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A LIM Protein Gene Homologue of *Protaetia brevitarsis*: cDNA Cloning and mRNA Expression

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A LIM protein gene homologue of the CRP (cysteine-rich protein) family in the whiter-spotted flower chafer, *Protaetia brevitarsis*, was cloned. The *P. brevitarsis* LIM protein cDNA encodes a 92 amino acid polypeptide with a predicted molecular mass of 10,030 Da and a pI of 8.57. The *P. brevitarsis* LIM protein contains the cysteine-rich consensus sequence of LIM domain and the glycine-rich consensus sequence observed in the cysteine-rich protein family 1 (CRP1). The potential nuclear targeting signal is retained. The deduced amino acid sequence of the *P. brevitarsis* LIM protein cDNA showed 92% identity to another beetle, *Apriona germari* LIM protein. Northern blot analysis showed that *P. brevitarsis* LIM protein is highly expressed in epidermis and midgut, but not in the fat body.

Key words: cDNA cloning, Insect, LIM protein, *Protaetia brevitarsis*

Introduction

The LIM domain defines a zinc-binding motif present in single or multiple copies in a wide variety of eukaryotic proteins that regulate cell growth and differentiation during development (Sadler *et al.*, 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995). The LIM motif was first identified in three developmentally regulated transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* Mec-3, from which the name LIM was derived (Way and Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990). The LIM motif is defined by a cysteine-rich consensus sequence (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Sadler *et al.*, 1992) and LIM domain proteins are divided into three evolutionarily conserved members of the CRP (cysteine-rich protein) family, CRP1, CRP2, and MLP (muscle LIM protein)/CRP3 (Liebhaber *et al.*, 1990; Sadler *et al.*, 1992; Weiskirchen and Bister, 1993; Arber *et al.*, 1994; Crawford *et al.*, 1994; Weiskirchen *et al.*, 1995). Members of CRP family of LIM proteins have been implicated in muscle differentiation (Arber *et al.*, 1994).

In insects, LIM protein genes have been isolated from several insect species such as Diptera, *Drosophila melanogaster* (Stronach *et al.*, 1996), Coleoptera, *Apriona germari* (Gui *et al.*, 2004), and Lepidoptera, *Epiblema scudderiana* (Bilgen *et al.*, 2001) and *Bombyx mori* (Hwang *et al.*, 2004). Two muscle-specific LIM proteins of the CRP family in *D. melanogaster*, referred to as muscle LIM proteins (Mlp) were identified and characterized: *Mlp60* encodes a protein with a single LIM domain linked to a glycine-rich region and *Mlp84B* with five tandem LIM-glycine modules (Stronach *et al.*, 1996). The temporal expression and spatial distribution of muscle LIM proteins in *Drosophila* are consistent with a role for Mlps in myogenesis, late in the differentiation pathway (Stronach *et al.*, 1996). Muscle LIM proteins are associated with muscle sarcomers and require dMEF2 (myocyte enhancer-binding 2 protein) for their expression during *Drosophila* myogenesis (Stronach *et al.*, 1999). In the goldenrod gall moth, *E. scudderiana*, furthermore, cold up-regulation and its pattern of *E. scudderiana Mlp* in the larvae suggest possible roles for the protein, such as in muscle maintenance over the winter or as a preparative function that could facilitate the rapid resumption of development and metamorphosis when environmental

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temperatures rise in the spring (Bilgen *et al.*, 2001).

The purpose of the present study was to elucidate the LIM protein gene in the whiter-spotted flower chafer, *Protaetia brevitarsis* (Coleoptera: Cetoniidae). In this paper, we reported the cDNA cloning and mRNA expression of the *P. brevitarsis* LIM protein gene.

Materials and Methods

Insects

The larvae of the white-spotted flower chafer, *P. brevitarsis*, were maintained at 28°C, 70% relative humidity, and photoperiod of 16 L : 8 D (Kim *et al.*, 2002) and final instar larvae were used for the experiment.

cDNA library screening, nucleotide sequencing and data analysis

A cDNA library constructed using whole bodies of *P. brevitarsis* larvae was used in this study. The clones harboring cDNA inserts were randomly selected and sequenced to generate the expressed sequence tags (ESTs). The plasmid DNA was extracted by Wizard miniprep kit (Promega, Madison, WI). The nucleotide sequence was determined by using a BigDyeTerminator cycle sequencing kit and an automated DNA sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program. MacVector (ver. 6.5, Oxford Molecular Ltd) was used to align the amino acid sequences of LIM protein.

RNA isolation and Northern blot analysis

The *P. brevitarsis* larva was dissected under the Stereomicroscope (Zeiss, Jena, Germany), individual samples such as midgut, fat body, and epidermis were harvested, and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the midgut, fat body, and epidermis of the larva by using the Total RNA Extraction Kit (Promega). Total RNA (10 μg/lane) from *P. brevitarsis* was denatured by glyoxalation (McMaster and Carmicharl, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The 800 bp LIM protein cDNA clone was labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA) for use as a probe for hybridization. After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and exposed to autoradiography film.

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-135                                                    gcacgaggggttgtc
     agtgacgggaagaactcgagtcccgaatcatctcaatcaaaaccatctggaagttgcatt
     ccggacgtcactactttaaacgaatctaacgactcaggccgaaactaaaacccagcaatc
   1 ATGCCTTTCAAACCAGTCGAAAACCCAAAATGCCCAAAGTGCGAAAAGTCAGTGTACGCC
   1 M  P  F  K  P  V  E  N  P  K  C  P  K  C  E  K  S  V  Y  A
  61 GCTGAAGAACGTGTGGCCGGTGGGTACAAGTTCCACAAGTCCTGCTTCAAATGCGGACTC
  21 A  E  E  R  V  A  G  G  Y  K  F  H  K  S  C  F  K  C  G  L
 121 TGCGGCAAGATGCTCGACTCTACCAACGTCACTGAACACGACAAGGAGCTGTTCTGCAAG
  41 C  G  K  M  L  D  S  T  N  V  T  E  H  D  K  E  L  F  C  K
 181 AACTGCCACGCCCGCAAATACGGACCAAAGGGATACGGTTTCGGTGGAGGTGCCGGCTGC
  61 N  C  H  A  R  K  Y  G  P  K  G  Y  G  F  G  G  G  A  G  C
 241 TTGAGCATGATGGACACCGGATCCCATCTCAGCCAGTAAtaatggaagcacatccgggac
  81 L  S  M  M  D  T  G  S  H  L  S  Q  *
 301 ttacatcaatccaataggcattcaatcaatcaacaatacgcatacaaaaacaagaggtgc
 361 acaatcatacaaacacaacacagtataaatttatataaataagcgttgaaataactcaaat
 421 tgtgtgcccngcaatccgtttcaatgtattttgaacatatatatattaaaaaaaaacgca
 481 aaaatatattattataatcaaaaaaatactcttaatttattattaattacttaatattat
 541 tattattactatcttttagttttataaaaaaaaataacaattttagatattatcatttcgt
 601 aatgttttcacgtggattatatacaaaaaaaaaacaaattagattttaattcgtatatta
 661 tttaaaggcgacccaaaatatactatcaaaaaatttttatgctatcttttgtaatattcga
 721 attgaggaataaaataatttgaattagtaaaaaaaaaaaaaaaaa
```

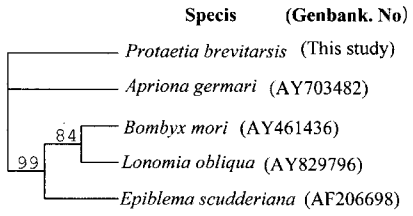
Fig. 1. The nucleotide and deduced amino acid sequences of the *P. brevitarsis* LIM protein cDNA. The start codon of ATG is boxed and the termination codon is shown by asterisk. In the cDNA sequence, the polyadenylation sequence AATAAA is underlined. This cDNA sequence has been deposited in GenBank under accession number DQ178131.

Results and Discussion

cDNA cloning, sequencing and alignment of *P. brevitarsis* LIM protein

In search of *P. brevitarsis* ESTs, we identified a cDNA showing high homology with previously reported LIM protein genes. The cDNA clone including the full-length open reading frame (ORF) was sequenced and characterized. The nucleotide and its deduced amino acid sequences of the cDNA encoding LIM protein are presented in Fig. 1. The entire length of *P. brevitarsis* LIM protein cDNA is 800 bp long, and contains an ORF of 276 nucleotides capable of encoding a 92 amino acid polypeptide with a predicted molecular mass of 10,030 Da and pI of 8.57. The ORF had both a start codon (ATG) and stop codon (TAA), indicating that the sequences contain the complete coding region. A putative polyadenylation signal, AATAAA, is located at nucleotide position 728-733.

Among the insect LIM available, *P. brevitarsis* LIM protein bears the strongest resemblance to the A. germari LIM protein (Gui *et al.*, 2004), but no clear phylogenetic relationship to other insect LIM proteins was detected (Fig. 2).



	GenBank No.	Percent similarity 1	2	3	4	5
1. *Protaetia brevitarsis*	This study		94	83	83	85
2. *Apriona germari*	AY703482	92		86	85	87
3. *Bomyx mori*	AY461436	77	81		96	88
4. *Lonomia obliqua*	AY829796	77	78	93		90
5. *Epiblema scudderiana*	AF206698	74	77	80	82	

Percent identity

Fig. 2. Phylogenetic relationships and pairwise identities and similarities of the deduced amino acid sequence of *P. brevitasis* LIM protein homologue among insect LIM protein sequences. The insect cuticle protein sequences were taken from the following sources: The sequences were taken from the following sources: *P. brevitarsis* (this study), *Apriona germari* (AY703482), *Bombyx mori* (AY461436), *Lonomia obliqua* (AY829796), and *Epiblema scudderiana* (AF206698).

Multiple alignment of the deduced protein sequence of *P. brevitarsis* LIM protein gene with available insect LIM protein sequences is shown in Fig. 3. The deduced protein sequence of *P. brevitarsis* LIM protein cDNA is comprised of a single LIM domain linked to a glycine-rich consensus sequence [GPKG(F/Y)G(F/Y)GXGAG] observed in CRP1 (Stronach *et al.*, 1996). As described previously in vertebrate CRPs, the *P. brevitarsis* LIM domain exhibits the cysteine-rich consensus sequence [$CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$] that contains a pair of zinc-finger-like structures (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Sadler *et al.*, 1992; Michelson *et al.*, 1993; Arber *et al.*, 1994). In addition, the potential nuclear targeting signal in *P. brevitarsis* LIM protein is retained with one conservative lysine to arginine substitution. Among the insect CRPs, *P. brevitarsis* LIM protein bears the strongest resemblance to another beetle *A. germari* LIM protein (Gui *et al.*, 2004). These similarities prompted us to that *P. brevitarsis* LIM protein cDNA in this study encodes a putative LIM protein of the CRP family.

mRNA expression of *P. brevitarsis* LIM protein

To confirm the expression of *P. brevitarsis* LIM gene at transcriptional level, Northern blot analysis was performed using mRNA prepared from fat body, midgut, and

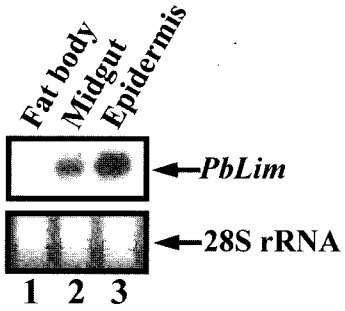


Fig. 4. Northern blot analysis of *P. brevitarsis* LIM protein. Total RNA was isolated from the fat body (lane 1), midgut (lane 2), and epidermis (lane 3) of *P. brevitarsis* larva, respectively. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (lower panel), transferred on to a nylon membrane, and hybridized with radiolabelled 800 bp *P. brevitarsis* LIM protein cDNA (upper panel). Transcripts of *P. brevitarsis* LIM protein gene are indicated on the right of the panel by arrow.

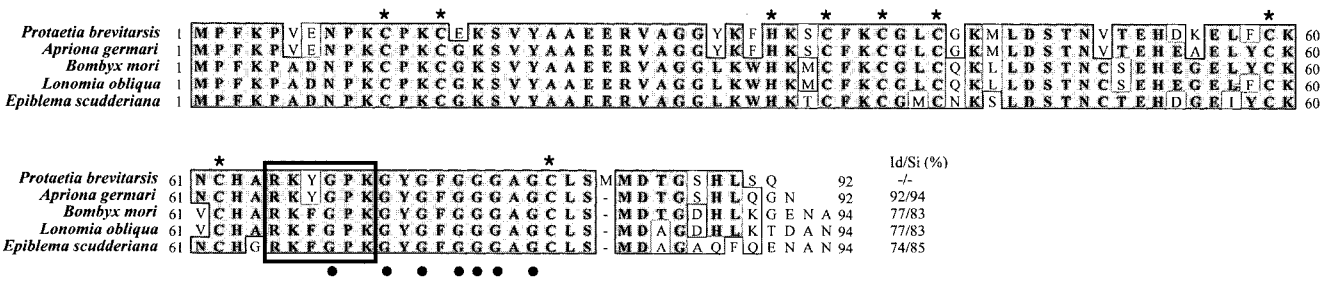


Fig. 3. Multiple alignment of the amino acid sequence of *P. brevitarsis* LIM protein with known insect LIM proteins. Residues are numbered according to the aligned insect LIM protein sequences, and invariant residues are shaded black. The conserved cysteine and histidine residues common to the LIM domain are shown by asterisk. Glycine residues in the glycine-rich region that follows the LIM domain are shown by solid circle. The putative nuclear targeting signal is boxed. The insect LIM protein sequences were taken from the following sources: *P. brevitarsis* (this study), *Apriona germari* (AY703482), *Bombyx mori* (AY461436), *Lonomia obliqua* (AY829796), and *Epiblema scudderiana* (AF206698). *P. brevitarsis* LIM protein sequence was used as reference for the identity / similarity (Id / Si) values.

epidermis, respectively (Fig. 4). Hybridization signal was detected as a single band in mRNA from epidermis and midgut. The signal of *P. brevitarsis* LIM protein transcripts showed a strong band in the epidermis and midgut, but not in the fat body. In *E. scudderiana* Mlp, intense hybridization signal of the EsMlp transcripts occurred from the body wall (muscle + exoskeleton), but EsMlp transcripts were not found in fat body (Bilgen *et al.*, 2001). Muscle LIM proteins in insects are associated with development and are crucial during myogenesis (Arber *et al.*, 1994; Stronach *et al.*, 1996; Bilgen *et al.*, 2001). Further biochemical and molecular biological studies are necessary to reveal the exact physiological role of *P. brevitarsis* LIM protein.

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