Binding of Cfserpin-1a to elastase

To examine whether or not the Cfserpin-1a protein interacts with any proteins in regulating cuticle degradation, several serine proteinases, including trypsin, chymotrypsin and elastase, were assessed for their binding activity with *Cf*serpin-1a protein under *in vitro* conditions. We found that the Cfserpin-1a protein could bind with porcine pancreatic elastase, a serine proteinase capable of breaching the insect cuticle (Segers et al., 1995), to form a protein complex (Fig. 5). This result provided another piece of evidence that Cfserpin-1a might play a role in cuticle degradation by interacting with serine proteinase in the integument. Whether or not elastase is actually present in the integument or the molting fluid and how serine proteinases interact with Cfserpin-1 proteins *in vivo* need to be further investigated.

4. Discussion

Insect serpins have been shown to be involved in the melanization cascade during the innate immune response (Kanost et al., 2004).

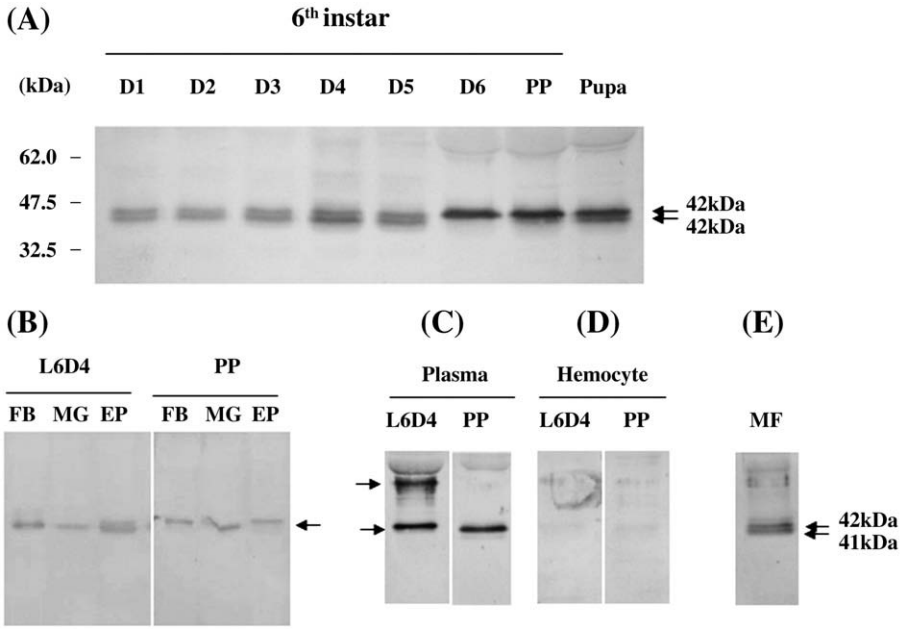

Fig. 4. Western blotting analysis of Cfserpin-1a, Cfserpin-1b, Cfserpin-1c and Cfserpin-1d proteins in whole insects (A), the epidermis, midgut and fat bodies (B), hemolymph (C and D) and molting fluid (E) of 6th instar larvae and pupae. L6D4: day 4 of 6th instar larvae; PP: pre-pupae: MF: molting fluid. The arrows indicate the main protein bands that were immunologically recognized by the anti-Cfserpin-1a antibodies.

Insects respond to injury or invasion by microorganism by activating prophenoloxidase through the action of a serine proteinase. The prophenoloxidase hydroxylates monophenols to become *O*-quinones via *O*-diphenols. Quinones polymerize to form melanin at the injury site or around the invading organisms to kill the invaders. Serpins have been shown to regulate the prophenoloxidase activation pathway (Jiang et al., 2003; Tong and Kanost, 2005).

In addition to their roles in the innate immune response, insect proteases have been suggested to be involved in cuticular protein degradation (Samuels and Paterson, 1995; Chamankhah et al., 2003; Hegedus et al., 2008). Insect cuticle is composed mainly of microfibers of chitin embedded in a protein matrix. Several proteases identified from insect molting fluid possess proteolytic activity and may contribute to degradation of the old cuticle during the molting process (Brookhart and Kramer, 1990). Samuels et al. (1993a) identified a cuticle degrading trypsin-like protease (MFP-1) from the molting fluid of *Manduca sexta*, which degraded cuticle proteins *in vitro* into 200–2000 Da peptides, which were probably further degraded by an aminopeptidase (MFP-2) in the molting fluid (Samuels et al., 1993b). These results supported the hypothesis that proteases are involved in degradation of the old cuticle. In this study, we cloned four cDNA variants of the *Choristoneura fumiferana* serpin-1 gene and studied their expression at RNA and protein levels to assess their likely role(s) during larval development and the larval–pupal molt. Because these cDNAs were isolated from an epidermis expression library screened using a mixture of antibodies generated against a mixture of molting fluid proteins, we hypothesized that these serpins may be involved in regulating cuticle degradation in the epidermis during the molting cycle. During the molting phase, lower levels of serpins allow serine proteinases to hydrolyze cuticular proteins so that the old cuticle can separate from the epithelial cells. After the insect molts and enters the intermolt phase, the increased level of serpins suppresses the action of serine proteinases, allowing the new cuticle to take shape. Additional evidence that supports this hypothesis is that although the cDNA variants of *Cfserpin-1* were expressed at most sampling time points, higher levels of transcripts were detected during the larval intermolt phases (Fig. 3). This was most obvious in the epidermis of 6th instar larvae undergoing a molt or during the 5th to 6th intermolt phase. Similar expression patterns were found in *Mamestra configurata* (Chamankhah et al., 2003) and *Manduca sexta* (Kanost et al., 1995). In *Mamestra configurata*, serpin-1 isoforms, as a group, were expressed in all of the developmental stages, with higher levels during the larval intermolt stages than during the molts, Serine protease activity in the hemolymph increased dramatically at the onset of the molt concomitant with a sharp decline in serpin gene expression (Chamankhah et al., 2003). Wounding and inoculation with bacteria did not induce the *Mamestra configurata serpin-1* gene expression but did lead to the appearance of different molecular weight forms. In *Manduca sexta*, the mRNA of the serpin-1 gene was abundant in the fat body of feeding 4th and 5th instar larvae, but disappeared abruptly at molts and the wandering stage (Kanost et al., 1995). The levels of serpin-1 proteins in the hemolymph were correlated with the abundance of mRNA in the fat body and 20-hydroxyecdysone down-regulated the expression of *Manduca sexta serpin-1* gene (Kanost et al., 1995). These results from different species indicate that the expression of the *serpin-1* gene fluctuates with the molting cycles. Another supporting evidence for the role of *Cfserpin-1* in cuticle metabolism comes from the results of the elastase-binding assay, which showed that Cfserpin-1 protein could bind to elastase (Fig. 5). Elastase is a serine proteinase that is capable of degrading cuticles in insects (Segers et al., 1995). Among the clones retrieved from the expression cDNA library of *Choristoneura fumiferana*, a number of serine proteases have been identified (unpublished data). It will be necessary to functionally test the inhibitory property of Cfserpin-1 proteins against these endogenous serine proteases and test their ability to form complexes as is the case with elastase.

Correlation between *Cfserpin-1* transcript accumulation and protein abundance is not consistent throughout development. This seems to be in conflict with the above evidence. For example, the mRNA transcripts were found to be most abundant during the 4th to 6th instar intermolts and decreased before and during ecdysis (e.g. prepupal stage, Fig. 3), but the proteins detected using anti-Cfserpin-1a antibody persisted in the tissues for a longer time than the transcripts and were at higher levels during the larval to pupal transition until the pupal stage (Fig. 4). This is in conflict with the above hypothesis that lower levels of serpins allow serine proteinases to hydrolyze cuticular proteins during the molting phase. In *Mamestra configurata*, proteinase activity goes up at the onset of the molt, which may suggest a drop in serpin levels (Chamankhah et al., 2003; Hegedus et al., 2008). Although discrepancies between transcript and protein levels are not uncommon, it should be further demonstrated whether the serpin-1 proteins are present at high levels during the prepupal stage, before the above hypothesis can be logically explained.

In *Mamestra configurata* larvae, the expression of the serpin-1 transcripts was restricted to the cuticle-associated remnants after removal of organs (carcass), fat body, foregut and hemocytes, while the proteins were found in the sub-cuticular layer, outside the muscle basement membrane, fat body and the hemolymph (Chamankhah et al., 2003). In *Manduca sexta*, the *serpin-1* gene was expressed at a high level in larval fat body and at a lower abundance in hemocytes (Kanost et al., 1995). In this study, the Cfserpin-1 proteins were detected in the midgut, fat body, epidermis, plasma of the hemolymph and molting fluid (Fig. 4B–E), indicating that the protein isoforms may be distributed to all the tissues after synthesis. Although the localization of serpin-1 proteins in different tissues and organs of various species needs to be further clarified, the distribution of the serpin-1 proteins in multiple tissues or organs of these species may imply that these proteins have pleiotropic functions.

The *N*-termini and central regions of the four cDNA variants of *Cfserpin-1* gene were highly similar to the corresponding regions (encoded by exons 1–8) of the *Manduca sexta* (Jiang et al., 1996) and *Mamestra configurata* (Hegedus et al., 2008) *serpin-1* genes, whereas the reactive center loop region found between residues 353 and 394 (or 395) of the *C*-termini were more divergent in sequences (Fig. 1B). The reactive loop regions of Cfserpin-1a and Cfserpin-1b were most similar to the exon 9Z of the *Manduca sexta serpin 1* gene and the *C*-terminal regions of Cfserpin-1c and Cfserpin-1d were most similar to the Exon 9G and 9D of the *Mamestra configurata serpin-1* gene, respectively. The different reactive center loop sequences of these *Cfserpin-1* variants are from alternative splicing of the transcripts of the exons 9 of the gene, as observed in the *Manduca sexta* and *Mamestra configurata serpin-1* genes (Jiang et al., 1996; Hegedus et al., 2008). In any case, differences among these cDNA variants may provide a mechanism for the gene products to regulate different serine proteinases in various processes or tissues. In addition, it remains to be known whether or not there are only four *Cfserpin-1* cDNA variants in *Choristoneura fumiferana*. The *Manduca sexta* serpin-1 gene generates 12 transcript variants forms through alternative splicing (Jiang et al., 1996; Jiang and Kanost, 1997). In *Mamestra configurata* 10 alternative splicing variants of *serpin-1* gene have been identified (Hegedus et al., 2008). Different numbers of the transcript variants of the *serpin-1* gene may provide another mechanism for the gene to play multiple functions in regulation of different serine proteinases in various processes.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Beliveau, C., Laforge, M., Cusson, M., Bellemare, G., 2000. Expression of a Tranosema rostrale polydnavirus gene in the spruce budworm, *Choristoneura fumiferana*. J. Gen. Virol. 81, 1871–1880.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Brookhart, G.L., Kramer, K.J., 1990. Proteinases in molting fluid of the tobacco hornworm, *Manduca sexta*. Insect Biochem. 20, 467–477.

Chamankhah, M., Braun, L., Visal-Shah, S., O'Grady, M., Baldwin, D., Shi, X., Hemmingsen, S.M., Alting-Mees, M., Hegedus, D.D., 2003. *Mamestra configurata* serpin-1 homologues: implications for a regulatory role for serpins in molting. Insect Biochem. Mol. Biol. 33, 355–369.

Chomyczynski, P., Sacchi, N., 1987. Methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N.T., Hoffmann, J.A., Kanzok, S.M., Letunic, I., Levashina, E.A., Loukeris, T.G., Lycett, G., Meister, S., Michel, K., Moita, L.F., Muller, H.M., Osta, M.A., Paskewitz, S.M., Reichhart, J.M., Rzhetsky, A., Troxler, L., Vernick, K.D., Vlachou, D., Volz, J., von Mering, C., Xu, J., Zheng, L., Bork, P., Kafatos, F.C., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. Science 298, 159–165.

Danielli, A., Kafatos, F.C., Loukeris, T.G., 2003. Cloning and characterization of four *Anopheles gambiae* serpin isoforms, differentially induced in the midgut by *Plasmodium berghei* invasion. J. Biol. Chem. 278, 4184–4193.

Gan, H., Wang, Y., Jiang, H., Mita, K., Kanost, M.R., 2001. A bacteria-induced, intracellular serpin in granular hemocytes of *Manduca sexta*. Insect Biochem. Mol. Biol. 31, 887–898.

Hegedus, D.D., Erlandson, M., Baldwin, D., Hou, X., Chamankhah, M., 2008. Differential expansion and evolution of the exon family encoding the serpin-1 reactive centre loop has resulted in divergent serpin repertoires among the Lepidoptera. Gene 418, 15–21.

Irving, J.A., Pike, R.N., Lesk, A.M., Whisstock, J.C., 2000. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res. 10, 1845–1864.

Jiang, H.B., Kanost, M.R., 1997. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from Manduca sexta. J. Biol. Chem. 272, 1082–1087.

Jiang, H., Kanost, M.R., 2000. The clip-domain family of serine proteinases in arthropods. Insect Biochem. Mol. Biol. 30, 95–105.

Jiang, H., Wang, Y., Kanost, M.R., 1994. Mutually exclusive exon use and reactive center diversity in insect serpins. J. Biol. Chem. 269, 55–58.

Jiang, H., Wang, Y., Huang, Y., Mulnix, A.B., Kadel, J., Cole, K., Kanost, M.R., 1996. Organization of serpin gene-1 from *Manduca sexta*. Evolution of a family of alternate exons encoding the reactive site loop. J. Biol. Chem. 271, 28017–28023.

Jiang, H., Wang, Y., Yu, X.Q., Zhu, Y., Kanost, M., 2003. Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. Insect Biochem. Mol. Biol. 33, 1049–1060.

Kanost, M.R., Prasad, S.V., Huang, Y.L., Willott, E., 1995. Regulation of serpin gene-1 in Manduca sexta. Insect. Biochem. Mol. Biol. 25, 285–291.

Kanost, M.R., Jiang, H., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol. Rev. 198, 97–105.

McMorran, A., 1965. A synthetic diet for the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Can. Entomol. 97, 58–62.

Potempa, J., Korzus, E., Travis, J., 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J. Biol. Chem. 269, 15957–15960.

Rutledge, R.G., Stewart, D., 2008a. A kinetic-based sigmoidal model for the polymerase chain reaction and its application to high-capacity absolute quantitative real-time PCR. BMC Biotechnol. 8, 47.

Rutledge, R.G., Stewart, D., 2008b. Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. BMC Mol. Biol. 9, 96.

Samuels, R.I., Paterson, I.C., 1995. Cuticle degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. Comp. Biochem. Physiol. 110B, 661–669.

Samuels, R.I., Charnley, A.K., Reynolds, S.E., 1993a. A cuticle degrading proteinase from the moulting fluid of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 23, 607–614.

Samuels, R.I., Charnley, A.K., Reynolds, S.E., 1993b. An aminopeptidase from the moulting fluid of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 23, 615–620.

Sasaki, T., 1991. Patchwork-structure serpins from silkworm (*Bombyx mori*) larval hemolymph. Eur. J. Biochem. 202, 255–261.

Scherfer, C., Tang, H., Kambris, Z., Lhocine, N., Hashimoto, C., Lemaitre, B., 2008. Drosophila Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. Dev. Biol. 323, 189–196.

Segers, R., Butt, T.M., Keen, J.N., Kerry, B.R., Peberdy, J.F., 1995. The subtilisins of the invertebrate mycopathogens *Verticillium chlamydosporium* and *Metarhizium anisopliae* are serologically and functionally related. FEMS Microbiol. Lett. 126, 227–231.

Song, K., Jung, M., Eum, J., Hwang, I., Han, S., 2008. Proteomic analysis of parasitized *Plutella xylostella* larvae plasma. J. Insect Physiol. 54, 1271–1280.

Tong, Y., Kanost, M.R., 2005. *Manduca sexta* serpin-4 and serpin-5 inhibit the prophenol oxidase activation pathway: cDNA cloning, protein expression, and characterization. J. Biol. Chem. 280, 14923–14931.

Veiga, A.B., Ribeiro, J.M., Guimaraes, J.A., Francischetti, I.M., 2005. A catalog for the transcripts from the venomous structures of the caterpillar *Lonomia obliqua*: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome. Gene 355, 11–27.