

Molecular Cloning and Characterization of a Vitellogenin of the Bumblebee *Bombus ignitus*

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A vitellogenin cDNA was cloned from the bumblebee *Bombus ignitus*. The cDNA encoding *B. ignitus* vitellogenin (BiVg) is 5473 bases long with an open reading frame of 1773 amino acid residues. BiVg possesses two consensus (RXXR/S) cleavage sites and has the conserved DGXR and GL/ICG motif near its C-terminus. The deduced amino acid sequence of *BiVg* cDNA showed significant similarity with hymenopteran Vgs (51% identity to *Apis mellifera* Vg, and 33~36% to other insect Vgs). The *BiVg* cDNA was expressed as a 200-kDa polypeptide in baculovirus-infected insect Sf9 cells. Northern and Western blot analyses showed the expression of BiVg in fat bodies of pupae and adults. In queens, the expression of BiVg was first detected in late pupal stage fat bodies, and secreted BiVg was also observed in late pupal stage hemolymph.

Key words: *Bombus ignitus*, Bumblebee, Hemolymph, Insect, Vitellogenin

Introduction

Vitellogenins (Vgs) are the precursors of the major egg yolk proteins, vitellins (Vts), in many oviparous vertebrates and invertebrates. In most insect species, Vgs are large molecules (~200 kD) that are synthesized in the fat body cell, in a tissue-, female- and stage-specific manner. These proteins are secreted into the hemolymph and sequestered by the developing oocytes via receptor-mediated endocytosis (Raikhel and Dhadialla, 1992; Sapping-

ton and Raikhel, 1998; Swevers *et al.*, 2005). During these processes, Vgs/Vts are modified by proteolytic cleavage, glycosylation, lipidation and phosphorylation and are used as a nutritional source for the developing embryo after incorporation into the oocytes (Bownes, 1986; Byrne *et al.*, 1989; Raikhel & Dhadialla, 1992; Hagedorn *et al.*, 1998; Giorgi *et al.*, 1999).

The Vgs have been reported extensively in a wide group of animals, both vertebrates and invertebrates, including insects. In insects, Vgs have been discovered in 25 insect species belonging to six different orders. In insects, the deduced Vg amino acid sequences show the highly conserved GL/ICG motif followed by nine cysteine residues, as occurs in all hymenopteran species and, as in other insect Vgs, a DGXR motif is located 18 residues upstream of the GL/ICG motif near the C-terminus (Chen *et al.*, 1997; Lee *et al.*, 2000a; Tufail *et al.*, 2000; Piulachs *et al.*, 2003). Vg synthesis in insect species is regulated at the transcriptional level by sex-, tissue- and hormone-mediated developmental specificities (Bownes, 1986). The regulation of Vg production has been studied in the honeybee *Apis mellifera*. Appearance of Vg in the hemolymph occurs during the pupal stage, but the timing is different between queens and workers. In queens, Vg appears in the mid-late pupal stage, whereas, in workers, it is detected in the late pupal stage (Engels *et al.*, 1990; Barchuk *et al.*, 2002; Piulachs *et al.*, 2003).

Although information on Vgs has been reported in some hymenopterans, including the honeybee, information on Vg of *B. ignitus* (Hymenoptera: Apidae), an important pollinator, is still unknown. In this study, we report the cloning and developmental expression profiles of the Vg gene at both the transcriptional and translational levels from the bumblebee *B. ignitus*.

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Materials and Methods

Insects

The bumblebees, *B. ignitus*, were reared under artificial conditions (28°C, 65% relative humidity, continuous darkness) in the Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Republic of Korea, as described previously (Yoon *et al.*, 2002, 2004).

cDNA library screening, nucleotide sequencing and data analysis

Clones harboring the cDNA insert were selected from expressed sequence tags (ESTs) from a cDNA library that had been generated using the entire bodies of *B. ignitus* (Choi *et al.*, 2006, 2008). The plasmid DNA was extracted using the Wizard mini-preparation kit (Promega, Madison, WI, USA) and sequenced using an ABI 310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST programs provided by NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). MacVector (ver. 6.5, Oxford Molecular Ltd.) was used to align the amino acid sequences of Vg. With the 14 GenBank-registered vitellogenin amino acid sequences, phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000). The accession numbers of the sequences in the GenBank are as follows: *B. ignitus* (this study: FJ606797), *A. mellifera* (AJ517411), *Solenopsis invicta* (AF512520), *Encarsia formosa* (AY553878), *Pimpla nipponica* (AF026789), *Plautia stali*-Vg1 (AB033498), *P. stali*-Vg2 (AB033499), *P. stali*-Vg3 (AB033500), *Riptortus clavatus* (U97277), *Graptosaltria nigrofusca* (AB026848), *Aedes aegypti* (U02548), *Anopheles stephensi* (DQ442990), *Anthonomus grandis* (M72980), *Bombyx mori* (D13160) and *Lymantria dispar* (U90756).

Production of recombinant protein

A baculovirus expression vector system (Je *et al.*, 2001), consisting of *Autographa californica* nucleopolyhedrovirus (AcNPV) and the insect cell line Sf9, was employed for the production of recombinant BiVg proteins. A cDNA fragment containing the full-length *BiVg* open reading frame (ORF) was excised from pBlueScript-*BiVg* by digestion with *SacI* and *NotI* and inserted into the same sites of the transfer vector pBacPAK9 (Clontech, Palo Alto, CA, USA) for expression of BiVg under the control of the AcNPV polyhedrin promoter. Five hundred nanograms of the construct (pBacPAK9-*BiVg*) and 100 ng of the AcNPV viral DNA (bAcGOZA) (Je *et al.*, 2001) were co-transfected into $1.0\text{--}1.5 \times 10^6$ Sf9 cells for 5 hours using

Lipofectin reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultivated in TC100 medium (Gibco BRL) at 27°C for 5 days. The recombinant AcNPV was propagated in Sf9 cells, and the titer was expressed as plaque-forming units (PFU) per milliliter according to standard methods (Je *et al.*, 2001).

SDS-polyacrylamide gel electrophoresis (PAGE)

Sf9 cells infected with the recombinant virus were washed three times with PBS, mixed with protein sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, and 0.125% bromophenol blue) and boiled for 5 min. The total cellular lysates were subjected to 7.5% SDS-PAGE (Laemmli, 1970). Following electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250.

Extraction of proteins

Fat bodies were extracted from *B. ignitus* specimens and placed in cold PBS containing 1 mM PMSF (phenylmethyl-sulfonylfluoride) and a few crystals of phenylthiourea. After washing, the gut regions were homogenized in PBS, and the homogenate was then centrifuged at $10,000 \times g$ for 10 min and the supernatant was stored at -70°C until use. Hemolymph was collected from *B. ignitus* specimens in cold test tube. A few crystals of phenylthiourea were added to the test tubes to prevent melanization. The collected hemolymph was centrifuged at $10,000 \times g$ for 10 min to remove hemocytes and cell debris, and the supernatant was stored at -70°C until used. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad). The protein samples from the gut were subjected to SDS-PAGE and Western blot analysis.

Polyclonal antibody production

The recombinant BiVg expressed in the baculovirus-infected insect cells was electroeluted from the gel, mixed with equal volume of Freund's complete adjuvant (a total of 200 μl , Sigma) and injected into Balb/c mice. Three successive injections were administered with antigens mixed with equal volumes of Freund's incomplete adjuvant (a total of 200 μl) at one-week intervals beginning one week after the first injection. Blood was collected 3 days after the last injection with antigens only and centrifuged at $10,000 \times g$ for 5 min. The supernatant antibodies were stored at -70°C until use.

Western blot analysis

Western blot analysis was performed using an enhanced chemiluminescence (ECL) Western Blotting Analysis System (Amersham Biosciences). The protein samples were mixed with the sample buffer, boiled for 5 min and loaded

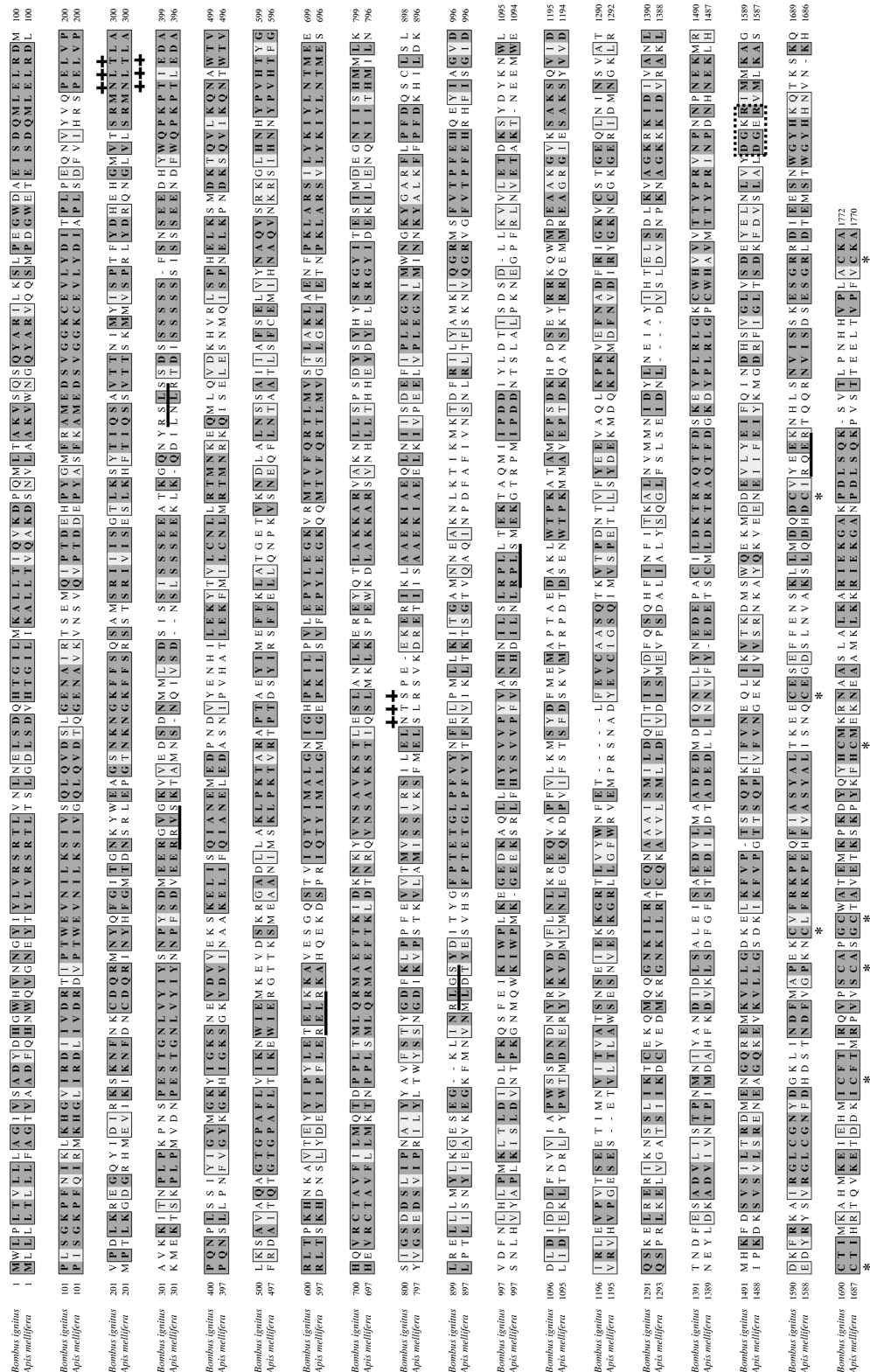


Fig. 1. Alignment of the amino acid sequences of *BiVg* and *A. mellifera* vitellogenin. Identical residues are shown in solid boxes. Dashes represent gaps introduced to preserve alignment. The conserved GLCG motif and DGXR motif are shown by a solid box and dotted box, respectively. Asterisks indicate conserved nine cysteine residues and the putative cleavage sites (RXRX/S) are indicated by underline. One glycosylation site, which may be effectively glycosylated according to NetNGly 1.0 Prediction Server, is denoted by +. GenBank accession numbers are *B. ignitus* (this study: FJ606797) and *A. mellifera* (AJ517411).

on a 10% SDS-PAGE gel as described above. The proteins were blotted onto a sheet of nitrocellulose transfer membrane (Schleicher & Schuell). After blotting, the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution, after which the membrane was incubated with antiserum solution (1:1000 v/v) at room temperature for 1 hr and then washed in TBST [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) Tween 20]. The membrane was incubated with 1:5,000 (v/v) diluted anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiographic films.

RNA isolation and Northern blot analysis

To confirm the expression profile in the fat body of pupal and adult stages, fat body tissues were collected at various time intervals and washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄,

1.5 mM KH₂PO₄, pH 7.4). Total RNA was isolated using a Total RNA Extraction Kit (Promega). Harvested total RNA (5 µg/lane) was separated on a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in hybridization buffer containing 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA. A short fragment of the cDNA at position 4741-5459 was labeled with [α-³²P]-dCTP (Amersham, Arlington Heights, IL, USA) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) and was then used 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 at 65°C and then exposed to autoradiography film.

Results and Discussion

Cloning, sequencing and data analysis of *BiVg* gene

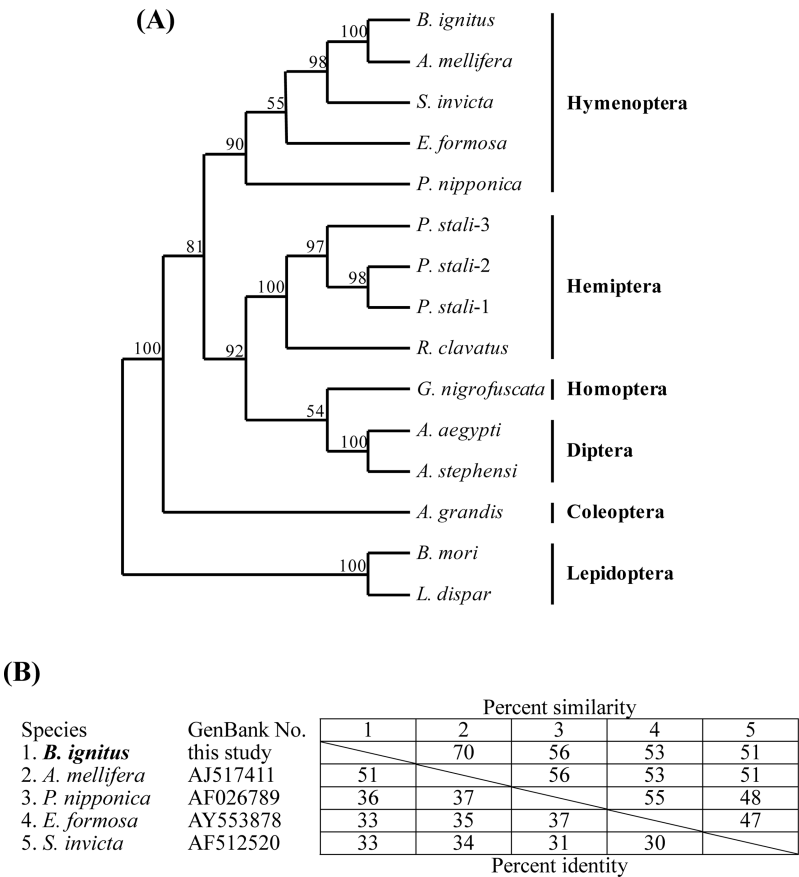


Fig. 2. Relationships among amino acid sequences of BiVg and known Vgs. (A) A maximum parsimony analysis for the amino acid sequences of BiVg and known Vgs derived from insects. The sequence sources are described in Materials and Methods. The tree was obtained by bootstrap analysis with the option of heuristic search, and the numbers on the branches represent bootstrap values for 1,000 replicates. (B) Pairwise similarities and identities of the deduced amino acid sequence of BiVg among Vg sequences.

After a detailed examination of *B. ignitus* ESTs, *BiVg* cDNA was identified as having homology to previously reported vitellogenin genes. The cDNA clone, including the full-length open reading frame (ORF), was sequenced and characterized. The *BiVg* cDNA is 5473 bases long and contains an ORF of 5319 nucleotides capable of encoding a 1773 amino acid polypeptide. The ORF had both a start (ATG) and stop codon (TAA), indicating that the sequence contained the complete coding region. A putative polyadenylation signal, AATAAA, was located at nucleotide positions 5415-5421. The calculated molecular mass and pI of BiVg were 201.8 kDa and 5.76, respectively. This *BiVg* cDNA sequence has been deposited in GenBank under accession number FJ606797.

A sequence alignment of the protein sequence deduced from *BiVg* cDNA with the *A. mellifera* Vg sequence is shown in Fig. 1. There were two putative cleavage sites at positions 369-372 (RSLS) and 918-921 (RLGS) from *B. ignitus*, but four putative cleavage sites are known in *A. mellifera* (Piulachs *et al.*, 2003). A GL/ICG motif site at position 1598-1601 and nine cysteine residues that followed at conserved locations near the C-terminus were present. It was noted that a DGXR motif, starting 18 residues upstream of the GL/ICG motif, was conserved among insect Vgs (Chen *et al.*, 1997; Comas *et al.*, 2000; Lee *et al.*, 2000a, b; Tufail *et al.*, 2000, 2001). Short serine-rich stretches were also found at positions 354-380. A glycosylation site, which may be effectively glycosylated according to NetNGly 1.0 Prediction Server, was well conserved.

A phylogenetic analysis using the deduced amino acid sequences of known insect Vg genes revealed that the Vgs can be divided into several groups (Fig. 2A). The deduced amino acid sequences of the Vg gene from hymenopteran insects, including the *BiVg* gene, together formed a subgroup, excluding the other orders. *BiVg* showed highest protein sequence identity to *A. mellifera* (51% identity), *P. nipponica* (36%), *E. formosa* (33%) and *S. invicta* (33%) (Fig. 2B).

Expression of the recombinant BiVg

To assess the *BiVg* gene, a 5473-bp fragment encompassing the *BiVg* cDNA was inserted into baculovirus transfer vector. Transfer vector pBacPAK9-BiVg was constructed by insertion of the *BiVg* gene such that it would be controlled by the AcNPV polyhedrin promoter of pBacPAK9 (data not shown). The baculovirus transfer vector was used to generate a recombinant virus expressing *BiVg*. The recombinant AcNPV, which we have termed AcNPV-BiVg, was produced in insect Sf9 cells by cotransfection with wild-type AcNPV DNA and the transfer vector.

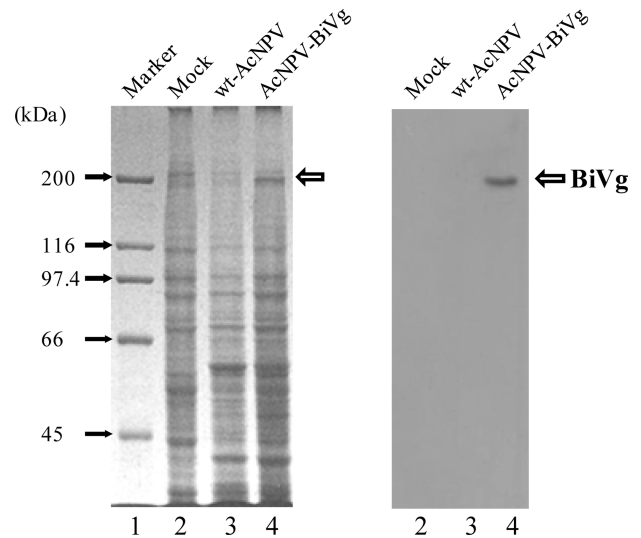


Fig. 3. Expression of the BiVg in baculovirus-infected insect cells. SDS-PAGE analysis of the BiVg expressed in recombinant baculovirus (AcNPV-AgCell)-infected insect cells. Sf9 cells were mock-infected (lane 2) or infected with the wild-type AcNPV (lane 3) and with the recombinant AcNPV (lanes 4) at a multiplicity of infection (MOI) of 5 PFU per cell. Cells were collected at 3 days post-infection (p.i.). Total cellular lysates were subjected to 7.5% SDS-PAGE (left) and Western blot analysis (right). The arrow to the right of the panel indicates the 200-kDa recombinant BiVg polypeptide. Molecular weight standards were used as size markers (lane 1).

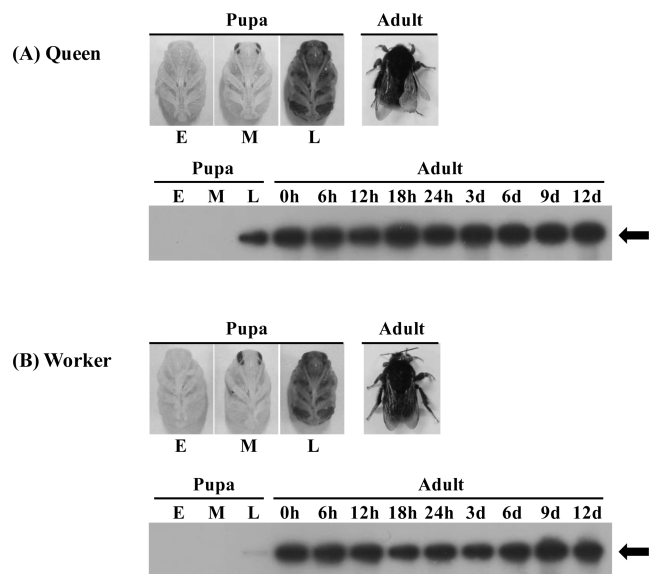


Fig. 4. Northern blot analysis of *BiVg* mRNA in *B. ignitus* queens (A) and workers (B). Fat body samples were collected at various time points, and the RNA was separated by 1.0% formaldehyde agarose gel electrophoresis, transferred on to a nylon membrane and hybridized with radiolabeled a 719-nt fragment of *BiVg* cDNA. Transcripts are indicated by the arrow on the right side of the panel.

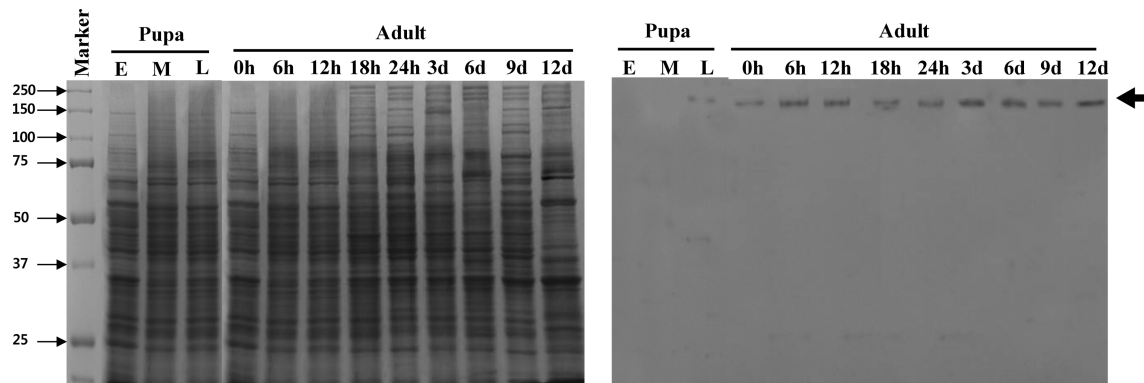


Fig. 5. Western blot analysis of BiVg in the fat body of *B. ignitus*. Protein samples were collected from the fat body at various time intervals. The protein samples were analyzed by 10% SDS-PAGE (left) and Western blot using the recombinant BiVg antibody (right).

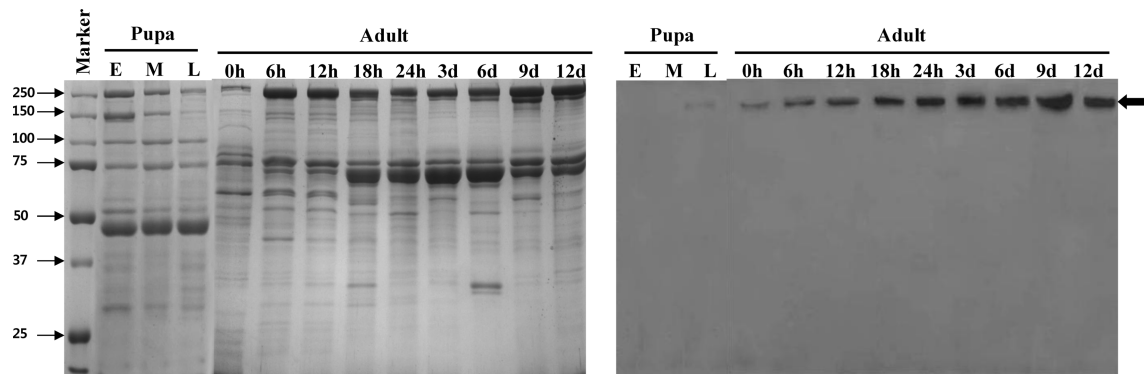


Fig. 6. Western blot analysis of BiVg in hemolymph of *B. ignitus*. Hemolymph was collected at various time points. The protein samples were analyzed by 10% SDS-PAGE (left) and Western blot using the recombinant BiVg antibody (right).

To examine the expression of BiVg by the recombinant virus in insect cells, the protein synthesis in Sf9 cells infected with the recombinant virus was analyzed by SDS-PAGE (Fig. 3A) and Western blot (Fig. 3B). The recombinant BiVg expressed by the *BiVg* cDNA was present as a single band of about 200 kDa in cells infected with the recombinant virus but not in cells infected with wild-type AcNPV or mock-infected cells.

Expression profiling of *BiVg*

To examine the expression of *BiVg* during development, we first performed Northern blot analysis. The expression of *BiVg* was first detected in the fat body during the late pupal stage of both queens and workers, but a low level was expressed in workers compared to queens (Figs. 4A and B). In adults, the expression level was increased at 0 hour from newly emerged adults, and a similar expression level was sustained until 12 days after the adult molt. This result is similar to a previous finding in that the *Vg* gene is stage-specifically detected in fat bodies from the honeybee (Piulachs *et al.*, 2003). In *A. mellifera* queens, vitellogenin mRNA was first detected in the mid-late pupal stage, whereas expression in workers was first detected in

late pupal stage (Piulachs *et al.*, 2003).

Expression profile of Bi-Vg

Consistent with the Northern blot data, Western blotting using the antibody for recombinant BiVg expressed in baculovirus-infected cells confirmed that the expression of BiVg protein was first observed in the fat body of the late pupal stage of queens and was continually detected during the adult stage (Fig. 5). To test when BiVg is secreted to the hemolymph, we performed Western blot analysis using antiserum to the recombinant BiVg. The detection of BiVg protein in the hemolymph also started in the late pupal stage of queens and gradually increased during the adult stage (Fig. 6).

In summary, we have cloned and characterized a vitellogenin gene, *BiVg*, in the bumblebee *B. ignitus*. Our study shows the stage-specific expression profile of BiVg by demonstrating that BiVg is first expressed in the fat body and hemolymph of the late pupal stage of queens and is then continually detected during adult stage.

Acknowledgments

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