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RESEARCH REPORT

Properties of the *Bombyx mori* insulin-like peptide (BmILP) gene

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Abstract

Insulin/Insulin-like growth factors play important roles in promoting proliferation, differentiation, growth and development of organisms. In this article, bioinformatics analysis was performed to locate and identify the *Bombyx mori* insulin-like peptide (BmILP) gene, which had two introns, three exons, and a predicted signal peptide at the front, and it also determined that BmILP was the same to the gene Bombyxin Z1, a member of the bombyxin family. Both the full-length BmILP gene and the gene without the signal peptide expressed mainly existed in inclusion body forms at different induction conditions and it was noted that the signal peptide had significant influence on the expression level. In addition, protein-protein interaction experiments suggested that there was interaction between BmILP and the autophagy-related protein Atg8, indicating that BmILP may play a role in immunity. It was found was that BmILP predominantly produced by the ovary among different tissues in the silkworm. Particularly, the adult females had higher expression levels than the adult males, and the expression level appeared to be the highest in the mated female moths, suggesting that BmILP may be a key factor in the regulation of egg maturation in *Bombyx mori*.

Key Words: *Bombyx mori*; insulin-like peptide; BmILP; signal peptide; Atg8; spatiotemporal patterns

Introduction

The insulin/insulin-like growth factor (IGF) signal pathway plays key biological roles in diverse organisms (Xu *et al*., 2009; Gronke *et al*., 2010a, b) and is considered to be the main metabolic pathway involved in establishing adult body size (Guirao *et al*., 2013). Since bombyxin was identified as the first insulin-like peptide in invertebrates in 1984 (Nagasawa *et al*., 1984), this brain neurosecretory hormone of the silkworm *Bombyx mori* (*B. mori*) has been of tremendous interest to scientists. Its gene structure, chemical nature, secretion control, distribution, as well as physiological functions have been studied widely (Mizoguchi *et al*., 2013). The three-dimensional structure of bombyxin-II, a representative molecular species of bombyxin, revealed that its overall main-chain structure was similar to that of human insulin (Nagata *et al*., 1995), and sequence analysis showed that its amino acid sequence had 40 % similarity with human insulin (Iwami, 2000), however, they had different biological functions (Nagata, 2010). In 2009, a novel insulin-related peptides was identified in the silkworm *Bombyx mori* named Bommo-IGFLP (BIGFLP, for *Bombyx mori* IGF-like peptide), which was mainly produced by the fat body, and was massively released during pupa-adult development (Okamoto *et al*., 2009). Secretion of this peptide was stimulated by ecdysteroids and the secreted peptide can promote the growth of adult-specific tissues (Okamoto *et al*., 2009).

In the silkworm genome, bombyxin genes have multiple copies. There are a total of 38 bombyxin genes, which exist in various forms arranged in the silkworm chromosome (Kondo *et al*.,1996). These genes have multiple families, of which seven (A-G families) have no introns and are clustered in unique distribution patterns and expressed predominantly in the central brain neurosecretory cells (Iwami, 2000). Five of the bombyxin families (V-Z) are similar to the vertebrate insulin genes, which have intervening introns at almost the same positions (Aslam *et al*., 2011). The bombyxin-V and -W mRNAs were expressed in the brain and bombyxin-X mRNA was expressed in fat bodies, and the bombyxin-Y mRNA was produced in both the ovary and brain of larval stages. Bombyxin-Z gene had a high level of expression in the follicular cells, indicating its role in

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reproduction (Kondo *et al*.,1996).

Insulin-like peptides (ILPs) are also present in many other invertebrates (Nijhout *et al*., 2002; Perillo *et al*., 2014). In the butterfly *Precis coenia*, bombyxin acted together with 20-hydroxyecdysone (20E) to stimulate the growth of wing imaginal disks and cell division (Perillo *et al*., 2014). Knockout of *Drosophila melanogaster* insulin-like peptide 3 (DILP3) resulted in a series of phenotypes, including developmental delay, reduced fecundity, reduced body size and increased lifespan (Gronke *et al*., 2010a). Loss of DILP8 can generate developmental instability, yield asymmetric individuals with normal-sized animals, and result in variant periods of maturation (Garelli *et al*., 2012). However, deletion of DILP6 and DILP7 showed no major metabolic defects in *Drosophila melanogaster* (Zhang *et al*., 2009). For the yellow fever mosquito *Aedes aegypti (A. aegypti)*, synthetic ILP3 stimulated dose-dependently ecdysteroid production by the ovaries and yolk uptake by oocytes (Brown *et al*., 2008). It also showed metabolic activity by elevating carbohydrate and lipid storage and acting as a critical regulator of egg production. The activity of ILP3 was dependent on the expression of the mosquito insulin receptor (Zhang *et al*., 2009).

Additionally, we hope to find interacting proteins with BmILP by the His-tag pull down test and identify its role in the insulin signaling pathway. Existing research showed that, in the process of development, bombyxin-II stimulated ecdysteroidogenesis was regulated by the adenosine 5' monophosphate-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3K), which were the downstream signal molecules of insulin signaling pathways (Gu *et al*., 2015). Phosphorylation of PI3K can activate the downstream signal molecules Akt and the Phosphorylated Akt stimulates ecdysone secretion by its downstream signal molecules p70 ribosomal protein S6 kinase (S6K), which can promote the molting of the silkworm. When the phosphorylation of PI3K was inhibited, ecdysone secretion will decrease (Brown *et al*., 2008; Nagata *et al*., 2008). However, for the lepidopteran insect *Manduca sexta*, inhibition of the phosphorylation of Akt had no apparent effect on ecdysone secretion, indicating that the regulation was specie-specific (Smith *et al*., 2014).

So far, the exact physiological functions of bombyxin is still vague, yet its structure similarity to insulin suggests functional importance. In this article, we primarily investigated BmILP using bioinformatics analysis and determined that it was the same peptide as bombyxin Z1. In addition, the effect of the signal peptide on the expression of BmILP gene in prokaryotes was evaluated. We also identified a protein that interact with BmILP, and examined the expression levels of the BmILP gene in specific tissues and different developmental stages, which may provide some insight into the biological functions of BmILP.

Materials and Methods

Materials and reagents

Bombyx mori strain 306 was preserved by the Institute of Life Sciences, Jiangsu University, and reared according to conventional methods in our lab. Total RNA extraction (TRIzol Reagent) and cDNA synthesis reagents (PrimeScriptTM RT Reagent Kit) were obtained from Invitrogen (CA, USA) and TaKaRa (Dalian, China), respectively. Enhanced ECL chemiluminescence detection kit and quantitative real-time PCR reagents were purchased from Vazyme (Nanjing, China). His-tag monoclonal antibody and Ni-NTA agarose were purchased from Proteintech (Chicago, USA) and Qiagen (Germany), respectively.

Bioinformatics analysis

The gene sequence of BmILP was obtained from the NCBI database (GeneBank: 100653512), followed by blast alignments with cDNAs of BmILP to obtain the contigs on the silkworm chromosome. Chromosome location analysis (http://silkworm.genomics.org.cn/silksoft/silkmap.html) and prediction of signal peptide (http://www.cbs.dtu.dk/services/SignalP) were achieved by online software as noted. Sequence alignments between cDNA and contigs were completed with the software DNAstar 6.0 to obtain the intron and exon sequences. Amino acids sequences of thirty-three bombyxin were downloaded from the NCBI database. Amino acids conservation analysis and phylogenetic tree construction were carried out by the software MEGA 5.0 and GENEDOC, while similarity analysis was performed with the software DNAstar Megalign. The RNAstructure 5.4 software was used to predict the secondary structure of the mRNAs of both the full-length gene and the gene without the signal peptide sequence.

RNA extraction and cDNA synthesis

The fifth instar larvae of the silkworm were dissected on ice and washed with cold phosphate buffered saline (PBS). Then the tissue sample was frozen immediately in liquid nitrogen and stored at -70 °C until extraction of RNA. The RNA extraction and cDNA synthesis were completed following the manufacturer's instructions rigorously.

Construction of recombinant plasmid

The ORF specific primers was designed through Primer 5.0 software according to the BmILP gene sequence from the NCBI database. The forward primer of the full-length gene was 5′ CGGGGTACCATGTTTCTGCTGTATTTCCTGAT 3′ with a Kpn I site (underlined), and the reverse primer was 5′ CGCGGATCCTCAGCAGTAATTAAGTAGATCATACG 3′ with a *Bam*H I site (underlined). For the BmILP gene without the signal peptide, the forward primer was 5′ AAGAATTCATGGACGTCCACGACAAAG 3′ with an *Eco*R I site (underlined), and the reverse primer was 5′ CGCAAGCTTTCAGCAGTAATTAAGTAGATC 3′ with a *Hind* III site (underlined). After one cycle of pre-denaturation at 94 °C for 5 min, the PCR reaction was carried out for 30 amplification cycles. Each cycle was set to denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. After cycle completion, nucleotide A was added to the 3′ termini at 72 °C and incubated for 10

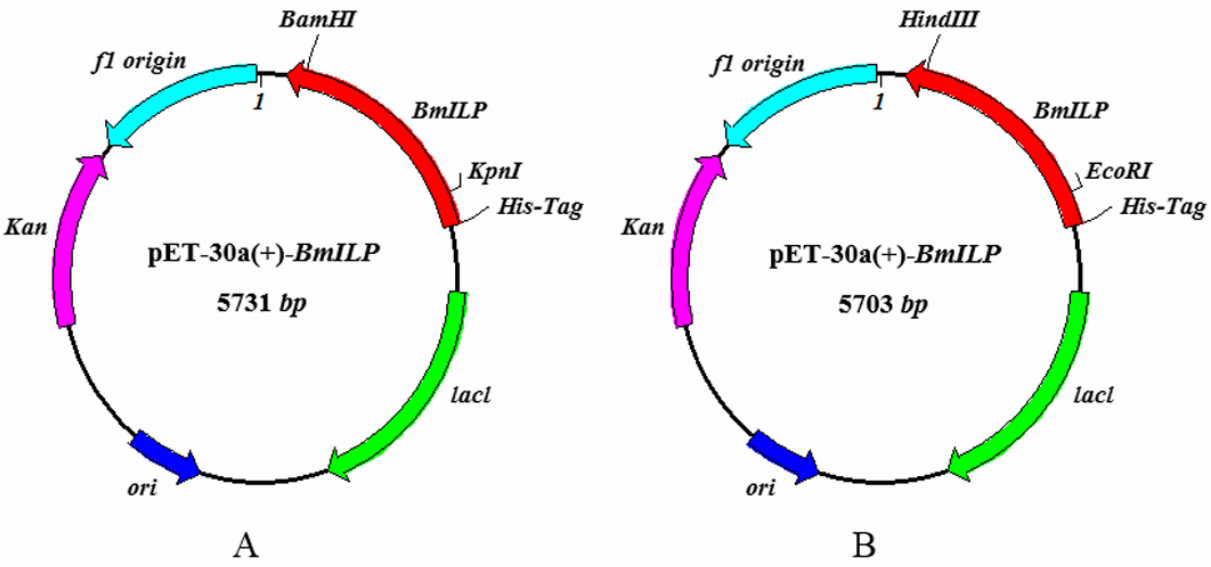

Fig. 1 Recombinant expression vectors. The recombinant plasmids of the full-length BmILP gene (A) and the gene without the signal peptide (B).

min. The PCR product was inserted into the pMD-18T vector and sequenced. After the correct sequence was obtained, it was ligated into the pET-30a(+) expression vector (Fig. 1). The recombinant plasmid was transformed into *E.coli* BL21 (DE3) by the heat shock method. DNA sequencing confirmed that BmILP was exactly fused to the N-terminal His-tag for protein expression.

Expression and solubility analysis of the fusion protein

The BL21 (DE3) containing the recombinant plasmid was inoculated into fresh LB medium and cultured at 37 °C for 2 - 3 h with powerful shaking (200 g per min). When the OD_{600} value reached 0.6, the expression of BmILP was induced with IPTG. The experiment was performed at different induction temperatures (16 °C, 28 °C, and 37 °C), induction periods (4 h, 8 h, and 12 h), and IPTG induction concentrations (0 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, and 1.0 mM) to obtain the optimum induction condition. The cells were collected by centrifugation (4500 g, 4 °C, 10 min). The supernatant was discarded and the precipitate was added bacterial lysate buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Trton-100, 1 mM lysozyme and 1 mM PMSF). The lysate was clarified by ultrasonication and then centrifuged at 12000 g under 4 °C for 10 min. The frequency and power of ultrasonication was fifteen times per minute and 200 w respectively. The total time was about half an hour. The supernatant was diverted into a new EP tube and the pellet was resuspended with lysis buffer. The supernatant and pellet were added with denaturant β-mercaptoethanol (20:1) separately, and were boiled at 100 °C for 10 min and harvested by centrifugation at 12000 g under 4 °C for 10 min. The supernatants from both samples were analyzed individually by SDS-PAGE.

Western Blotting

The proteins in the gel were blotted onto a polyvinylidene difluoride (PVDF) membrane at 250 mA for 55 min, and the membrane was incubated with 10 ml TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20) containing 2 % (w/v) skim milk at room temperature for 1 h followed by incubation with the mouse anti-6×His monoclonal antibody (1:1000 dilution) at room temperature for 2 h. The mouse monoclonal antibody was diluted with TBST buffer containing 2 % (w/v) skim milk. The membrane was washed with the TBST buffer for 5 min three times before incubation with the second antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, 1:1000 dilution) at room temperature for 2 h. The second antibody was diluted with TBST buffer. After washing three times with PBST for 5 min, the bands were visualized by 200 µl ECL chromogenic reagent under Tanon 5500 automatic chemiluminescence imaging analysis system.

Purification of the fusion protein and mass spectrometry analysis

After ultrasonication, the pellet of the cell lysate was harvested by centrifugation (12000 g, 4 °C, 10 min) and dissolved with 5 ml lysis buffer B (100 mM NaH_2PO_4,10 mM Tris-HCl, 8 M urea, pH = 8.0) at 4 °C for 1 h. The supernatant collected by centrifugation (12000 g, 4 °C, 10 min) was loaded onto a gravity column, which was filled with 500 µl Ni-NTA agarose and conditioned with lysis buffer B in advance. The column was gently rotated overnight at 4 °C. The flow-through was collected from the gravity column for SDS-PAGE analysis. The column was washed with 500 µl buffer B twice, and each time the flow-through was collected for the SDS-PAGE analysis. The column was then eluted sequentially with 500 µl of buffer C, D, and E, which

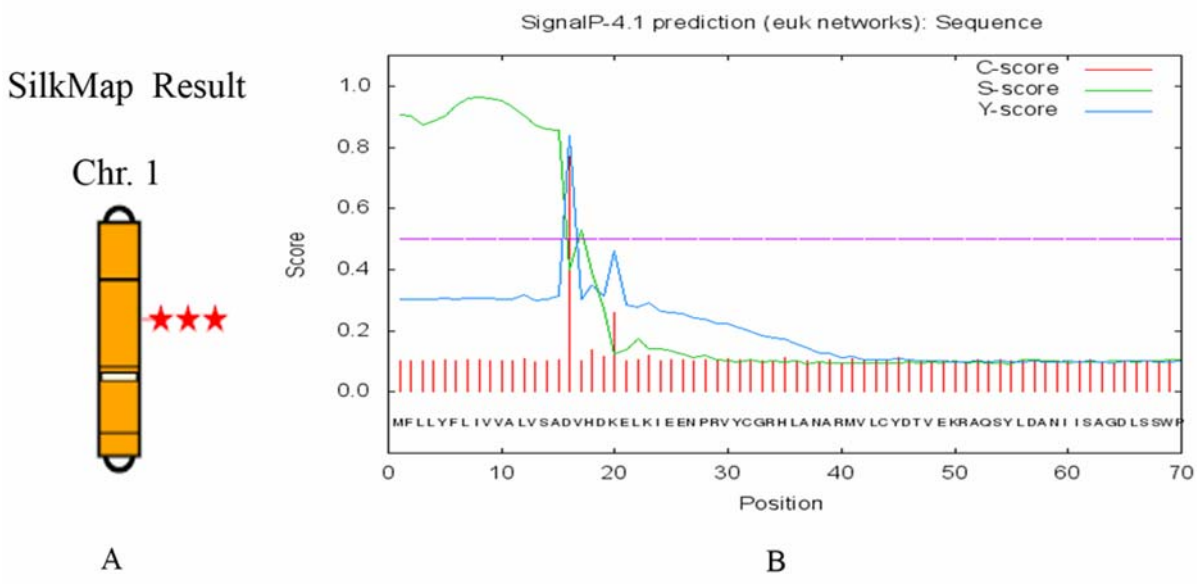

Fig. 2 Chromosomal localization of the BmILP gene (A), and the predicted signal peptide sequence from 1 to 15 amino acids (B).

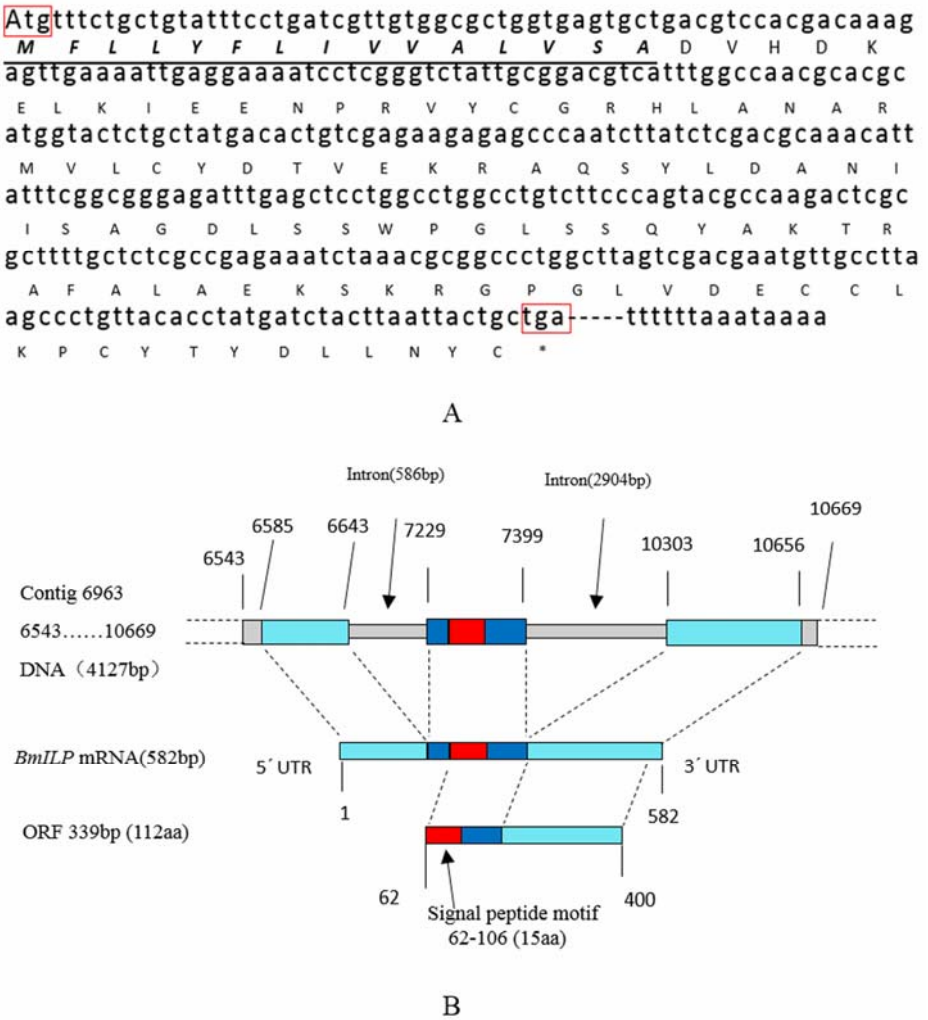

Fig. 3 (A) The BmILP ORF gene sequence and its coding amino acid sequence. The gene had 339 bp, and the underlined sequence refers to the signal peptide, which contained 15 amino acids. The initiation and stop codons were labeled with red boxes. (B) Structure analysis of the BmILP gene, which contained two introns, three exons and a signal peptide motif. The signal peptide motif was coded by nucleotides No.62-No.106 on the cDNA.

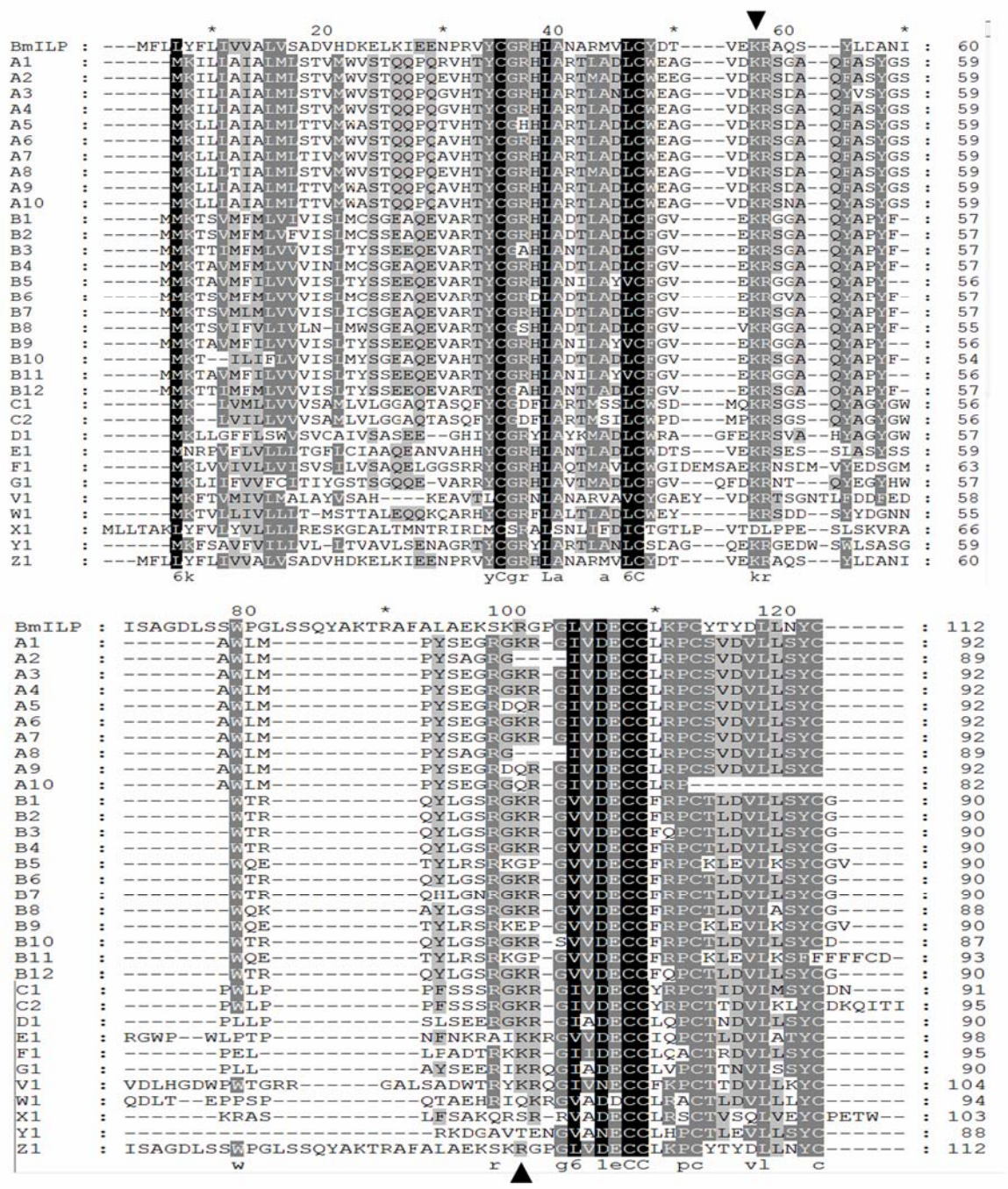

Fig. 4 Conservation analysis of the bombyxin family and BmILP. The cysteine (C) is highly conserved and is the site for disulfide bond formation for BmILP and all the bombyxin family proteins. The black arrow refers to protease cleavage sites to produce mature peptides.

all contained the same buffer content to buffer B but had different pH values at 6.3, 5.9, and 4.5, respectively. The elution was repeated four times for each buffer and each eluent was collected for the SDS-PAGE analysis. The specific band in the SDS-PAGE gel corresponding to the BmILP protein was digested with trypsin according to the in-gel digestion protocol (Li *et al*., 2006). The resulting digest was prepared for mass spectrometry analysis using a MALDI-TOF instrument (Bruker, Germany).

His-tag pull down

The His-tag was used to conduct the pull-down test to find the interaction protein with the BmILP mature peptide without the signal peptide. The previously purified BmILP inclusion body protein was dialyzed for renaturation with decreasing concentrations of urea in the dialysate (100 mM NaH_2PO_4, 10 mM Tris-HCl, 4 M down to 0 M urea) to decrease the urea concentration in the sample from 8 M to 0 M. Label a sufficient number of Pierce spin

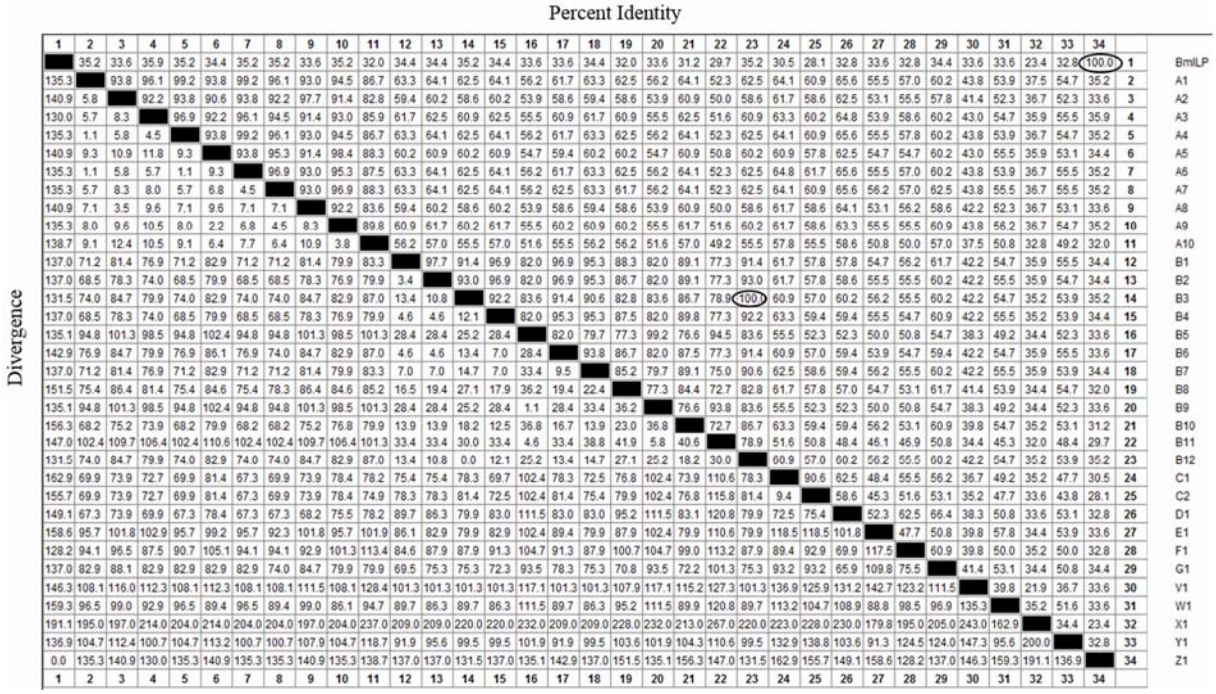

Fig. 5 Similarity analysis between the bombyxin family and BmILP. The black elliptic regions indicate high degree of amino acid similarity between BmILP and bombyxin Z1 and between bombyxin B3 and B12.

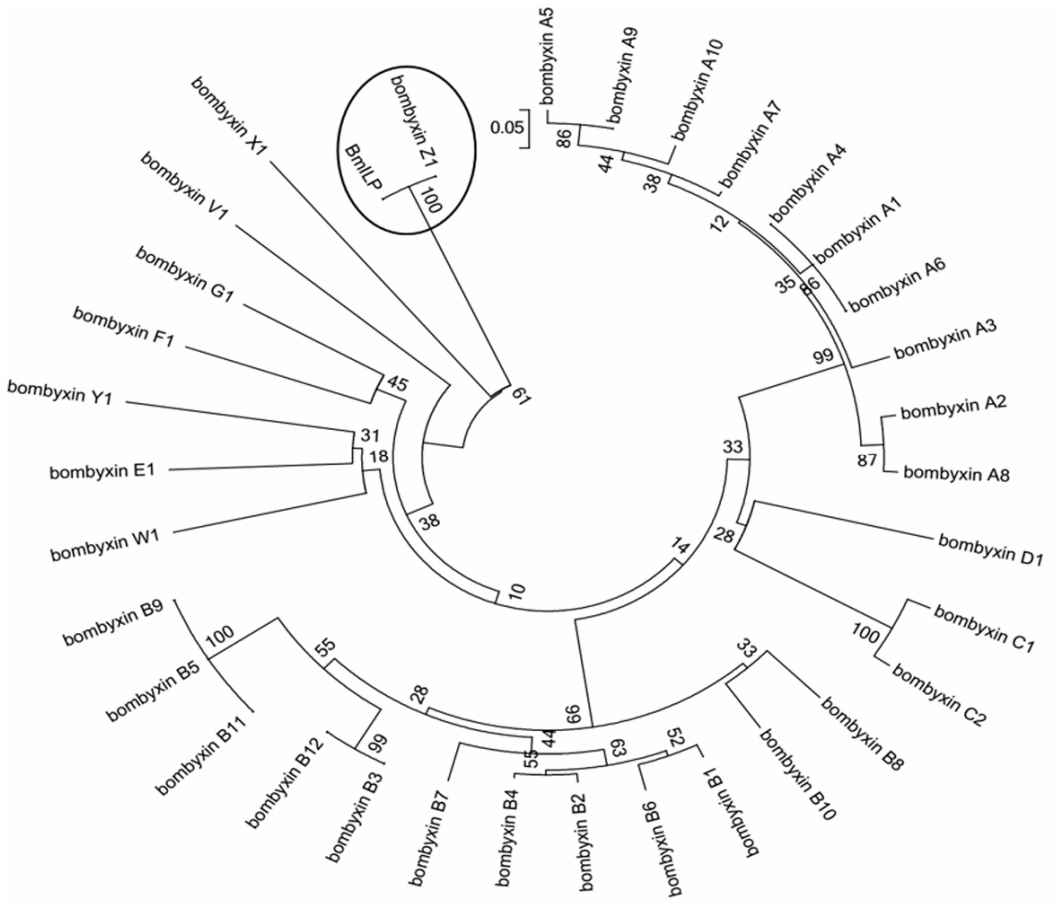

Fig. 6 The neighbor-joining tree of the bombyxin family and BmILP. The scale bar indicates unit length for 5% amino acid sequence difference. The black elliptic region indicates that BmILP and bombyxin Z1 are the same family.

columns, including a sample, a non-treated resin control and an empty vector group. Pipette 150 µl of Ni-NTA agarose into each labeled spin column. The column was then preconditioned four times with 200 µl of the wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1 % NP-40, 5 % glycerin, 10 mM imidazole, pH 7.4) each time and centrifuged at 1300 g for 60 s. Addition of 400 µl prepared 6×His-tagged fusion bait protein was made onto the column and incubated at 4 °C for 12 h with gentle rocking. The flow-through was discarded through centrifugation at 1300 g for 60 s and placed on ice. The column was washed with 200 µl of wash buffer for five times followed with the addition of 400 µl of prepared prey protein sample that was extracted from the silkworm and incubated at 4 °C for 10 h on a rotating device. The column was centrifuged at 1300 g for 60 s, and the flow-through was discarded. The spin column was washed with the wash buffer for four times, and the first time wash buffer was collected for SDS-PAGE analysis. The column was eluted twice with the elution buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1 % NP-40, 5 % glycerin, 290 mM imidazole, pH 7.4) and the first time eluent was collected for SDS-PAGE analysis.

Quantitative real-time PCR

The specific primers of the full-length BmILP gene were 5′ GTCCACGAC AAAGAGTTGAAAA 3′ and 5′ ATCTCCCGCCGAAATAATG 3′ for the forward and reverse primer, respectively. For the reference gene α-tublin, the specific primers were 5′ CTCCCTCCTCCATACCCT 3′ and 5′ ATCAACTACCAGCCA CCC 3′ for the forward and reverse primer, respectively. The PCR reaction was carried out for 1 cycle of pre-denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 10 s and 60 °C for 30 s, 1 cycle at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s in a 7300 Real PCR System. Each sample was done in triplicate.

Results

Chromosomal localization and structure analysis of the BmILP gene

The BmILP gene having the correct sequence was analyzed with the NCBI database and the silkworm genome database. The results indicated that the gene was located on the silkworm genome chromosome 1 (Fig. 2A). In the front of the gene was a signal peptide from 1 to 45 bp as shown by the signal peptide online prediction software (Fig. 2B). The DNA and mRNA lengths of BmILP were 4127 bp and 582 bp, respectively. Its ORF contained 339 bp and coded for a protein having 112 amino acid residues (Fig. 3A). The predicted molecular weight of BmILP is about 12.6 kDa. Comparison of gene sequences between the coding sequence and the complete genome demonstrated that BmILP contained two introns and three exons (Fig. 3B), whereas the absence of introns was found in bombyxin families A-G (Iwami, 2000).

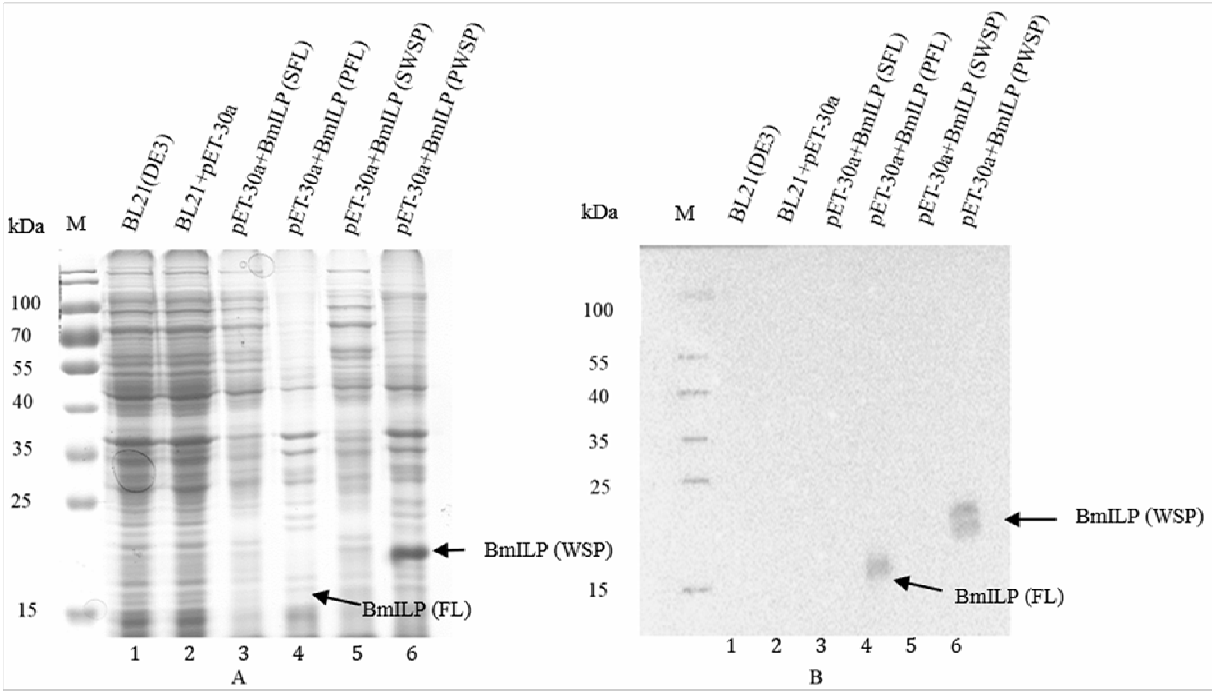

Fig. 7 Prokaryotic expression of the full-length (FL) BmILP gene and the gene without the signal peptide (WSP) by SDS-PAGE (A) and western blotting (B) analysis. Lane 1: BL21 (DE3). Lane 2: BL21 (DE3) containing pET-30a(+) empty vector. Lane 3 and 4: supernatant of the expression of the full-length BmILP gene (SFL) and precipitate of the expression of the full-length BmILP gene (PFL), respectively. Lane 5 and 6: supernatant of the expression of the BmILP gene without the signal peptide (SWSP) and precipitate of the expression of the BmILP gene without the signal peptide (PWSP), respectively.

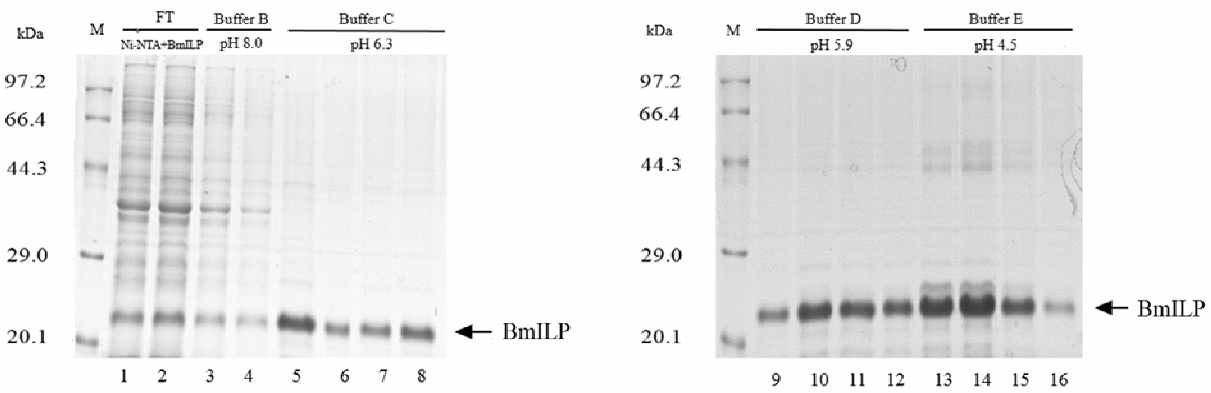

Fig. 8 SDS-PAGE analysis on the purification of the recombinant protein. Lane 1-2: the flow-through (FT) obtained after 12-16 h of incubation upon loading the protein sample onto the Ni-NTA agarose gravity column. Lane 3-4: 1st-2nd washing using buffer B, respectively. Lane 5-8: 1st-4th eluent using buffer C, respectively. Lane 9-12: 1st-4th eluent using buffer D, respectively. Lane 13-16: 1st-4th eluent using buffer E, respectively.

Conservation and similarity analysis of the bombyxin protein family and phylogenetic tree construction

The amino acid sequences of 33 bombyxin family proteins were downloaded from the NCBI database. The family contains 10 members of bombyxin A, 12 members of bombyxin B, 2 members of bomyxin C and only one member for each of bombyxin D, E, F, G, V, W, X, Y, and Z (Kondo *et al*., 1996; Iwami, 2000). Conservation analysis showed that BmILP was similar to the members of the bombyxin family, which all contain cysteines (C) at the same positions and are highly conservative. Previous studies have shown that these cysteines were sites for disulfide bond formation, which was essential for the formation of mature peptides (Narhi *et al*., 1993). It was also noted that the protease cleavage sites lysines (K) and arginines (R), cleavage of which mature peptides form, were highly conserved throughout the bombyxin family and BmILP (Fig. 4). Amino acid similarity analysis of bombyxin showed that the similarity between members of the same family can be extremely high. The amino acid similarity between B3 and B12, and between BmILP and bombyxin Z1 were both 100%, indicating that bombyxin B3 and B12, as well as BmILP and bombyxin Z1 were the same peptides (Fig. 5). This was further confirmed by the neighbor-joining tree, which demonstrated that all members of the bombyxin family were highly conserved during evolution (Fig. 6).

Effect of the signal peptide on the expression of BmILP genes in prokaryotes and peptide solubility analysis

Figure 7 shows the results of SDS-PAGE and western blotting analysis. Protein expression of the full-length BmILP gene only appeared in the precipitation no matter which induction condition was used, although the expression level was fairly weak (Fig. 7). However, the expression level of the BmILP gene without the signal peptide was much higher, and mainly existed in the inclusion body (Fig. 7). These observations indicate that the signal peptide can affect the expression of the BmILP gene in prokaryotic systems, and the expression level would increase dramatically after removing the signal peptide.

Secondary structure analysis of the mRNA of the full length gene and the gene without the signal peptide

The mRNAs of the full-length BmILP gene and the gene without the signal peptide were analyzed by the RNAstructure software. The results showed that the signal peptide sequence of the 5' end domain of the full-length mRNA was located inside the annular area and formed a complex and stable secondary structure (Fig. S1A), which was unfavorable for binding to the translation initiation factor and thereby hindered the initiation of the translation. On the other hand, the 5' terminal region of mRNA without the signal peptide was completely exposed, which was beneficial to the initiation of translation (Fig. S1B). This was consistent with our observation that removing the signal peptide significantly increased the expression level of the BmILP gene.

Purification of the recombinant protein and mass spectrometry identification

After 12 - 16 h of incubation upon loading the protein sample onto the Ni-NTA agarose column, the recombinant protein became bound to the agarose while impurity proteins were removed in the flow-through, although it was found that the flow-through also contained some unbound protein (Fig. 8, Lane 1 - 2). The buffer B flow-through contained a small quantity of the fusion protein, and very little impurity proteins were observed (Fig. 8, Lane 3 - 4). A fair amount of fusion proteins was obtained with buffer C (Fig. 8, Lane 5 - 8) and an increasing amount was obtained with buffer D (Fig. 8, Lane 9 - 12). These observations indicate that lowering the buffer pH can increase fusion protein contents in the flow-through. The optimal elution condition was when elution buffer E (Fig. 8, Lane 13 - 16) was used, which had the lowest pH among the different buffers. The purified protein was identified

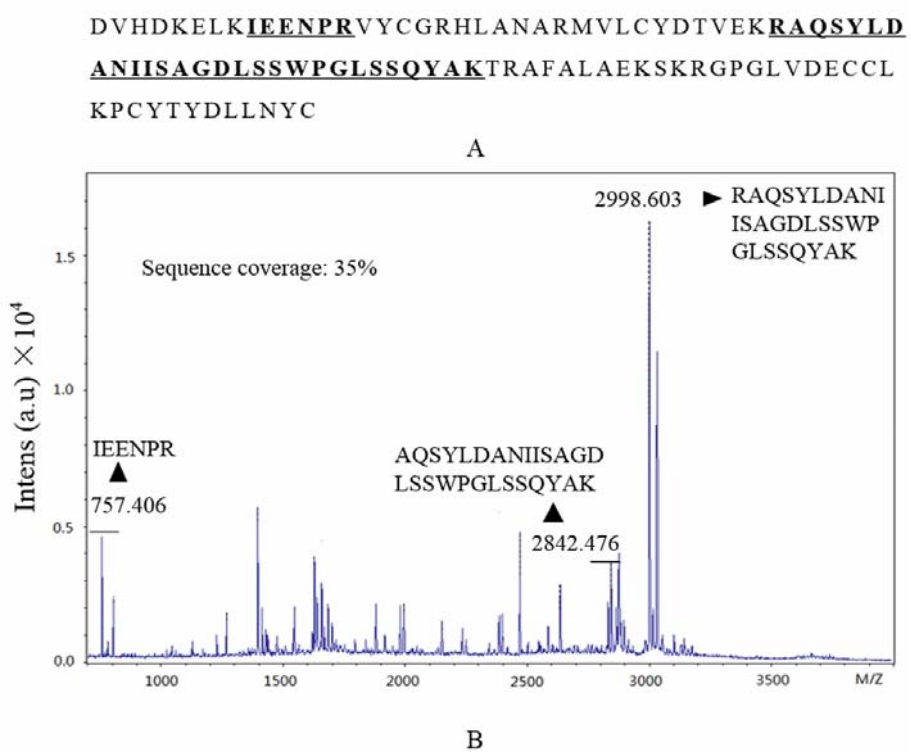

Fig. 9 MALDI-TOF mass spectrometry analysis of the purified recombinant protein. The amino acid sequence of the recombinant fusion protein BmILP is displayed in panel A with the mass spectrometry identified sequences underlined. The identified sequences were labeled on corresponding mass spectrometry peaks in panel B.

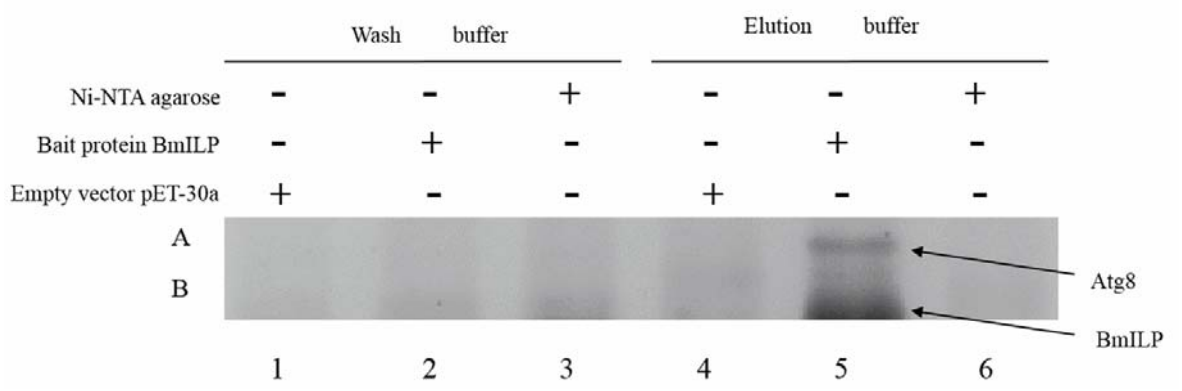

Fig. 10 SDS-PAGE analysis of the His-tag pull down experiment. Lane 1, 2, 3 and lane 4, 5, 6 are from the wash buffer and elution buffer, respectively. Lane 1 and 4 are from the control group having empty vector pET-30a(+), and Lane 2 and 5 are from the experimental group of the fusion protein BmILP. Lane 3 and 6 are the control group of Ni-NTA agarose. A5 was the prey protein Atg8, and D5 was the fusion bait protein BmILP.

by MALDI-TOF mass spectrometry to confirm the amino acid sequence and to compare with the predicted one based on the DNA sequence (Fig. 9).

The His-tag pull down analysis

Figure 10 shows the results of the His-tag pull down analysis. It was apparent that a prey protein was “pulled down” by the bait protein BmILP under appropriate elution conditions (Fig. 10, Lane 5), while the prey protein was absent in the control groups, indicating that there existed an interacting protein with BmILP. The interacting protein from Lane A5 was digested with trypsin and analyzed by MALDI-TOF mass spectrometry, and then compared across the NCBI database. The results suggested that this prey protein was very likely to be the autophagy-related protein Atg8 of the silkworm (Fig. S2), which was in accordance with previous studies that bombyxin acts synergistically with 20E, a hydroxy ecdysteroid that controls the expression of the *Atg8* genes, to regulate the growth of the imaginal disc (Nijhout *et al*., 2007; Tian *et al*., 2014).

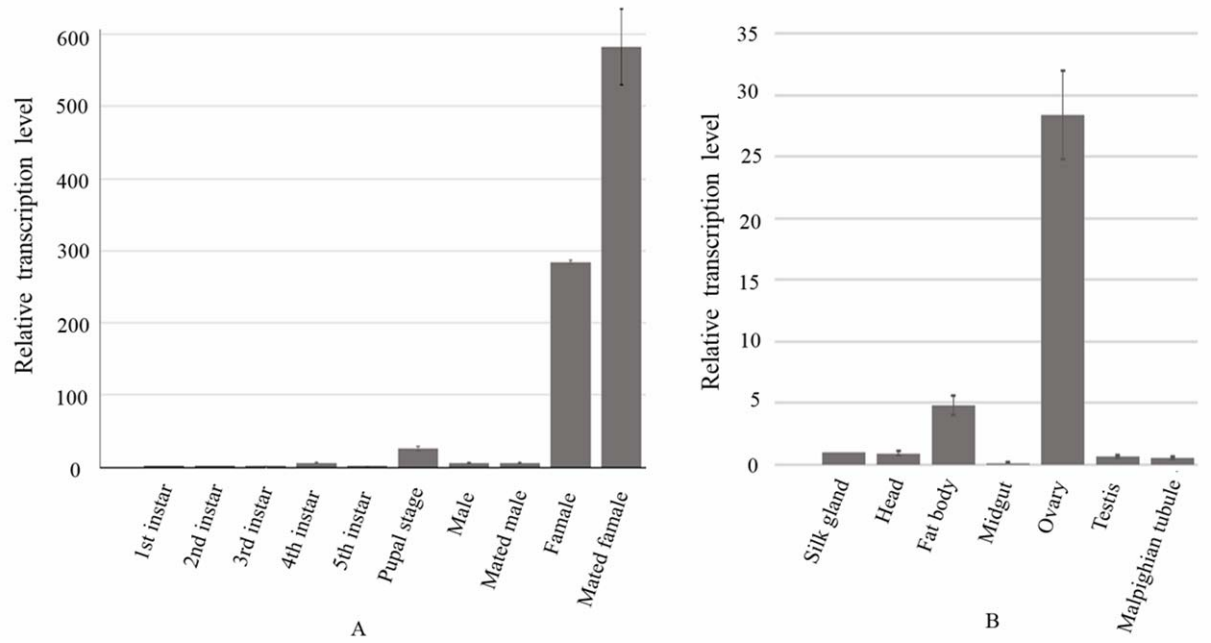

Fig. 11 Transcription phase analysis of the BmILP gene at different developmental stages (A) and tissues (B). In panel A, the horizontal axis shows different developmental stages, sexes, and mating conditions of the silkworm. The vertical axis shows the transcription levels relative to the 1st instar. In panel B, the horizontal axis refers to different tissues from the third day of the fifth instar larvae. The vertical axis shows the transcription levels relative to the silk gland. The analysis was done in triplicates with standard error noted for each bar.

Transcription phase analysis of the BmILP gene in various tissues and developmental stages

In order to understand physiological functions of BmILP in the silkworm, it is necessary to evaluate the expression levels of the BmILP gene in various tissues and different developmental stages. Real-time fluorescent quantitative PCR analysis showed that the total transcription level of the BmILP gene was very low at the development phases of the instar larvae, which lasted from the first instar larvae to the fifth instar larvae (Fig. 11A). The level started to increase at the pupal stage and continued to increase until it reached the highest at the adult stage. It was found that female moths possessed much higher levels of expression than the male moths. In addition, the transcription level of the BmILP gene in mated female moth was more than twice the level of those unmated ones (Fig. 11A). Among the different tissues of the silkworm, ovary had the highest transcription level of the BmILP gene, followed by the fat body. The transcription level was very low in the silk gland, head, testis, malpighian tube, and the midgut had the lowest level of transcription (Fig. 11B).

Discussion

Studies have shown that for the genes of the bombyxin family, 26 genes are located on chromosome 11, 2 of them on chromosome 9, 3 on chromosome 1, and 7 unknown (Kondo *et al*., 1996). Bioinformatics analysis indicated that the BmILP gene had two introns and was located on chromosome 1, while the insulin gene also has two introns, suggesting that they may have similar structural features. Analysis of amino acid conservation showed that BmILP and the bombyxin family were highly conservative and contained cysteines at various sites, where disulfide bonds form. The formation of disulfide bonds are vital for the activity of bombyxin (Nagata *et al*., 1992). Comparison of the prokaryotic expression of the BmILP gene having full-length and the gene without the signal peptide showed that the signal peptide had significant suppression on the expression level, which may be caused by the formation of the complex secondary structure on the 5′ end of the mRNA, thereby hindered the initiation of the translation (Gong *et al*., 2009).

The His-tag pull down experiments indicated that there might be interactions between BmILP and the autophagy-related protein Atg8 of the silkworm. According to literature, Atg8 was crucial for the formation of the autophagy body, and participated in the ubiquitin pathway (Hu *et al*., 2010). Autophagy is very important for the growth of organisms and development of disease. In the fat body of the silkworm, 20E (hydroxy ecdysteroids) regulates the expression of the Atg8 genes positively, thereby inducing autophagy (Narhi *et al*., 1993). In *Manduca sexta*, bombyxin acts synergistically with 20E to regulate the growth of the imaginal disc (Tian *et al*., 2014). Studies have shown that insulin-related peptide-binding protein 2 (BmIBP2) of the silkworm is a member of the immunoglobulin superfamily, and the BmIBP2 has high homology to the binding protein 7 (IGFBP7) of insulin growth factors (Ruan *et al*., 2007; Gao *et al*., 2012). IGFBP7 induces

apoptosis by generating a tumor suppressor and BmIBP2 plays an important role in the immune response of silkworm to *Bombyx mori* cypovirus (BmCPV) infection (Ruan *et al*., 2007; Hu *et al*., 2010). Based on these previous findings, we suggest that BmILP is an integral part of the immune system of the silkworm via involving in procedures of apoptosis induction.

Previous researchers found that ILPs regulate the synthesis of ovarian steroid hormone in *Phormia regina* (Manière *et al*., 2004), and this hormone plays a key role in reproduction control. In *Aedes aegypti*, ILPs activate several signaling pathways, which are necessary for egg maturation (Vogel *et al*., 2015), suggesting that ILPs possess important biological functions in females. Our experiments showed that the transcription levels of the BmILP gene varied at different growth stages and tissues of the silkworm. It was also noted that the adult female moth had much higher transcription level than the adult males, and the level was the highest in mated female moths. We therefore speculate that the BmILP polypeptide may play a critical role in the process of egg production of female moths, increasing spawning and promoting egg maturation. The expression level of the BmILP in ovarian was the highest among different tissues, suggesting the significance of this protein for the growth and development of the gonads in *B. mori*.

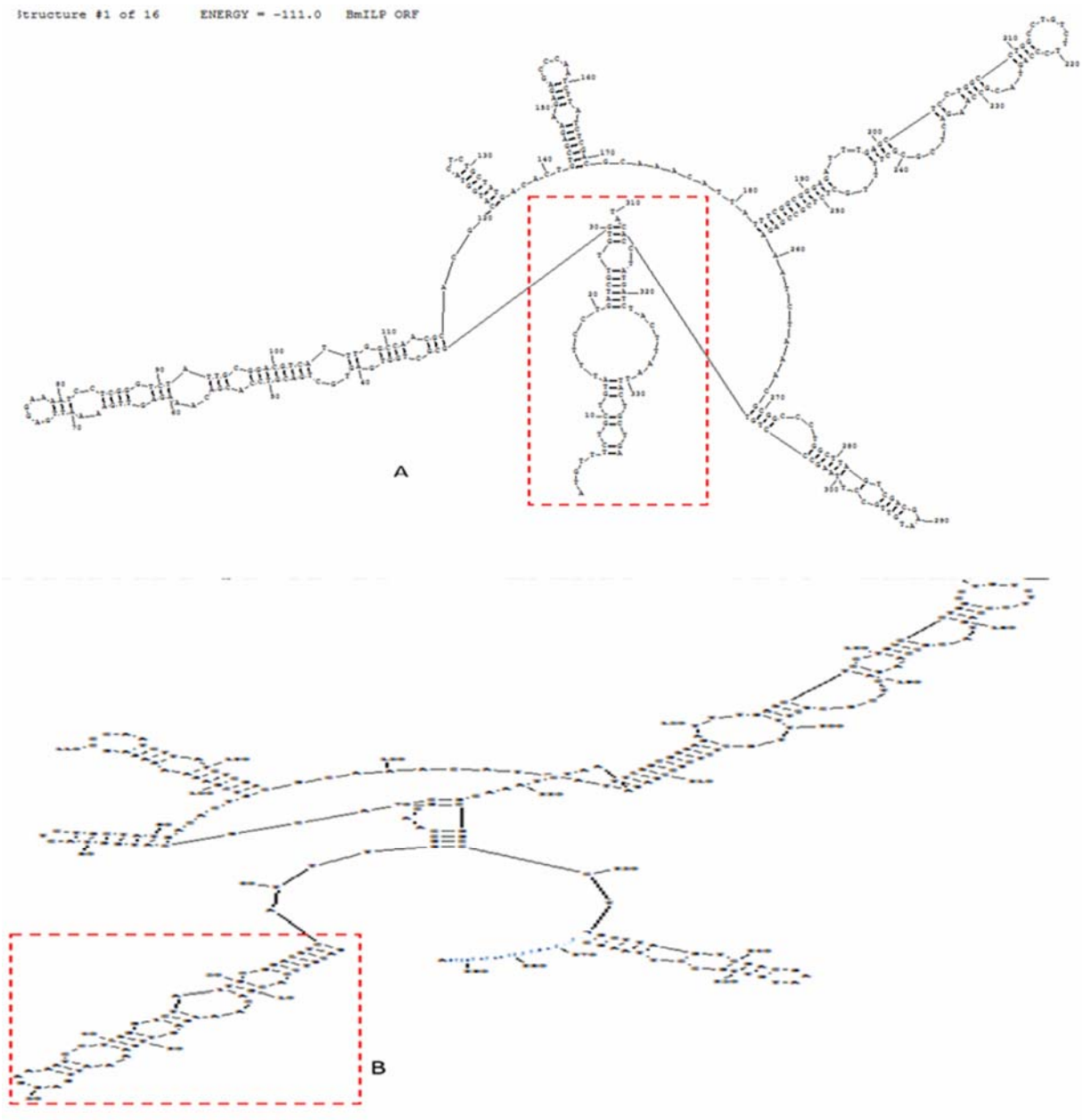

Fig. S1 The secondary structures of the mRNAs of the full-length BmILP gene (A) and the gene without the signal peptide (B). The red dotted box in each panel indicates 5' terminal region of the gene.

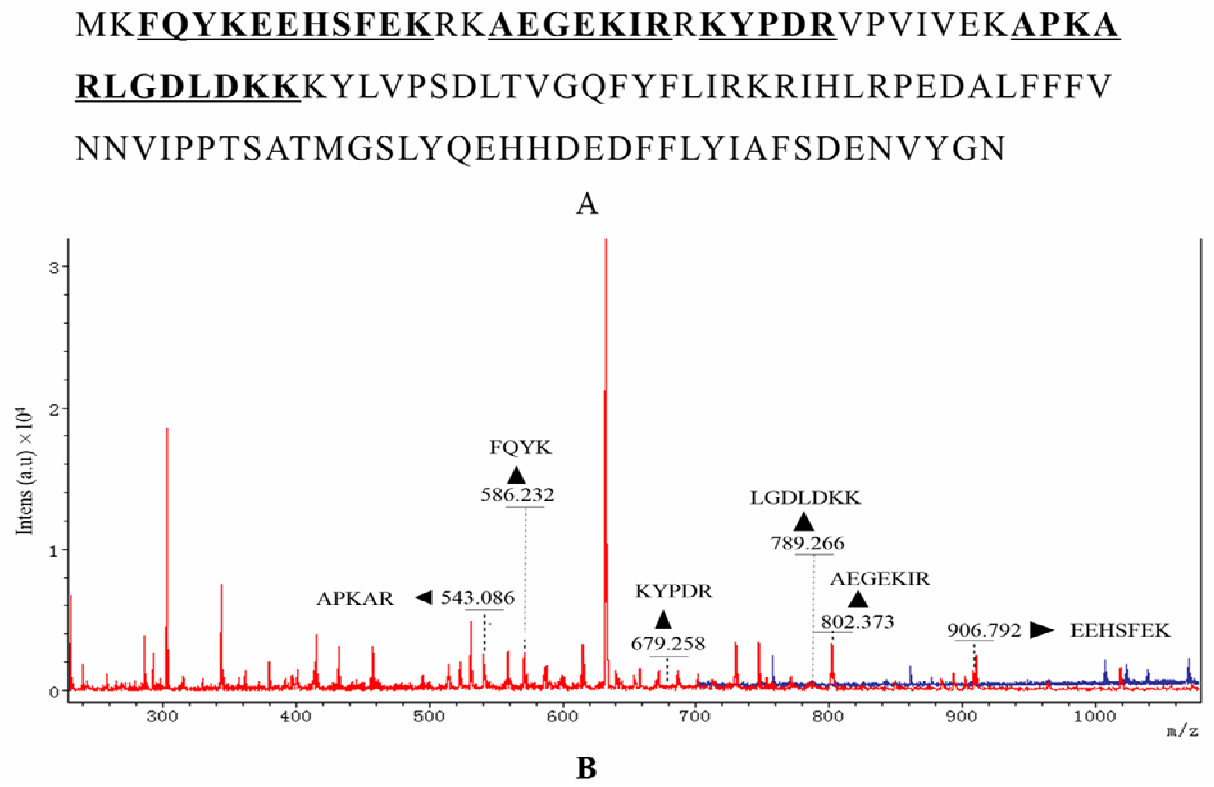

Fig. S2 Mass spectrometry analysis of the prey protein from lane A5 in the Fig. 10. The amino acid sequence of the autophagy-related proteins Atg8 of the silkworm is displayed in the figure A. The peptides that were identified by mass spectrometry were labeled on top of the peaks in the figure B and underlined for the corresponding sequence.

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