

Characterization of a female-specific protein from the wild silkworm *Actias selene*

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Abstract

In *Actias selene* (Hubner) (Lepidoptera Saturniidae) there is at least one female specific protein limited to the fat body. Sodium dodecyl sulfate polyacrylamide gel electrophoresis performed on the fat body proteins from male and female pupas of *A. selene* and a female-specific protein with a molecular weight of about 24 kDa was revealed. This protein was purified for determination of the N-terminal amino acid sequence. Conventional polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) PCR were also carried out to clone the cDNA encoding 24 kDa protein (*As-24K*) based on the amino acid residues. The *As-24K* cDNA consists of 832 bp and the amino acid sequence shares 71% similarity with the 24 kDa proteins from *Antheraea pernyi* (Guerin-Meneville) and *Antheraea yamamai* (Guerin-Meneville) (Lepidoptera Saturniidae). In female pupa western blotting using antiserum against *As-24K* revealed that 24 kDa protein was restricted to the fat body. It was not found in any other pupal tissues.

Key words: female-specific protein, fat body, protein expression.

Introduction

Female-specific proteins are important for the growth and development of insects. Up to now, few female-specific proteins have been discovered in fat body (Telfer, 1953; Telfer and Williams, 1953; Telfer and Melius, 1963; Pan *et al.*, 1969; Hays and Raikhel, 1990; Yokoyama *et al.*, 1994), haemolymph (Ryan *et al.*, 1985; 1986), egg (Ono *et al.*, 1975; Zhu *et al.*, 1986) or colleterial glands (Amornsak *et al.*, 1992) from various insects. These female-specific proteins such as vitellogenin (Engelmann, 1979; Liu *et al.*, 2001; 2003; Tufail and Takeda, 2008), storage protein (Tojo *et al.*, 1978; 1980; Bean and Silhacek, 1989; Yokoyama *et al.*, 1993) and glue proteins (Liu *et al.*, 2005) are mainly involved in egg production (Yokoyama *et al.*, 1994).

Actias selene (Hubner) (Lepidoptera Saturniidae) is an important wild silk-spinning insect mainly located in China, Japan, India and Southeast Asian countries. In our previous study, a female-specific protein, vitellogenin, had been identified from the fat body in *A. selene* (Qian *et al.*, 2011). Here, we report the characterization of another 24 kDa female-specific protein from this insect, hereafter referred to as *As-24K*, and the absence of expression of *As-24K* in pupal tissues other than fat body.

Materials and methods

Experimental insects

A. selene larvae were collected from *Salix babylonica* L. and reared in the room with willow tree leaves until pupation.

Protein extraction

Fat body was sampled from male and female pupae respectively. Total proteins were extracted from fat body using a ReadyPrep protein extraction kit (Bio-rad) according to the instructions. Quantification for total protein was performed using the Bradford method (Bradford, 1976).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The proteins from the fat body were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 5% stacking gel and 12% separating gel by the method of Laemmli (1970). Molecular sizes were estimated using middle-molecular-weight markers (Bioass Inc.). Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Electroblotting and protein sequencing

After electrophoresis, the bands containing the 24 kDa were cut out and eluted with a Model 422 Electro-Eluter (Bio-Rad) according to the instructions. The prepared samples were subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Sigma) by an electrophoretic transfer system (Bio-Rad). Subsequently, the membrane was stained by Coomassie blue and the band corresponding to 24 kDa was excised for N-terminal amino acid sequencing by Edman degradation (Edman, 1950).

RNA extraction

One hundred microgram of fat body from a female pupa was collected for RNA extraction. Total RNA was extracted using Trizol Reagent (Invitrogen) according to the instructions. The total RNA concentration was determined by Nano Drop 2000 (Thermo) and electrophoresis was used to check the RNA integrity.

RNA extraction

Total RNA from fat body was reverse transcribed into cDNA with a First-Strand System Kit (Promega) following the manufacturer's protocol. Oligonucleotide primers (F₁: 5'-CTGAAGTCCTACGATTATGT-3'; R₁: 5'-CGAGATACCATTTGGAAGAC-3') were designed with Primer premier 5.0 software package and used for the conventional PCR and RACE PCR. Conventional PCR with primers F₁R₁ was performed using PCR amplification Kit (Takara) and the amplification program

consisted of 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 54 °C for 35 s, 72 °C for 45 s and a final elongation step of 72 °C for 5 min. RACE PCR with primers RC3 (5'-GCTGGTATACGATTTCGAGAACAT-3') and RC5 (5'-CAACTTGGGTCAGTTCAGTTTCC-3') were conducted using the SMART RACE cDNA Amplification Kit (Clontech). Generated DNA fragments were analyzed on 1% agarose gels, then subcloned into the PGEM T easy cloning vector (Promega) and sequenced at Invitrogen, Shanghai.

Antibody preparation

Antiserum was prepared according to the method described by Harlow and Lane (1999). The New Zealand white rabbits were immunized with 100 µg of eluted *As-24K* protein homogenized in complete Freund's adjuvant for three times at 2-week intervals. A boost injection was given for another week and antiserum was collected after the last immunization, this antiserum was used for the verification of *As-24K* by western blotting.

Enzyme-linked immunosorbent assay

Antibody titer was determined from the immunized rabbits by enzyme-linked immunosorbent assay (ELISA) (Zhu and Wu, 2008). *As-24K* protein was diluted in 0.05M carbonate buffer and coated onto a 96-well plate (Nune, Denmark) and then incubated overnight at 4 °C with blocking solution (5% skimmed milk powder in phosphate-buffered saline). After washing with PBST (phosphate-buffered saline containing 0.1% Tween 20), each well was filled with rabbit serum of different dilutions and incubated at 37 °C for 1h. The plates then

were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase for 30 min at room temperature. Finally, TMB/H₂O₂ substrate was added to each well and Optical density was measured at 450 nm with Elix 800 Universal micro-plate reader (Biotek Instruments).

Western blotting

Total proteins from mid-intestine, malpighian tube, blood, fat body and ovary were subjected to SDS-PAGE and then transferred onto PVDF membrane (Sigma) by an electrophoretic transfer system (Bio-Rad). Membranes were blocked with 1% BSA for 2 h at room temperature. After washing with PBST, membranes were subsequently incubated with primary antibodies for 2 h and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG antibody (Sigma) for 1 h at room temperature. Final detection was performed with an HRP-DAB Detection Kit (Tiangen) (Zhu and Wu, 2008; Liu *et al.*, 2011). The beta actin immunoreactive signal used as loading control.

Results

SDS-PAGE of fat body proteins

A protein of 24 kDa molecular weight was discernable in fat body proteins from *A. selene* female pupa, but not in male pupa (figure 1A). This result suggested that this protein is a female - specific fat body protein. Subsequently, fractions containing this 24 kDa protein were cut out for gel elution and a single band was obtained by SDS-PAGE (figure 1B).

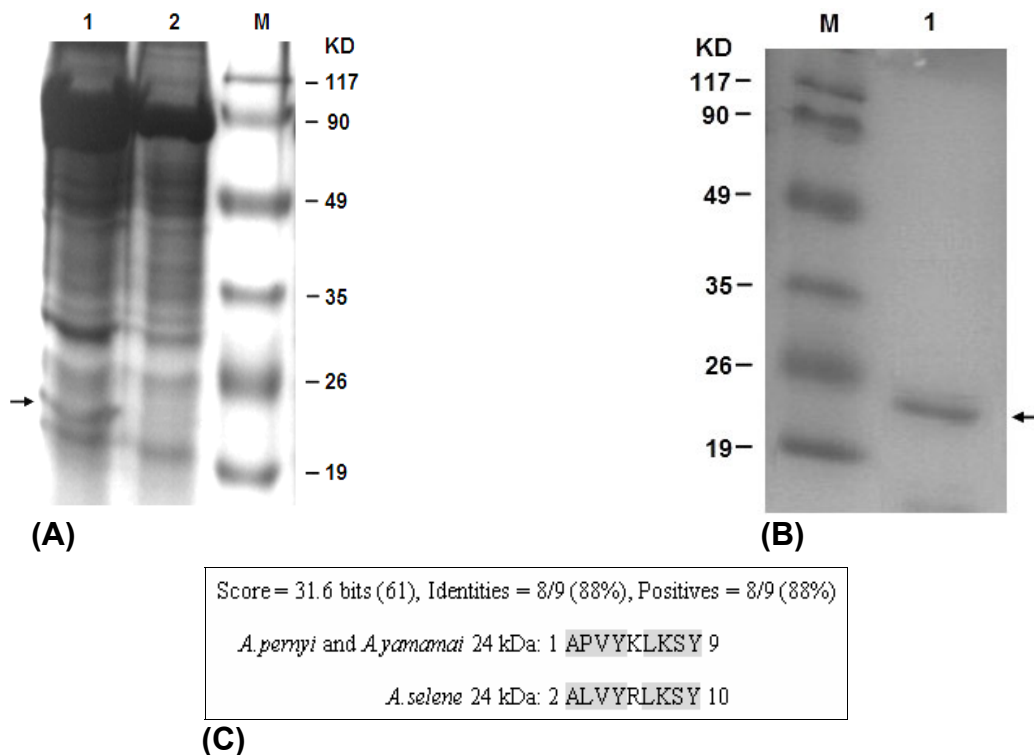


Figure 1. (A) SDS-PAGE of fat body proteins from pupa. A female-specific 24 kDa fat body protein was revealed. Lane 1, Fat body proteins from female pupa; Lane 2, Fat body proteins from male pupa. (B) Elution of 24 kDa protein fractions. (C) Alignment of N-terminal amino acid sequence of the *As-24K* using ExPASy BLAST. Identical residues are shaded with grey colour.

Partial sequence of *As-24K*

As shown in figure 1C, the partial amino acid residues in the N-terminal of *As-24K* protein exhibits 88% identity with female-specific fat body proteins isolated from *Antheraea pernyi* (Guerin-Meneville) and *Antheraea yamamai* (Guerin-Meneville) (Lepidoptera Saturniidae).

Cloning and sequence analysis of *As-24K* cDNA

The RNA extracted from fat body of female pupa was used for *As-24K* cloning. A cDNA fragment of about 300 bp was obtained by conventional PCR with F₁R₁ and RACE-PCR was performed with the primers RC3 and RC5 to clone full-length cDNA (figure 2). The cDNA (GenBank accession number JN400271) contains a 25-base 5'-untranslated sequence, an open reading frame of 624bp and a 183-base 3'-untranslated sequence (figure 3). Comparison of *As-24K* with 24 kDa proteins from *A. pernyi* and *A. yamamai* revealed 71% similarity in the entire sequence (figure 4A). Interestingly, this *As-24K* is also homologous to the glutathione S-transferase (GST) proteins from other insects according to the phylogenetic analysis (figure 4B).

Western blotting

The partial eluted *As-24K* proteins were used for the preparation of antiserum against rabbit and the titer of anti-*As-24K* antibody (about 1:8400) was determined by

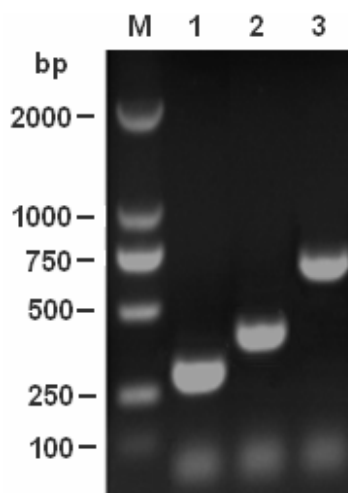


Figure 2. PCR amplification results of *As-24K* cDNA. Lanes 1-3, PCR products using primer pairs with F₁R₁, RC5 and RC3, respectively. M, marker.

ELISA. *As-24K* was detected in female fat body proteins by Western blotting while no immunoreactive band was found in male fat body proteins (figure 5). This result suggested that *As-24K* was a female-specific protein.

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1  gatttattgtaaatcgtctacaaacATGGCGCCCGTCTACAGACTCAAGTCCTACTATTA
                                     F1
1                                     M A P V Y R L K S Y Y Y
61  TGTCGGTGATGCAGAATCAATTAGGTATCTTCTCGCTTATGCTGGTATACGATTCGAGAA
                                     RC3
13  V G D A E S I R Y L L A Y A G I R F E N
121 CATTGTCCTCAAGAAGGATGTAACATGGCCTGAGCTACAGAAAACTCTACCGTTCGGCAA
33  I V L K K D V T W P E L Q K T L P F G K
181 ACTGCCGGTGTGGAAAGTCGATAATAAGGTGTTGTTCCAGAGCATACCCATTGACTTGGTA
53  L P V L E V D N K V L F Q S I P L T W Y
241 TGTTGCAAAAGGATCTTGGACTGTCCTCGAGTGACATCTTTGAAGAGGCGGAATTTGGTTC
73  V A K D L G L S S S D I F E E A E F G S
301 AATCTCTATAACCGTACATGAATTAGGCGAAAAAGTCGTCCAATGGTITGTCGAAGAAGA
                                     R1
93  I S I T V H E L G E K V V Q W F V E E D
361 CAAATATAAAAAAGAAAAATTGGAAACTGAACTGACCCAAAGTTGTTTTCCCAAATACCT
                                     RC5
113 K Y K K E K L E T E L T Q V V F P K Y L
421 GACTGACTTCGAAATCATTGCGCAGAAGAAAGGCGGATATTTGGAGGAAAGAAAATATC
133 T D F E I I A Q K K G Y F G G K K I S
481 CTGGTCTGACATTTACTTCGCGGCTATCCTTGTAAGTGTGCAAACCTATGGAATAAGGA
153 W S D I Y F A A A I G L V T V Q T L W N K D
541 TCTTCTTCAGAAGCAATATCCACTGCTGTACCAAATCTACAGGGATGTTACACATACD
173 L L Q K Q Y P L L Y Q I Y R D V H N I P
601 TGCAATCAAGGCCTACGTCACCAAATACGTGCCCGGCTACAAATATTAAttatttgctgt
193 A I K A Y V T K Y V P G Y K Y *
661 tcatttcacatataatacatatatttatataatatacatgcttatagaatcctacaat
721 acattatgtaggatatttctgtttcttattatcatatatactatttaattatatgtataca
781 ttttaccogttaaataatattttattttttcattcaaaaaaaaaaaaaaaaaaaaaa

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Figure 3. Nucleotide and amino acid sequence of *As-24K*. The primer sites for PCR are underlined and asterisk indicates the stop codon.

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MAPVYRLKSYYYVGD AESIRYLLAYAGIRFENIVLKKDVTWPELQRTL PFGKLPVLEVDN 60
MAPVYKLKSYDYVGV AESIRYLFAYGGIQYENIVLKKDITIIIEVQRTI PFGKLPVLAVND 60
MAPVYKLKSYDYVGV AESIRYLFAYGGIQYENIVLKKDITITEVQRTI PFGKLPVLAVND 60

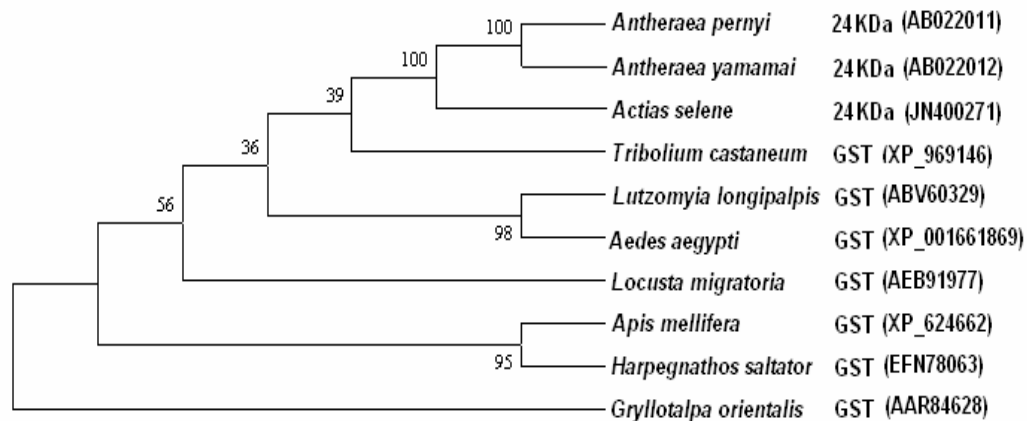
KVL FQSIPLT WYVAKDLGLS S SDIFEEAEFGSISITVHELGEKVVQWFVEEDKYKKEKLE 120
TILFHPVPICWYVGKNVGLTSDIDIEEAELGAISAAIFDLGEKVFQWYLEEDKYKKEKLE 120
TILFHPVPMCWYVGKNVGLTSDIDIEEAELGAISAAVFDLGEKVFQWYLEEDKYKKEKLE 120

TELTQVVF PKYLDFEIIAQKKG YFGGKKISWSDIYFAAILVTVQTLWNKDLLQKQYPL 180
SELQEDILPKYLDFEIIAQKKG YFGGKKISWSDVCFAGVLVTVQGVWKKDVLQKQYPL 180
SELQEDIY PKYLDFEIIAQKKG YFGGKKISWSDVCFAGVLVTVQGVWKKDVLQKQYPL 180

LYQIYRDVHNI PAIKAYVTKYVPGYKY 207 A. selene Similarity
LYQIYRDVHNI PAIKAYVSKNVATYKY 207 A. pernyi 71%
LYQIYRDVHNI PAIKAYVSKNVATYKY 207 A. yamamai 71%

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(A)



(B)

Figure 4. Phylogenetic analysis of *As-24K* based on the amino acid sequences. (A) Identical and similar amino acids conserved were represented in black and gray, respectively. (B) The phylogenetic tree was constructed by MEGA (version 4.0) program using the maximum parsimony method and bootstrap values (1000 repetitions) of the branches are indicated. GST represents glutathione S-transferase.

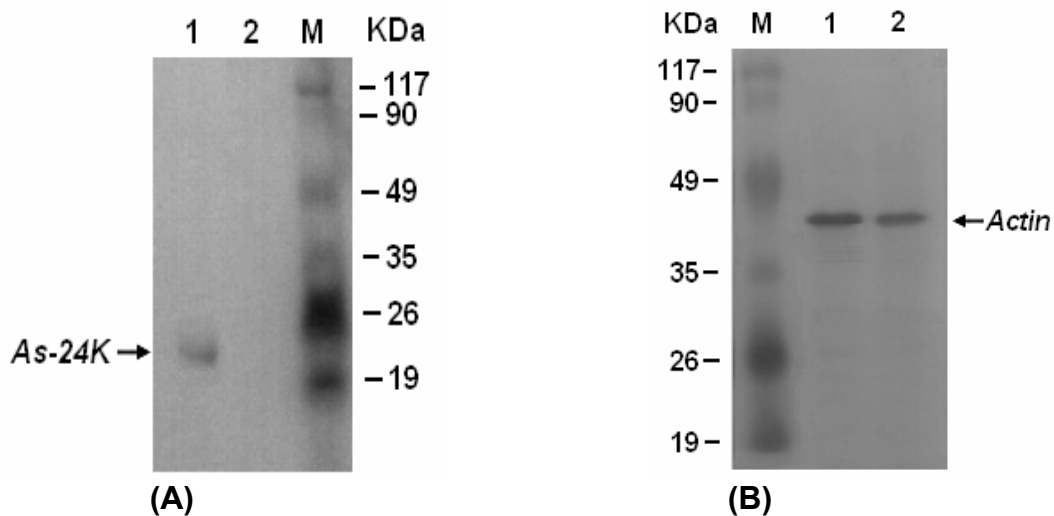


Figure 5. Western blot analysis of *As-24K* protein. (A) A 24 kDa protein was detected by Western blotting. No immunoreactive band was found in the fat body proteins from male pupa using antiserum. (B) Western blotting of actin protein was used as a control. Lane 1, fat body proteins from female pupa; Lane 2, fat body proteins from male pupa.

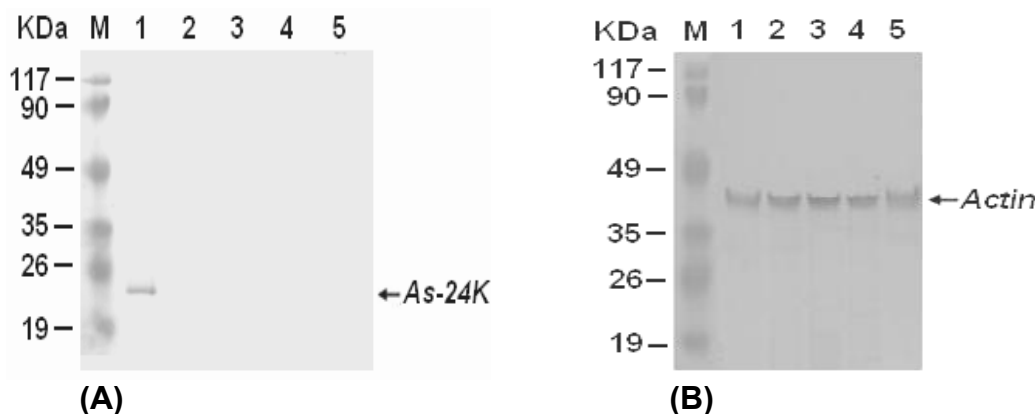


Figure 6. Expression of *As-24K* in different developmental tissues. **(A)** Lanes 1-5, Expression of *As-24K* in fat body, mid-intestine, blood, malpighian tube and ovary in female pupae, respectively. Expression of beta actin was used as an internal control **(B)**. M, prestained protein marker.

Expression of *As-24K* in different tissues

Total proteins were prepared from pupal fat body, mid-intestine, haemolymph, malpighian tubes and ovaries, and immediately analyzed by SDS-PAGE. Western blotting was carried out to detect the expression of *As-24K* using antiserum. *As-24K* expression was restricted to fat body and was not detectable in any other examined tissues (figure 6). These results suggest that the *As-24K* is a female-specific fat body protein.

Discussion

In this paper, a new 24 kDa female-specific protein was determined from *A. selene* and two conserved domains predicted by SMART (<http://smart.embl-heidelberg.de/>), GST (Glutathione S-transferase)-N (5-75 aa) and GST-C (84-192 aa), were found in *As-24K*. Phylogenetic analysis indicated *As-24K* was highly homologous to *A. pernyi* and *A. yamamai* 24 kD as far as the amino acid sequences are concerned. However, the *As-24K* also shows some similarity with glutathione S-transferases reported for several other insects (figure 4). Since Glutathione S-transferase plays a key role in detoxifying exogenous compounds (Yu, 1996) and is related with the resistance of insects (Fournier *et al.*, 1992; Kostaropoulos *et al.*, 2001; Ranson *et al.*, 2001; Willoughby *et al.*, 2006; Gui *et al.*, 2009), whether these 24 kD proteins are enzyme-like proteins remains to be explored.

The lack of expression of *As-24K* in different pupal tissues also suggested that it is restricted to the fat body, which is different from the expression profile of vitellogenin of *A. selene* in our previous study (Qian *et al.*, 2011). Furthermore, this 24 kD like protein could not be taken up by ovaries and is different from other female-specific proteins according to Yokoyama *et al.* (1994). All this suggest that *As-24K* is not a member of vitellogenins.

The results of this paper and studies by other authors (Yokoyama *et al.*, 1994) suggest that 24 kD protein was limited to the female fat bodies and its function remains unknown. Considering that some proteins secreted by female fat body was closely related with the egg devel-

opment of insect (Telfer and Melius, 1963; Tufail and Takeda, 2008), so whether this 24 kD protein is also involved in the embryonic development need to be further investigated.

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