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Molecular Cloning and Expression of a Novel Cuticle Protein Gene from the Chinese Oak Silkmoth, *Antheraea pernyi*

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In our research to identify gene involved in the cuticle protein, we cloned a novel cuticle protein gene, ApCP15.5, from the Chinese oak silkmoth, *Antheraea pernyi*, larvae cDNA library. The gene encodes a 149 amino acid polypeptide with a predicted molecular mass of 15.5 kDa and a pI of 9.54. The ApCP15.5 contained a type-specific consensus sequence identifiable in other insect cuticle proteins and the deduced amino acid sequence of the ApCP15.5 cDNA is most homologous to *Tenebrio molitor*-C1B (43% protein sequence identity), followed by *Locusta migratoria*-76 (42% protein sequence identity). Northern blot and Western blot analyses revealed that the ApCP15.5 showed the epidermis-specific expression. The expression profile of ApCP15.5 indicated that the ApCP15.5 mRNA expression was detected in the early stages after larval ecdysis and larval-pupal metamorphosis, and its expression level was most significant on the first day of larval ecdysis and pupal stage. The ApCP15.5 was expressed as a 15.5 kDa polypeptide in baculovirus-infected insect cells.

Key words: *Antheraea pernyi*, cDNA, Chinese oak silkmoth, Cuticle protein, Insect

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Introduction

The insect cuticle is an extracellular structure composed mainly of chitin and proteins that are synthesized and secreted by epidermal cells. The cuticle is highly diverse in appearances, in mechanical properties and in biological functions (Andersen *et al.*, 1995). Insect cuticle proteins have been identified and characterized in various species (Snyder *et al.*, 1982; Rebers and Riddiford, 1988; Charles *et al.*, 1992; Binger and Willis, 1994; Jensen *et al.*, 1997; Nakato *et al.*, 1997; Rondot *et al.*, 1998; Shofuda *et al.*, 1999; Togawa *et al.*, 2001; Sawada *et al.*, 2003; Kim *et al.*, 2003). Especially, larval cuticle proteins (LCPs) designated as LCP17, LCP18, LCP22 and LCP30 are well characterized in the silkworm, *Bombyx mori*. Biosynthesis of cuticle proteins is controlled stage-dependently and is regulated by the hormones ecdysteroid and juvenile hormone (Nakato *et al.*, 1994, 1997; Braquart *et al.*, 1996; Hiruma *et al.*, 1997; Kramer and Wolbert, 1998; Shofuda *et al.*, 1999). Interestingly, three cDNAs encoding larval cuticle proteins were cloned from the mulberry longicorn beetle, *Apriona germari*, and their cuticle protein mRNAs are differentially expressed in epidermis after larval ecdysis (Kim *et al.*, 2003).

Silkworm, *B. mori*, is well-known industrial insect, which produces natural fiber silk. The wild silkmoth also is a lepidopteran insect with a long history of significant agricultural value. Although the silkworm, *B. mori*, has been intensively studied, the genetic information in the wild silkmoths is still limited at the molecular and biochemical levels. No cuticle protein genes have been reported from the wild silkmoths.

In order to obtain further genetic information on the cuticle protein from wild silkmoth, we have cloned a novel cDNA encoding cuticle protein homologue of the larvae of the Chinese oak silkmoth, *Antheraea pernyi*. In this paper, the cloning, expression and characterization of *A. pernyi* cuticle protein gene (ApCP15.5) are described. The *A. pernyi* cuticle protein cDNA was expressed in baculovirus-infected insect cells and its antibody was produced for further study.

Materials and Methods

Insects

The larvae of the Chinese oak silkmoth, *Antheraea pernyi*, were reared indoors with the fresh leaves of Japanese oak, *Quercus acutissima*, at room temperature under the laboratory condition of a natural photoperiod.

cDNA library screening, nucleotide sequencing and data analysis

Total RNAs were isolated from the whole body of *A. pernyi* larvae following the procedure of Total RNA extraction kit (Promega). Poly(A)+ mRNA was purified using oligo(dT) columns of Quick mRNA isolation kit (Stratagene). A cDNA library was constructed from poly(A)+ mRNA isolated from the whole body of *A. pernyi* larvae by Uni-ZAP XR vector and Gigapack III Gold Packing Extract (Stratagene). The cDNA was ligated into *Eco*RI-*Xho*I sites of Uni-ZAP XR vector. Ligated library was transformed into *E. coli* XL1-Blue MRF' strain. *E. coli* XL1-Blue MRF' strain was infected by the Uni-ZAP XR library harboring *A. pernyi* cDNAs and cultured on the NZY agar medium. Each plaque was suspended in SM buffer [5.8 g/l NaCl, 2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 M TrisCl (pH 7.5) and 0.01% gelatin solution] containing 0.02% (v/v) chloroform and stored at 4°C for 1 day. The pages were eluted into SM buffer. The pBluescript phagemids were *in vivo* excised from the Uni-ZAP XR vector using an ExAssist helper phage. *E. coli* strain, SOLR cell (Stratagene), was infected by the excised phagemids and plated on LB-Amp medium (50 μg/ml ampicillin). Plasmid DNA from the overnight culture was isolated. The size of inserted cDNA was estimated with a 1% agarose gel electrophoresis after treatment of restriction enzymes (*Eco*RI and *Xho*I). For DNA sequencing, plasmid DNA was extracted by Wizard mini-preparation kit (Promega). Sequence of the 5' end of each cDNA clone was determined using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were translated into 6 reading frames and compared using the DNASIS and BLAST programs provided by the NCBI. GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program (www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd) was used to align the amino acid sequences of cuticle protein gene. With the GenBank-registered cuticle protein sequences, phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000).

RNA isolation and Northern blot analysis

The larvae of *A. pernyi* were dissected under the Stereomicroscope (Zeiss, Jena, Germany), individual samples such as fat body, midgut, 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 whole body, midgut, fat body, and epidermis of the *A. pernyi* larvae by using the Total RNA Extraction Kit (Promega). Total RNA (10 μg/lane) from the *A. pernyi* larvae was denatured by glyoxalation (McMaster and Carmichael 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 *A. pernyi* cuticle 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.

Cell culture and virus

The insect Sf9 cells (Vaughn *et al.*, 1977) were maintained at 27°C in TC100 medium (GIBCO BRL LIFE Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS; GIBCO BRL LIFE Technologies) as described by standard methods (O'Reilly *et al.*, 1992). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV were propagated in Sf9 cells. The titer was expressed as plaque forming units (PFU) per ml (O'Reilly *et al.*, 1992).

Construction of baculovirus transfer vector and recombinant virus

The 632 bp ApCP15.5 cDNA from pBlueScript-GoPrx was subcloned between *Eco*RI and *Xho*I sites of pBacPAK9 (Clontech, Palo Alto, CA) to produce transfer vector pBacPAK9-ApCP15.5. In the transfer vector, the ApCP15.5 cDNA is under the control of the AcNPV poly-

hedrin promoter. One microgram of BacPAK6 viral DNA (Clontech), five μg of pBacPAK9- ApCP15.5 in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty microliters of 100 μg/ml Lipofectin™ (GIBCO BRL LIFE Technologies) were gently mixed with the DNA solution and the mixture was incubated at room temperature for 30 min. The cells ($1.0 - 1.5 \times 10^6$ cells per 35-mm cell culture dish) were washed twice with 2 ml serum-free TC100 medium and refed with 1.5 ml serum-free TC100 medium. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 hrs, TC100 medium containing antibiotics and 10% FBS was added to each dish and incubation at 27°C was continued. At 5 days of postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C. Recombinant AcNPV was plaque purified on 6-well plates seeded with 1.5×10^6 Sf9 cells as described by O'Reilly *et al.* (1992).

SDS-polyacrylamide gel electrophoresis (PAGE)

Insect Sf9 cells were mock-infected or infected with the wild-type AcNPV and recombinant AcNPV in a 35-mm cell culture dish (1×10^6 cells) at a multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27°C, cells were harvested at 3 days p.i. For SDS-PAGE (Laemmli, 1970) of cell lysates, samples were washed twice with PBS and mixed with protein sample buffer and boiled. The total cellular lysates were subjected to 10% SDS-PAGE. After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250.

Preparation of polyclonal antiserum and Western blot analysis

The recombinant ApCP15.5 (about 10 μg) extracted from the gels was mixed with equal volume of Freund's complete adjuvant (a total of 200 μl; Sigma, St. Louis, MO) and injected into Balb/c mice, respectively. Three successive injections were performed with one-week interval beginning a week after the first injection with antigens mixed with equal volume of Freund's incomplete adjuvant (a total of 200 μl, Sigma). Blood was collected 3 days after the last injection and centrifuged at 13,000 rpm for 5 min. The supernatant antibodies were stored at –70°C until use. For Western blot analysis, 10% SDS-PAGE was performed as described above. Proteins were blotted to a sheet of nitrocellulose transfer membrane (Schleicher & Schuell) (Towbin *et al.*, 1979). The blotting was performed in transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 30 volts overnight at 4°C. After blotting, the membrane was blocked by incubation in 1% BSA solution for 2 hrs at room temperature. The blocked membrane was incubated with antiserum solution (1:1000 v/v) for 1 hr at room temperature and washed in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20). Subsequently, the membrane was incubated with anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Pharmacia Biotech) and finally exposed to autoradiography film.

Results and Discussion

Cloning, sequencing, and characterization of ApCP15.5 cDNA

A cDNA library was constructed using whole bodies of A. pernyi larvae. Sequencing of randomly selected clones harboring cDNA inserts was performed to generate A. pernyi ESTs (expressed sequence tags). One clone, which is 632 bp-long had a full-length coding sequence similar to that of previously reported cuticle proteins (Andersen et al., 1993, 1997). The nucleotide and deduced amino acid sequences of a cDNA encoding a putative member of the insect cuticle protein family are presented in Fig. 1. The ApCP15.5 cDNA contains an ORF of 447 bp encoding 149 amino acid residues with a predicted molecular mass of 15.5 kDa and pI of 9.54, and we designated ApCP15.5.

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-33                                          GCGAGCTTTTAAGGGTTTGTACTGCTGGAG
  1 ATGATGTTAAAACTTCTTCTGTTTTGTTGTGCCATGGTGGCAGTCCATGGCGGAGCGCTT
  1 M  M  L  K  L  L  L  F  C  C  A  M  V  A  V  H  G  G  A  L

 61 ATTTCTCCAGTGTACGGTGCTCCTTATAGCTATGGTTCTTGGAATCCATACAGCTCGTAT
 21 I  S  P  V  Y  G  A  P  Y  S  Y  G  S  W  N  P  Y  S  S  Y

121 CCATCAACACCTGCATTGGCTTCACAGCATTCTAACACGTACAGGTCTCCGTTTAATCTA
 41 P  S  T  P  A  L  A  S  Q  H  S  N  T  Y  R  S  P  F  N  L

181 GGACAGGTATCTACATACTCGAAATCTGTTGACACCCCATTCTCTAGTGTGCGTAAGGCA
 61 G  Q  V  S  T  Y  S  K  S  V  D  T  P  F  S  S  V  R  K  A

241 GACATTCGCGTTAGCAACCCTGGCGTTGCAGTATCCCCTGCTTACAGCGGTTTTGCTGCT
 81 D  I  R  V  S  N  P  G  V  A  V  S  P  A  Y  S  G  F  A  A

301 CCATACGTCTCTCATGTCGGTTTGGCCGCGCCACTAACAGCTCCCGTCGCTAAAGTTGCC
101 P  Y  V  S  H  V  G  L  A  A  P  L  T  A  P  V  A  K  V  A

361 ACTGGACTCTTAGGTGTGGCATACTCAGCAGCTCCTGCTGTGTCCCACATGACATATACC
121 T  G  L  L  G  V  A  Y  S  A  A  P  A  V  S  H  M  T  Y  T

421 AACGGACTCGGCCTTGCATATGCCTGGTAATTAACGCACGCAACGTCGATCATAATTGAT
141 N  G  L  G  L  A  Y  A  W  *

481 TCCATTGTATTGTTTAAAGATTCCTTTTTATTTGTTACCTGATAGTTGTTAACATTATTT

541 CATTTACCTAACTTTAGTTATATTACAATAAATATTAAAACTAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide and deduced amino acid sequences of ApCP15.5 cDNA. The start codon of ATG is boxed and the termination codon is shown by asterisk. The polyadenylation signal is shaded. The GenBank accession number is AY438329.

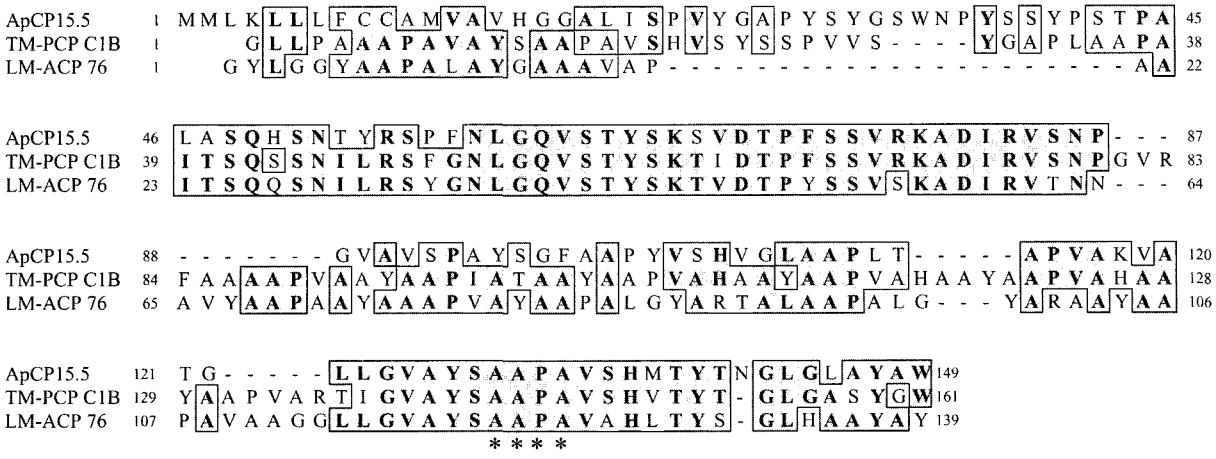

Fig. 2. Multiple sequence alignment of the deduced protein sequence of ApCP15.5 cDNA with related insect cuticle protein sequences. The identical residues are shown in solid boxes. Asterisks indicate the cuticle protein consensus sequence of AAPA/V motif. The abbreviation and GenBank accession number for the cuticle protein sequences analyzed are: ApCP15.5, *A. pernyi* cuticle protein (AY438328; this study); TM-PCP C1B, *T. molitor* cuticle protein (P80684); LM-ACP 76, *L. migratoria* cuticle protein (P45588).

This cDNA sequence has been deposited in GenBank under accession number AY438329.

A multiple sequence alignment of the deduced protein sequence of ApCP15.5 cDNA among related cuticle protein sequences is shown in Fig. 2. Alignment of these ApCP15.5 sequences with those of cuticle proteins from two species indicates the extent of the identity that exists. The ApCP15.5 contained cuticle protein consensus sequence of AAPA/V motif, present in a hydrophobic region near the C-terminal end (Andersen *et al.*, 1995). This consensus sequence was conserved among 3 cuticle protein sequences from dipteran, coleopteran and orthopteran species, suggesting that ApCP16.4 is a putative member of the insect cuticle protein family. In addition, the ApCP15.5 showed 43% protein sequence identity to the *Tenebrio molitor* C1B and 42% to the *Lucusta migratoria* ACP76 (Fig. 3).

Expression of ApCP15.5 mRNA

To confirm the tissue-specific expression of the ApCP15.5 at transcriptional level, Northern blot analysis was performed using mRNA prepared from epidermis,

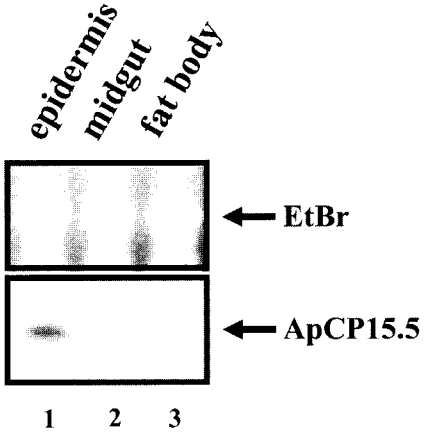

Fig. 4. Tissue-specific expression of ApCP15.5 mRNA. Total RNA was isolated from the whole body (lane 1), epidermis (lane 2), midgut (lane 3) and fat body (lane 4) on the first day of larval ecdysis of *A. pernyi* larva, respectively. The RNAs were separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred on to a nylon membrane, and hybridized with the radiolabelled ApCP15.5 cDNA probe (lower panel). Transcripts of ApCP15.5 are indicated on the right of the panel by arrow.

Species	GenBank No.	Percent similarity			
			1	2	3
ApCP15.5	**AY438329**	1		52	52
TM-PCP C1B	P80684	2	43		56
LM-ACP 76	P45588	3	42	47	
		Percent identity			

Fig. 3. Pairwise identities and similarities of the deduced amino acid sequence of ApCP15.5 with insect cuticle protein sequences. The abbreviation and GenBank accession number for the cuticle protein sequences aligned are described in Fig. 2 legend.

midgut and fat body (Fig. 4). Hybridization signal was detected as a single band of mRNA from the whole body as a positive control and epidermis, indicating that the ApCP15.5 is expressed only in the epidermis. The result is consistent with the previous reports in that the cuticle protein gene is tissue-specifically expressed in epidermis (Rebers *et al.*, 1997; Nakato *et al.*, 1997; Shofuda *et al.*, 1999; Togawa *et al.*, 2001; Kim *et al.*, 2003; Sawada *et al.*, 2003).

To verify the expression profile of ApCP15.5 after larval ecdysis and larval-pupal metamorphosis, total RNA was prepared from the epidermis with various intervals and analyzed by Northern blot hybridization (Fig. 5). The ApCP15.5 mRNA is expressed as a high level on the first day of larval ecdysis and after larval-pupal metamorphosis. The level of ApCP15.5 mRNA dramatically declined at the third day and disappeared at the fourth day after larval ecdysis and larval-pupal metamorphosis.

The insect cuticle protein gene is controlled stage-dependently and is regulated by the ecdysteroid and juvenile hormone (Nakato *et al.*, 1994, 1997; Braquart *et al.*, 1996; Hiruma *et al.*, 1997; Kramer and Wolbert, 1988; Shofuda *et al.*, 1999). The expression of larval cuticle protein mRNA was detected at early stage after the molt (Nakato *et al.*, 1997; Shofuda *et al.*, 1999; Sawada *et al.*, 2003). Cuticle proteins are intensively studied from some insects such as *B. mori* and *D. melanogaster*. Especially, five LCP genes are cloned from *B. mori* and the developmental profiles and/or hormone regulation of each LCP gene are investigated (Nakato *et al.*, 1994, 1997; Shofuda *et al.*, 1999; Sawada *et al.*, 2003). In *B. mori*, a large amount of two LCP mRNAs, LCP17 and LCP22, was detected on the day of the fourth molt, and the level of expression was maintained until the fourth day of the final larval instar (Nakato *et al.*, 1997). Level of LCP18 mRNA is higher expressed from the day of each larval ecdysis to the second day than that of the fourth day (Shofuda *et al.*, 1999). The strongest signal of BMCPA (*B. mori* cuticle protein A-A-P-A/V-repeat) mRNA was recognized on the second day of the fifth-instar (Sawada *et al.*, 2003). Larval cuticle proteins are made in dipteran and lepidopteran larvae continuously as the larva feeds and grows (Locke, 1998). The mRNAs for these proteins are therefore usually continually present during the intermolt and then shut off during the molt in response to the high ecdysteroid (Riddiford *et al.*, 1986; Rebers and Riddiford, 1988; Hiruma *et al.*, 1991). Lamp and Willis (1994) reported that the cuticle protein (HCCP*66*) found in the rigid cuticles of both larvae and pupae of the giant silkmoth, *Hyalophora cecropia*. Taken these results together, our result of ApCP15.5 is the first to show expression of cuticle protein in wild silkmoth, *A. pernyi*, and clearly shows that there are likely different mechanisms of control during larval and pupal stages.

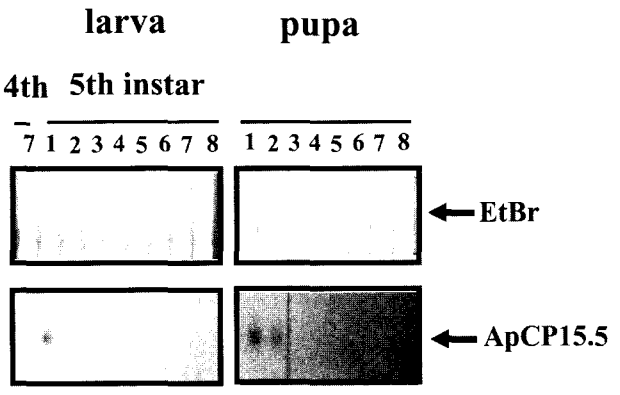

Fig. 5. Expression profiles of ApCP15.5 mRNA after larval ecdysis and larval-pupal metamorphosis. Total RNA was isolated from the epidermis after larval ecdysis and larval-pupal metamorphosis. Developmental stage is indicated on the top of each lane. The RNAs were separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred on to a nylon membrane, and hybridized with the radiolabelled ApCP15.5 cDNA probe (lower panel). Transcripts of ApCP15.5 are indicated on the right of the panel by arrow.

Expression of ApCP15.5 cDNA in baculovirus-infected insect cells

To assess ApCP15.5 cDNA, the 447 bp for ApCP15.5 cDNA was inserted into baculovirus transfer vector. The

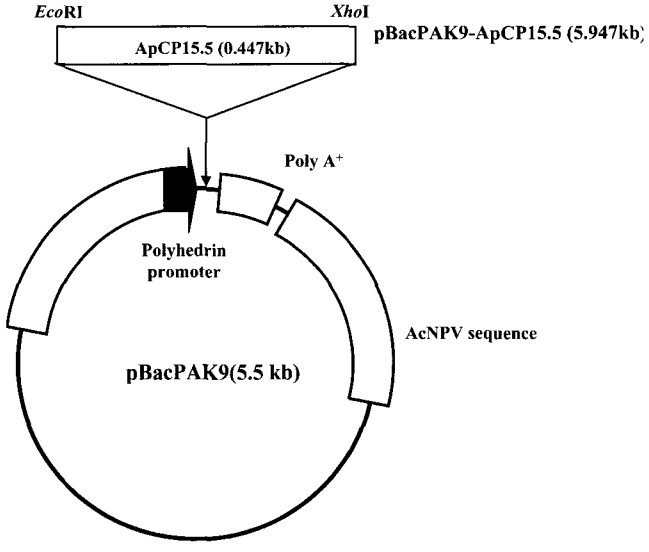

Fig. 6. Structure of the transfer vector used to generate the recombinant baculovirus. The transfer vector pBacPAK9-ApCP15.5 was constructed by insertion of the *A. pernyi* cuticle protein gene into pBacPAK9 under the control of AcNPV polyhedrin promoter.

baculovirus transfer vector was used to generate a recombinant virus expressing ApCP15.5. Transfer vector pBacPAK9-ApCP15.5 was constructed by insertion of ApCP15.5 cDNA under the control of AcNPV polyhedrin promoter of pBacPAK9 (Fig. 6). Recombinant AcNPV, which we have termed AcNPV- ApCP15.5, was produced in insect Sf9 cells by cotransfection with wild-type AcNPV DNA and the transfer vector.

To examine the expression of ApCP15.5 cDNA by recombinant virus in insect cells, SDS-PAGE and Western blot analysis were performed to analyze the protein synthesis in Sf9 cells infected with the recombinant virus (Fig. 7). The recombinant ApCP15.5 was present as a single band of about 15.5 kDa polypeptide in the cells infected with the recombinant virus, but not in the cells infected with the wild-type AcNPV or mock-infected cells.

Expression of ApCP15.5 in *A. pernyi* larva

Tissue specific expression of ApCP15.5 was analyzed from the protein samples of epidermis, midgut, fat body, and hemolymph of *A. pernyi* larva. Each protein sample was subjected to 10% SDS-PAGE and Western blot analysis (Fig. 8). A signal band of 15.5 kDa was detected specifically from the epidermis in the Western blot analysis,

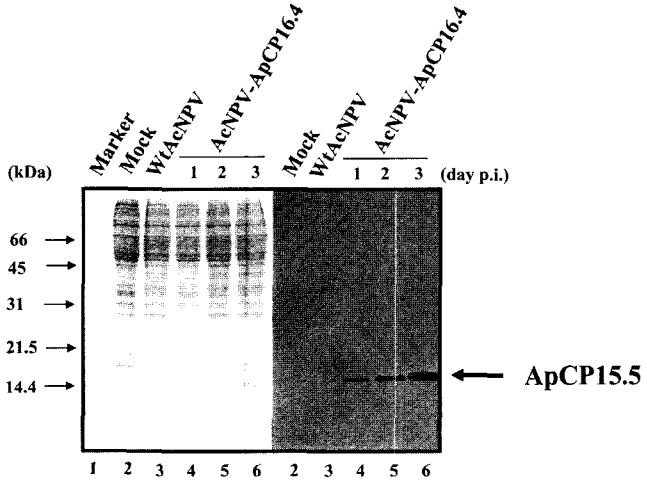

Fig. 7. SDS-PAGE and Western blot analysis of the recombinant ApCP15.5 expressed in baculovirus-infected insect cells. Sf9 cells were mock-infected (lane 2) or infected with wild-type AcNPV (lane 3) and recombinant AcNPV (lanes 4, 5 and 6) at a MOI of 5 PFU per cell. Cells were collected at 1 (lane 4), 2 (lanes 2 and 5) and 3 (lane 6) days p.i. Total clellular lysates were subjected to 10% SDS-PAGE (left panel), electroblotted and incubated with recombinant ApCP15.5 antibody (right panel). Molecular weight standards (lane 1) were used as size marker. The recombinant ApCP15.5 is indicated by arrow on the right side of the panel.

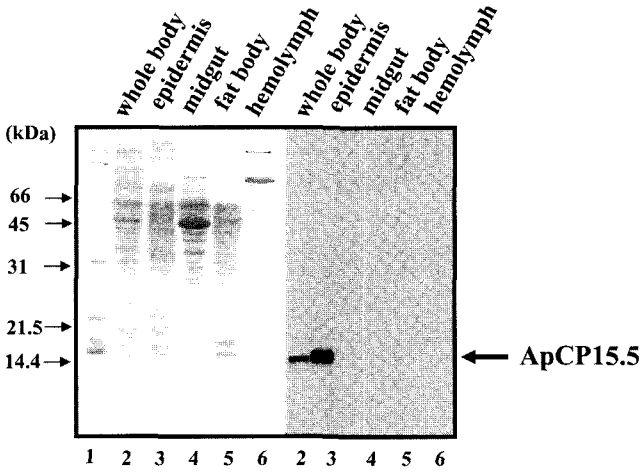

Fig. 8. Expression of ApCP15.5 in *A. pernyi* larva. The protein samples were collected from whole body (lane 2), epidermis (lane 3), midgut (lane 4), fat body (lane 5), and hemolymph (lane 6) of *A. pernyi* larva. The protein samples were subjected to 10% SDS-PAGE (left panel), electroblotted and incubated with recombinant ApCP15.5 antibody (right panel). Molecular weight standards (lane 1) were used as size marker. The ApCP15.5 in *A. pernyi* larvae is indicated by arrow on the right side of the panel.

but not in the midgut, fat body and hemolymph protein samples. The result is in good agreement with the Northern blot hybridization result that ApCP15.5 showed the tissue-specific expression in the epidermis.

In conclusion, we have cloned a novel cDNA encoding cuticle protein from the wild silkmoth, *A. pernyi*. We hope that the molecular characterization of cuticle protein in *A. pernyi* will expand the understanding of insect cuticle proteins.

Acknowledgements

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