

Protein Expression of the *Bombyx mori* Decapentaplegic Gene using the Baculovirus Expression Vector System

Seong Tae Jeong and Seung-Won Park*

Department of Biotechnology, Catholic University of Daegu, Daegu, Korea

The *Bombyx mori* decapentaplegic gene is one of the conserved genes in vertebrate and invertebrates. The TGF- β superfamily contains conserved polypeptide growth factors that play important roles in different cellular processes such as proliferation, apoptosis, differentiation and cell-fate determination. The *B. mori* dpp gene shares genetic homology with *hBMPs* and *Drosophila dpp*. Until now, only few studies have been conducted to examine the functions of *B. mori* dpp; and hence, its function is not yet well understood. In this study, the baculovirus expression vector system (BEVS) was used for expression of the recombinant *B. mori* dpp protein and in which the recombinant baculovirus is recovered in the host *Sf9* cells. The selected pure recombinant baculovirus containing *B. mori* dpp gene (rBV-egfp-Bm dpp) was used to increase the effective protein purification by using His-tag extraction strategy. After selection of recombinant baculovirus, recombinant *B. mori* dpp proteins were extracted from the re-infected cells with pure rBV-egfp-Bm dpp. Herein, we summarize the efficient expression and purification of *B. mori* dpp proteins from the insect cells using the BEVS. This recombinant protein could be suitable for functional test and various application studies.

Key Words: BEVS, *Bombyx mori* decapentaplegic, Recombinant protein

INTRODUCTION

Bombyx mori decapentaplegic (*dpp*) is a member of the transforming growth factor beta (TGF- β) superfamily. The largest TGF- β superfamily subgroup shares genetic homology with *human BMPs* (*hBMPs*) and silkworm *dpp* genes (1~3). The TGF- β superfamily contains conserved polypeptide growth factors that play important roles in different cellular processes such as proliferation, apoptosis, differentiation and cell-fate determination (1~3). In addition, *hBMPs* are functionally interchangeable with *Drosophila dpp* (1~3). *B. mori* dpp may induce osteoblastic differentiation

in mammalian cells (3).

BEVS continue to be used widely for the expression of a variety of recombinant proteins in insect cells (4, 5). This include membrane bound, secreted proteins, nuclear, cytosolic, and mitochondria (4, 5). Recombinant baculovirus infected insect cells also provide a valuable system to study the virus capsid assembly processes such as the production of virus-like particles (VLPs) (4, 5). BEVS is being increasingly utilized for the development of vaccine candidates based on the production of conventional recombinant antigens (4, 5). Co-expression of protein-modifying enzymes using multiple recombinant baculovirus can be used to increase in production of functional recombinant proteins

Received: May 22, 2015/ Revised: August 17, 2015/ Accepted: August 21, 2015

*Corresponding author: Seung-Won Park, Ph.D. Department of Biotechnology, Catholic University of Daegu, Daegu 712-702, Korea.
Phone: +82-53-850-3176, Fax: +82-53-850-3459, e-mail: microsw@cu.ac.kr

**This work was supported by research grants from the Catholic University of Daegu in 2013.

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>).

produced by infected insect cells (4, 5). Therefore, the BEVS has long been used to produce for the research and industrial purpose including gene therapy and development of vaccine.

In this study, we have developed recombinant baculovirus continuously expressing the *B. mori dpp* gene under the control of an *AcNPV polyhedrin (polh)* promoter. To isolate the recombinant dpp protein from cells overexpressing the *B. mori dpp* gene, recombinant baculovirus is infected and recovered in the *Sf9* cells. Finally, *B. mori dpp* protein was efficiently expressed and extracted from the insect cells using the BEVS. This recombinant protein can be used in further studies for osteoblastic functional test of *B. mori dpp* protein in mammalian cells.

MATERIALS AND METHODS

Cell culture

Insect cells of the *Spodoptera frugiperda* cell line *Sf9* were grown in Grace' insect Medium (Gibco, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (v/v) (Gibco) and 1% gentamycin (w/v) at 27°C in an incubator.

Construction of recombinant baculovirus shuttle vector

All recombinant DNA manipulations were performed using standard techniques (6). Total RNAs were isolated from the fat body of 3-day-old 5th instar larvae using the *TRIzol* reagent according to the manufacturer's instructional guides (Invitrogen, CA, USA). The amount of total RNAs was determined spectrophotometrically by measuring the absorbance at 260 nm. RNAs were stored at -70°C until use. After purification, oligo dT-primed cDNAs were prepared from 2 µg of total RNAs using the High-Capacity cDNA Archive kit (Applied Biosystems, CA, USA). The reaction was allowed to proceed for 2 h at 37°C. The 1,285-bp full-length cDNA of *B. mori dpp* was amplified using the gene specific primers (forward: 5'-agatcaggatccAGAGATTCGT-TGTTATGT-3' and reverse: 5'-ctcgagtgcggccgcGGCGTG-CCTTCGTCATCG-3'). Top-Taq PreMix (CoreBio Systems, Seoul, Korea) polymerase was used for cDNA amplification. The annealing temperature was 55°C, and the PCR reaction

was conducted for 35 cycles. The PCR fragment was cloned into the pGEMT-Easy vector (Promega, WI, USA). The resulting plasmid, referred to as pSRDA-1, was used for DNA sequencing (CoreBio Systems). Sequence data of the DNA fragments were analyzed by using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov>). To generate the *B. mori dpp* expression vector, the *dpp* gene was excised as a 1,324-bp fragment following *Bam*HI/*Not*I restriction enzyme digestion from the pSRDA-1 plasmid DNA. This fragment was inserted into the *Bam*HI/*Not*I restriction site of the pBAC-gus4X-egfp BEVS shuttle vector (Novagen, Darmstadt, Germany) in a direct orientation with respect to the *polh* promoter to generate pBAC-gus4X-Bm dpp (Fig. 1).

Recombinant baculovirus production

Cells were seeded in a 6-well tissue culture plate at 5×10^5 cells/well with 2 ml of unsupplemented Grace's insect Medium (GiBco) without antibiotics and serum. The cells were incubated overnight at 27°C to allow the cells to fully attach to the bottom of the plate. On the next day, the recombinant pBAC-gus4X-Bm dpp shuttle vector and baculovirus genomic DNA were transfected into *Sf9* cells using cellfection®II Reagent (Invitrogen). The cells were incubated at 27°C for 72 h until they exhibited cytopathic effects (CPE). Subsequently, 2 ml of culture medium was collected from each well and centrifuged in order to remove cells and large debris.

Genomic DNA PCR and Reverse transcription

Two PCR primers were used to confirm that the inserted *EGFP* (forward: 5'-GGTGAGCAAGGGCGAGGAGCT-3' and reverse: 5'-TCTTGAAGTTCACCTTGATGCCG-3') and *dpp* (forward: 5'-GACGAGCGACCGAAGACG-3' and reverse: 5'-CGAAGTCGACAAACAGTG-3') genes used were same as previous experiments. The genomic DNA was isolated from selected cell clones using the *TRIzol* reagent (Invitrogen). A 5 µl aliquot of genomic DNA from each sample was mixed with *Taq* DNA polymerase in PCR buffer and amplified for 35 cycles at an annealing temperature of 52°C. To detect the specific expression patterns of the

candidate genes in the recombinant baculovirus infected cells, total RNAs were treated with *DNase* I for 15 min at 37°C to remove the genomic DNA. After purification, oligo dT-primed cDNAs were synthesized from 5 µg of total RNAs using the High-Capacity cDNA Archive kit (Applied Biosystems). The reaction was allowed to proceed for 2 h at 37°C. The specific primers were used for RT-PCR. The annealing temperature was 52°C for 30 cycles. The PCR products were analyzed using a 0.7% agarose gel.

Western blotting analysis

Samples were prepared for Western blot analysis in the following manner. *Sf9* cells were infected with BAC-gus4X-Bm dpp in 6-well plates. After 72 h, cells were lysed in 1x Laemmli buffer [125 mM Tris, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.001%

bromophenol blue, pH 6.8] and heated to 100°C for 5 min prior to electrophoresis. All samples were run on SDS-10% polyacrylamide gel electrophoresis. The 6X His-specific monoclonal antibody was obtained from the Clontech Co. (Otsu, Japan). The presence of the *B. mori* dpp protein was detected using the ECL Western blotting analysis system (Amersham Pharmacia Biotech. Ltd., UK).

RESULTS AND DISCUSSIONS

The 1,285-bp full-length cDNA of *B. mori* dpp was successfully amplified and cloned into the pGEMT-Easy vector. Based on gene sequencing analysis, the authenticity of the *B. mori* dpp full-length cDNA sequence was confirmed. Subsequently, to generate the recombinant baculovirus that could produce a *B. mori* dpp-expressing virus, the

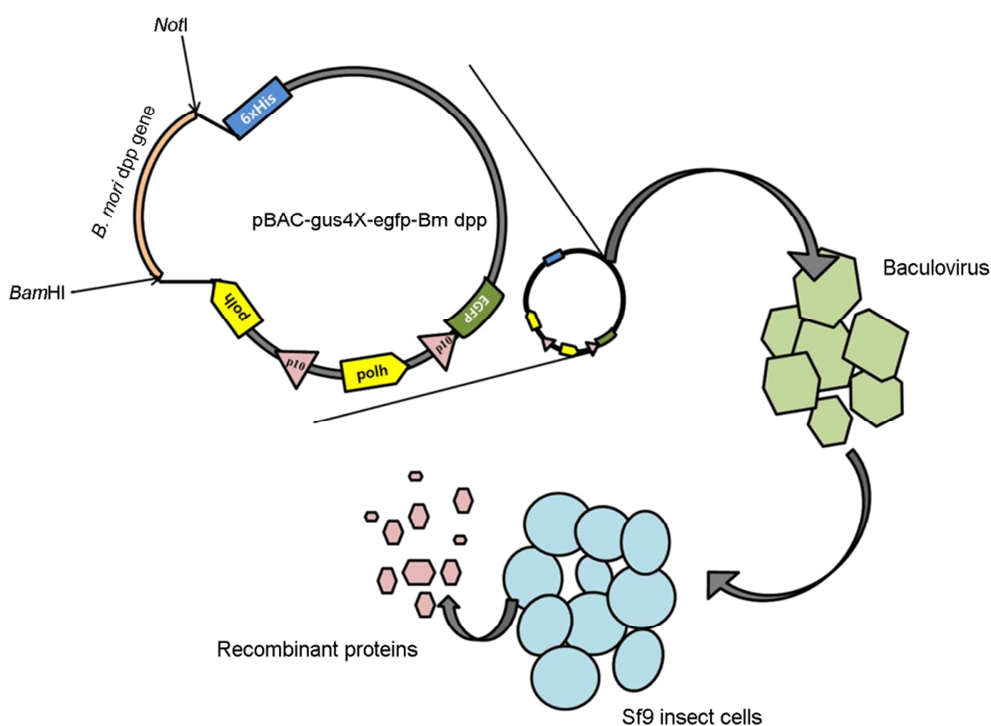


Figure 1. Cloning of the *Bombyx mori* dpp expression vectors. The 1,324-bp full-length cDNA of *B. mori* dpp was synthesized using total RNA obtained from fat bodies of 3-day-old of 5th instar larvae. *B. mori* dpp gene cassette inserted into the pBACgus4X-egfp plasmid DNA with *Bam*H I and *Not* I restriction enzymes is terminated by stop codon of vector and added 6X His amino acids to 3'-end region. pBACgus4X-egfp vector contain the 0.7-kb EGFP downstream of the other p10 promoter. This fragment was inserted into the pBACgus4X-egfp vector in a direct orientation with respect to the *polh* promoter to generate the pBAC-gus4X-Bm dpp plasmid DNA.

recombinant baculovirus transfer plasmids were generated by inserting expression cassettes encoding *B. mori dpp* and the *EGFP* reporter gene into the standard baculovirus transfer vector pBAC-gus4X-egfp. The orientation of the genes was manually designed, so that the direction of transcription was convergent (Fig. 1). *B. mori dpp* gene expression was controlled by the *polh* promoter, and, therefore, the protein expression should be at high levels in insect cells during the late infection. The *EGFP* reporter gene was driven by the *p10* promoter so that it should be expressed in insect cells, allowing easy detection of viral recovery. The detailed procedure for construction of recombinant pBAC-gus4X-Bm dpp plasmid DNA is shown in Fig. 1.

Recombinant baculovirus was obtained by using homologous recombination strategy. AcNPV is one of the most familiar baculovirus used for gene expression. The gene of interest is cloned into a transfer plasmid containing the insect cell-specific promoter flanked by baculovirus DNA derived from a nonessential locus, the polyhedrin gene. The target gene is inserted into the AcNPV viral genome by homologous recombination after transfection into *Sf9* cells. The titer was of recombinant baculovirus varied from 5 to 11×10^7 plaque-forming units per milliliter.

To confirm the specific viral genes expression pattern in the recombinant baculovirus infected cells, *Sf9* cells infected with rBV-egfp as wild-type or with rBV-egfp-Bm dpp as recombinant virus were incubated for 72 h. Amplification of *EGFP* and *dpp* genes in the rBV-infected insect cells were examined by performing PCR using genomic DNA as a template. As shown in Fig. 2A and B, 500-bp and 213-bp fragments were amplified from the genomic DNA of cells infected with rBV-egfp or rBV-egfp-Bm virus. A 213-bp fragment of the *B. mori dpp* gene was amplified from only rBV-egfp-Bm dpp recombinant virus infected sample that was transfected with the pBAC-gus4X-Bm dpp plasmid DNA. These results clearly demonstrate that the recombinant baculovirus infected cells contain *EGFP* and *dpp* genes in the viral genome.

RT-PCR analysis was performed to determine whether the cells infected with the rBV-egfp-Bm dpp virus expressed recombinant *B. mori dpp* protein. To verify that we per-

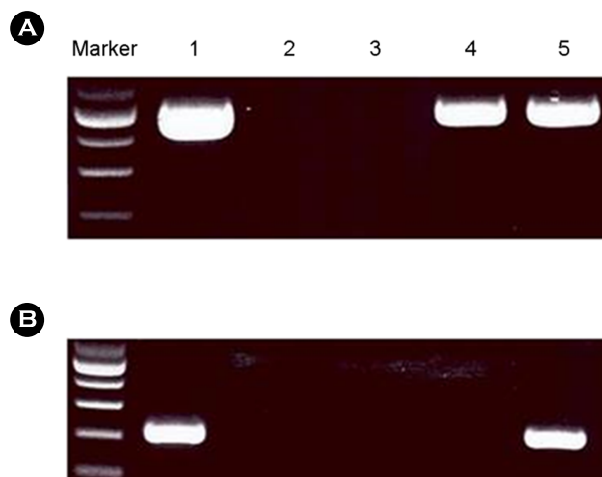


Figure 2. Genomic DNA PCR analysis of *Sf9* cells transfected with the rBV-egfp or rBV-egfp-Bm dpp recombinant viruses. (A) To evaluate *dpp* expression in cells transfected with plasmid DNA, we performed genomic DNA PCR analysis by using a specific primer pair. An approximately 500-bp *egfp* gene PCR fragment was amplified from the genomic DNA of *Sf9* cells infected with rBV-egfp or rBV-egfp-Bm dpp recombinant viruses. (B) An approximately 213-bp *B. mori dpp* gene PCR fragment was amplified from the genomic DNA of *Sf9* cells infected with rBV-egfp or rBV-egfp-Bm dpp recombinant viruses. 1: positive control (plasmid DNA); 2: negative control; 3: *Sf9* cells infected without rBV; 4: *Sf9* cells infected with rBV-egfp; 5: *Sf9* cells infected with rBV-egfp-Bm dpp.

formed 35 cycles of PCR to amplify RNA transcripts derived from control, cells infected with the the rBV-egfp, and rBV-egfp-Bm dpp. As shown in Fig. 3, 213-bp fragment of *B. mori dpp* gene was amplified from an RNA transcript obtained from cells infected with rBV-egfp-Bm dpp but not from the RNA transcripts obtained from the cells infected with rBV-egfp and without rBV. This result indicates that the target cells have expressed the *B. mori dpp* gene, and it could be used in further studies for functional test of *B. mori dpp* protein in the cells.

Since it was previously reported that expression of *Drosophila dpp* is functionally interchangeable with mammalian BMPs (3), we used a strategy in which the expression of *B. mori dpp* in insect *Sf9* cells was dependent on infection with recombinant baculovirus. To determine whether the *B. mori dpp* protein has been successfully expressed from the recombinant virus and incorporated into virus particles, we

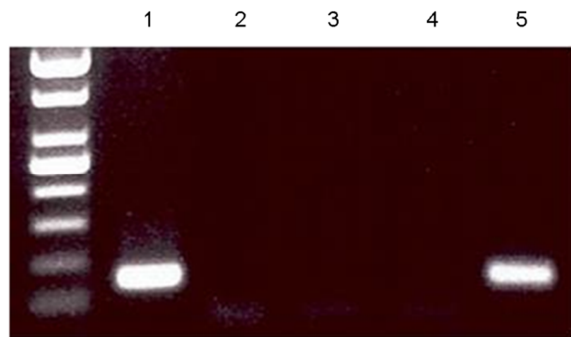


Figure 3. RT-PCR analysis of *dpp* expression with RNA extracted from different cells of rBV infected and uninfected. To determine whether the *B. mori dpp* gene was expressed in the target cells, we performed RT-PCR analysis. A 213-bp fragment of the *B. mori dpp* gene was amplified from only rBV-egfp-Bm *dpp* recombinant virus infected sample that was transfected with the pBAC-gus4X-Bm *dpp* plasmid DNA. 1: positive control (plasmid DNA); 2: negative control; 3. *Sf9* cells infected without rBV; 4: *Sf9* cells infected with rBV-egfp; 5. *Sf9* cells infected with rBV-egfp-Bm *dpp*.

examined infected recombinant baculovirus cells by Western blot analysis (Fig. 4). About 72 h after recombinant baculovirus infection, the insect cells were analyzed by Western using 6X His-specific monoclonal antibody. Fig. 4 shows a comparison of *B. mori dpp* expression in rBV-egfp-Bm *dpp* or rBV-egfp infected *Sf9* cells. *B. mori dpp* protein was not detected in rBV-egfp infected cells but detected in cells infected with either rBV-egfp-Bm *dpp*.

In this study, we have generated *B. mori dpp* carrying baculovirus by cloning the *B. mori dpp* gene in the baculovirus genome. The *B. mori dpp* gene shares genetic homology with *hBMPs* and *Drosophila dpp*. However, functional analyses of this gene have not been conducted, so far (3, 7). To investigate the biological roles of the *B. mori dpp* gene, we constructed a pBAC-gus4X-Bm *dpp* expression cassette vector under the control of the *polh* promoter for producing a recombinant protein in the *Sf9* cells. Expression of *B. mori dpp* was controlled by the *polh* promoter, so that it would be expressed at high levels in the infected insect cells. *Sf9* cells infected with rBV-egfp-Bm *dpp* produced the *B. mori dpp* protein. Co-expression of protein-modifying enzymes using multiple recombinant baculovirus can be used to

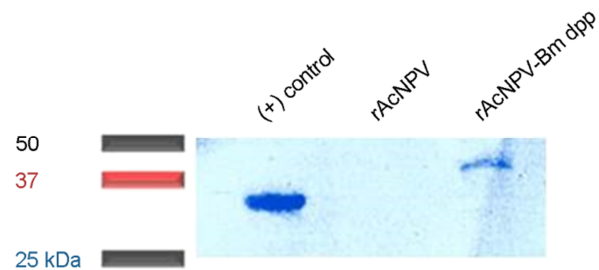


Figure 4. Western blot analysis of *B. mori dpp* protein induction in *Sf9* cells infected with the rBV-egfp-Bm *dpp* recombinant baculovirus. To confirm of recombinant *B. mori dpp* protein purification condition, selected recombinant rBV-egfp-Bm *dpp* from A was infected in *Sf9* cells and harvested at 72 h post infection. The specificity of each antibody is indicated each rBV-egfp-Bm *dpp* with *B. mori dpp* gene. And positions and molecular weights of protein size markers are indicated on the left (37 kDa; red bar).

enhance the production of functional recombinant proteins produced by infected insect cells. In near future, the baculovirus surface display continues to evolve as a useful research tool (4).

Previous studies have reported that the bone-forming activity of the *Drosophila dpp* protein can be demonstrated by measuring the specific activity of ALP and the calcium content in subcutaneous rat implants (3, 7). Further studies examining the *B. mori dpp* should be conducted to produce recombinant protein, such as bio-medical protein, which can be used to produce bone growth formation biomaterials. The recombinant *B. mori dpp* protein will be used in further studies for various functional analyses.

REFERENCES

- 1) Chen Y, Riese MJ, Killinger MA, Hoffmann FM. A genetic screen for modifiers of *Drosophila* decapentaplegic signaling identifies mutations in *punt*, *Mothers against dpp* and the BMP-7 homologue, 60A. *Development* 1998;125:1759-68.
- 2) Künnapu J, Shimmi O. Evolutional imprints on the sequences of BMP2/4/DPP type proteins. *Fly (Austin)* 2010;4:21-3.
- 3) Park SW, Goo TW, Choi GH, Kang SW, Kim SW, Kim

- SR. Functional analysis of *Bombyx mori* Decapentaplegic gene for bone differentiation in a mammalian cell. *Int J Indust Entomol* 2013;27:159-65.
- 4) Park SW, Lee HK, Kim TG, Yoon SK, Paik SY. Hepatocyte-Specific Gene Expression by Baculovirus Pseudotyped with Vesicular Stomatitis Virus Envelope Glycoprotein. *Biochem Biophys Res Commun* 2001; 289:444-50.
- 5) Jin JY, Park CJ, Park SW, Paik SY. Protein Expression of the Human Norovirus Capsid Gene using the Baculovirus Expression System. *J Bacteriol Virol* 2011;41:183-7.
- 6) Russell D, Sambrook J. *Molecular cloning: a laboratory manual*, 3rd Ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2000.
- 7) Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM. *Drosophila* transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. *Proc Natl Acad Sci U S A* 1993; 90:6004-8.
-