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# Expression of Cocoonase in Silkworm (*Bombyx mori*) Cells by Using a Recombinant Baculovirus and Its Bioactivity Assay

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**Abstract**

In this research, the cocoonase gene was cloned by RT-PCR as an 860 bp fragment, including the signal peptide and the core sequence of cocoonase gene. In order to investigate the function of signal peptide, recombinant transfer vector pBacPAK8-Cocoonase-EGFP were constructed by fusing with enhanced green fluorescent protein (EGFP) gene to observe under fluorescence microscope. The purified pBacPAK8-Cocoonase-EGFP DNA was co-transfected with linear virus Bm-BacPAK6 DNA into BmN cells. The homologous recombination occurred in the cells and then the recombinant virus Bm-BacPAK8-Cocoonase-EGFP was obtained. BmN cell was infected with the recombinant virus Bm-BacPAK8-Cocoonase-EGFP, and fluorescent signal was detected in most of the cells under fluorescence microscope at 72 hrs postinfection. Then BmN cells were harvested. Both SDS-PAGE and Western-blotting analysis indicated that the cocoonase was expressed successfully in silkworm (*Bombyx mori*) baculovirus expression vector system. Furthermore, referred to Astrup methods,used fibrin plate process confirmed that expression product in vitro had cellulolytic activity. We conclude that silkworm expression system can be used successfully to express functional cocoonase.

**Keywords:** Cocoonase, *Bombyx mori*, Recombinant baculoviruses, Expression product

## 1. Introduction

Cocoonase is a protease produced by silk moths during adult development and used for hydrolyzing and softening the end of the cocoon to permit exit of the adult moth. Studies on the cocoonase have significance not only in the understanding of the physiological process of the silkmoth's escape from cocoon, but also in the exploitation of silk protein. The full-length sequence of the cocoonase gene has released in the National Center for Biotechnology Information (GenBank accession No. EF428980) and the full-length cDNA of the cocoonase gene from *Bombyx mori* is 1047 bp with an ORF of 780 bp, 54 bp nucleotide sequence in 5′UTR (untranslated region), 210 bp nucleotide sequence in 3′UTR and termination codon TAA. The protein which was encoded by the cocoonase gene contains 260 amino acids. The protein molecular weight is about 27.6 kDa and its isoelectric point is 8.89. An alignment of the cDNA sequence with the silkworm genome sequences revealed that there were 4 introns and 5 exons within the open reading frame in the cocoonase gene from *B. mori*.

The baculovirus expression vector system (BEVS) is an effective recombinant protein production system which utilizes the recombinant virus carrying the gene of interest to infect cultured cells or host larvae (Wu *et al*., 2004, 155 462–466). In this paper, recombinant pBacPAK8-Cocoonase-EGFP was expressed very well using *B. mori* nuclear polyhedrosis baculovirus expression vector system. Infected BmN cell with recombinant virus, and fluorescent signal was detected in most of the cells by fluorescent microscope showing the successful expression of cocoonase-EGFP fusion protein in BmN cells. Then BmN cells were harvested at 72 hrs postinfection. Protein from the cells was analysed by SDS-PAGE and Western-blotting, indicated that the cocoonase was expressed successfully in silkworm (*B. mori)* baculovirus expression vector system. More importantly, we have confirmed that expression product in vitro had cellulolytic activity.

## 2. Materials and methods

### *2.1 Materials*

The silkworm *B.ombyx mori*, *E. coli* (strain TG1) and *B. mori* nuclear polyhedrosis virus were inbred in our lab. Transfer vector (pBacPAK8-EGFP vector) was supplied by our lab.Restriction enzyme, T4 DNA ligase, PCR reagents and pMD18-T were obtained from TaKaRa Company (Dalian); primer and other reagents were obtained from Shanghai Sangon Bio-technology Corpotation.

#### 2.2.1 RT-PCR

A pair of specific primers was designed based on the sequence we obtained. The forward primer (5'-GCAGGATCCATGGAAAAGTTGTATCTGTTTAT-3') contained a *Bam*H I restriction enzyme site (underlined), and the reverse primer (5'-TATGGTACCTAGGCCCGCCGTTGATTTTAT-3') contained a *Kpn* I restriction enzyme site (underlined). cDNA was prepared from midgut RNA with M-MLV reverse transcriptase and an oligodT primer. PCR reaction was carried out with Taq polymerase for 35 amplification cycles (94°C for 45 sec; 58°C for 45 sec; 72°C for 1min). PCR product was examined by electrophoresis in 1% agarose gel with the ethidium bromide staining.

#### 2.2.2 Cloning and sequencing

The PCR product was ligated into pMD18-T vector using T4 DNA ligase and then transformed into *E. coli* (TG1 strain). Plasmid was purified with MiniBEST Plasmid Purification Kit (Takara). The sequencing was performed using an automatic sequence: CEQ8000 (Beckman company).

#### *2.2.3 Construction of transfer vector*

The plasmid pMD18-T/Cocoonase was digested with *Bam*H I and *Kpn* I, and then purified. The purified fragment was ligated with the *Bam*H I-*Kpn* I digested pBacPAK8-EGFP vector and transformed into *E. coli* (TG1 strain). The transformants harboring the recombinant plasmid were confirmed by restriction enzyme analysis (Fig. 1).

#### 2.2.4 Generation of recombinant baculoviruses

The lipofection technique was used to cotransfect into monolayers of BmN cells with the recombinant transfer vectors, all generated as described above, and *Bsu*36I triple-cut *B. mori* nuclear polyhedrosis virus DNA (Felgner *et al*., 1987, 84 7413-7417). Recombinant baculoviruses were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described elsewhere (Kitts *et al*., 1993, 14 810-817).

#### 2.2.5 Detection of the expression of Cocoonase-EGFP fusion protein

BmN cells transfected with recombinant baculoviruses were examined under fluorescent microscope for expression of Cocoonase-EGFP 72 hrs post transfection.

#### 2.2.6 Protein analysis

Confluent monolayers of BmN cells in 35-mm tissue culture dishes were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 5. At 48 hrs post infection, cells were harvested and lysed in 150 μl of lysis buffer (50

mM Tris-HCl, pH 8, 200 mM NaCl, 1% Triton X-100). Twenty microliters of the protein samples were boiled for 6 min in 5× sample buffer [2.3% sodium dodecyl sulfate (SDS), 2.5% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue], and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie blue.

2.2.7 Western blot analysis

Western blot analysis of proteins was performed following the standard protocol as described previously (Kar *et al*., 2004, 324 387-399).

2.2.8 Assay for cellulolytic activity of expression product in vitro

Referred to *Astrup* methods (Park *et al*., 1999, 32 239-246; Lee *et al*., 2001, 11 845-852),used fibrin plate process to measure cellulolytic activity of expression product. Collected BmN cells were added lysis buffer solution and sonicated by ultrasonic wave while ice bathed. After centrifugated, took 20 μl and 40 μl supernatant into the hole of nutrient medium and incubated at 37°C for 48 hrs.

## 3. Results

### *3.1 Cloning and identification of cocoonase*

PCR amplification of the midgut cDNA was performed using the two specific primers. After electrophoresis in 1% agarose gel, we obtained a band about 860 bp, which was consistent with the expected molecular mass. The PCR product was ligated into pMD18-T vector and confirmed by restriction endonuclease digestion and DNA sequencing. The cocoonase fragment could be isolated from the pMD18-T vector after the recombinant plasmid was digested with *Bam*H I and *Kpn* I (Fig. 2).

### *3.2 Construction of recombinant transfer vector*

The plasmid pMD18-T/Cocoonase was digested with *Bam*H I and *Kpn* I and ligated with pBacPAK8-EGFP which was also digested with the same restriction enzymes to generate pBacPAK8-Cocoonase-EGFP. The cocoonase fragment could be isolated from the pBacPAK8-Cocoonase-EGFP vector after the recombinant plasmid was digested with *Bam*H I and *Kpn* I (Fig. 3, lane 1). The EGFP fragment could be isolated from the pBacPAK8-Cocoonase-EGFP vector after the recombinant plasmid was digested with *Kpn* I and *Eco*R I (Fig. 3, lane 2). The recombinant plasmid pBacPAK8-Cocoonase-EGFP was successfully constructed (Fig. 3).

### *3.3 Expression of Cocoonase-EGFP fusion protein*

BmN cells transfected with recombinant baculoviruses showed cytopathic effect at 72 hrs post transfection. To detect the expression of Cocoonase-EGFP fusion protein, these cells were examined by fluorescent microscope. Fluorescent signal was detected in most of the cells (Fig. 4), showing the successful expression of Cocoonase-EGFP fusion protein in BmN cells.

### *3.4 SDS-PAGE and Western blotting analysis*

BmN cells were infected with recombinant baculoviruses BacPAK8-Cocoonase-EGFP and virus BacPAK6 as a control,and cells were harvested at 72 hrs post infection. Protein from the cells was analysed by SDS-PAGE. Recombinant baculoviruses BacPAK8-Cocoonase-EGFP includes a 57 kDa band that is absent from virus BacPAK6 (compared lanes 1 and 2). This corresponds to the size expected for the Cocoons-GFP fusion protein. Immunoblot analysis with antiserum specific for GFP confirmed that this band includes EGFP and cocoonase (Fig. 5).

### *3.5 Assay for cellulolytic activity of expression product in vitro*

Used fibrin plate process to measure cellulolytic activity of expression product, the protein of BmN cells which infected with pBacPAK8-Cocoonase-EGFP had apparently dissolved plaque, however, the protein of BmN cells which infected with BacPAK6 had not yet dissolved phenomenon (Fig. 6). It confirmed that expression product in vitro had cellulolytic activity.

## 4. Discussion

The baculovirus expression system has been proven to be a most effective and versatile eukaryotic expression tool (Maeda, 1989, 34 351-371) and it has been used to express many recombinant proteins using insects as bioreactors (Sehgal *et al*., 2003, 27 27-34). Recombinant proteins are functional properties similar to those of their native counterparts. Baculovirus-infected insect cells perform many of the posttranslational modifications higher eukaryotes do, and this advantage makes this expression system become a valuable tool for production of biologically active proteins.

Cocoonase is an important protease produced by silk moths during adult development and used for hydrolyzing and softening the end of the cocoon to permit exit of the adult moth. In this study,we successfully cloned cocoonase gene and constructed recombinant transfer vector pBacPAK8-Cocoonase-EGFP. After infected BmN cell with the

recombinant virus, we observed BmN cells by a fluorescence microscope and detected fluorescent signal in most of the cells. Both SDS-PAGE and Western-blotting indicated that the Cocoonase was expressed successfully in silkworm (*Bombyx mori)* baculovirus expression vector system. Furthermore, we successfully confirmed that expression product in vitro had cellulolytic activity. To further study of the cocoonase both in the understanding of the physiological process of the silkmoth's escape from cocoon and in the exploitation of silk protein to lay the foundation.

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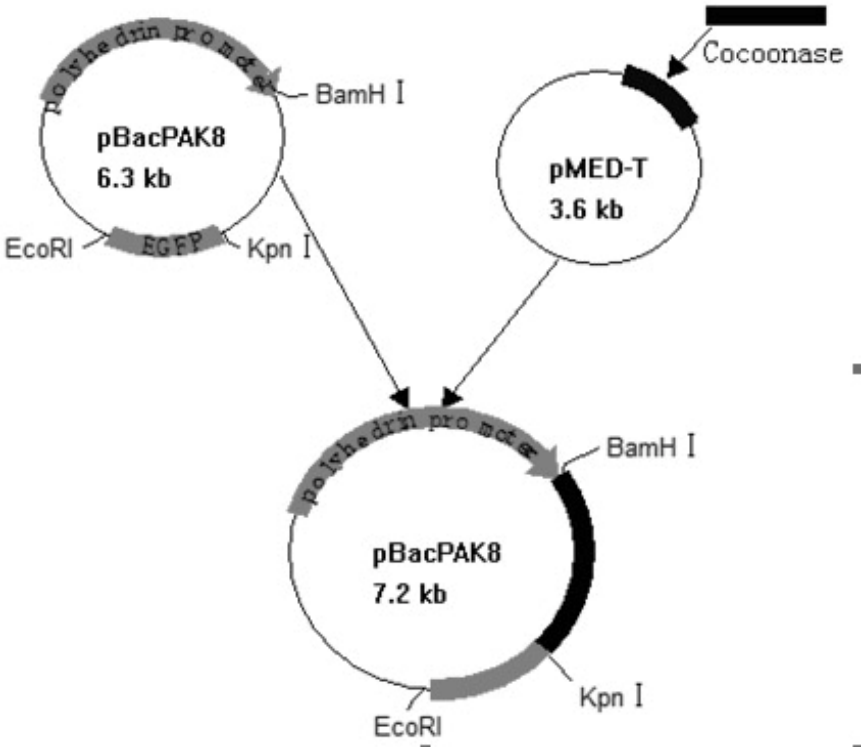


Figure 1. The construction of pBacPAK8-Cocoonase-EGFP.

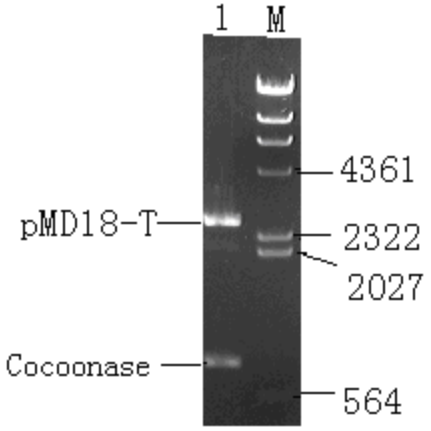


Figure 2. Identification of the recombinant plasmid pMD18-T/Cocoonase. Llane 1, pMD18-T/Cocoonase digested with *Bam*H I and *Kpn* I generated two fragments: pMD18-T (2.6 Kbp) and cocoonase (860 bp); M, DNA molecular mass maker.

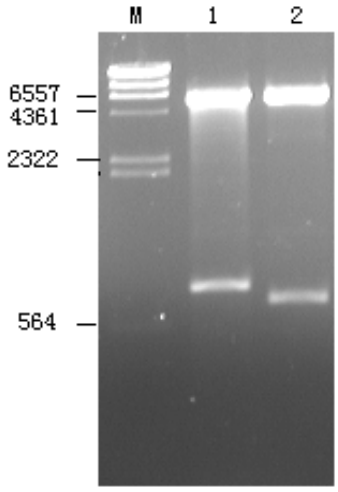


Figure 3. Identification of the transfer vector pBacPAK8-Cocoonase-EGFP. Lane 1, pBacPAK8-Cocoonase-EGFP digested with *Bam*H I and *Kpn* I generated two fragments: pBacPAK8-EGFP (6.3 Kbp) and cocoonase (860 bp); Lane 2, pBacPAK8-Cocoonase-EGFP digested with *Kpn* I and *Eco*R I generated two fragments: pBacPAK8-Cocoonase (6.36 Kbp) and EGFP (800 bp); M, DNA molecular mass maker.

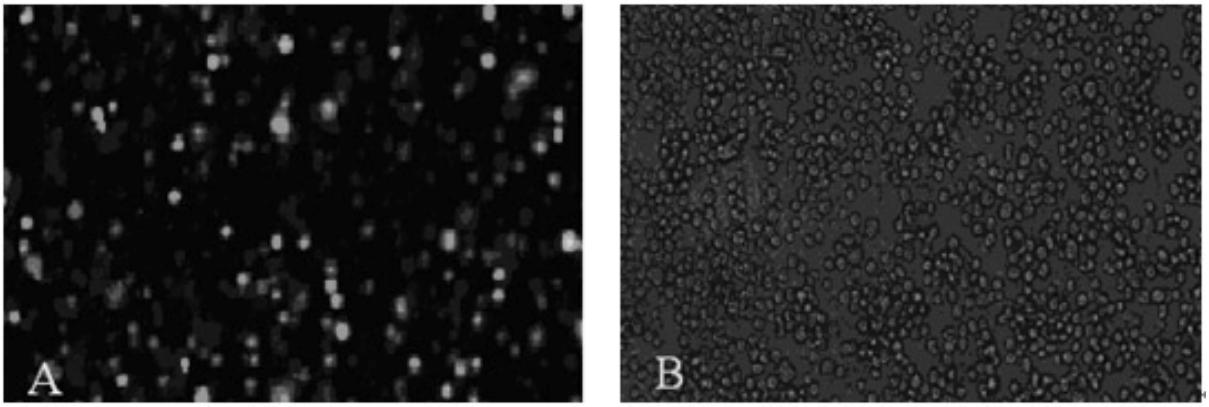


Figure 4. Detection of the expression of cocoonase-EGFP fusion protein. BmN cells were infected with recombinant baculovirus expressing the Cocoonase-EGFP fusion protein and EGFP fluorescence was detected with fluorescence (A) and light (B) microscope.

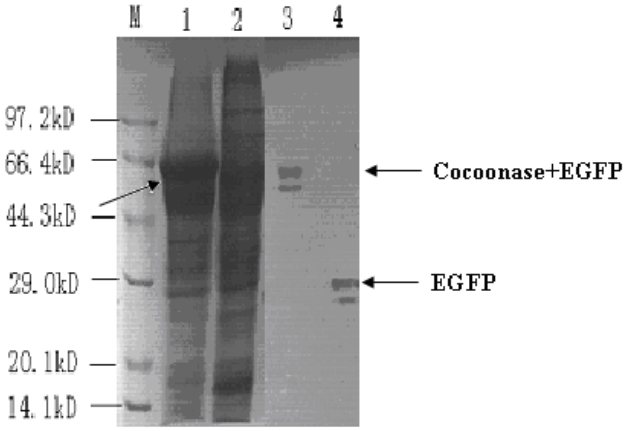


Figure 5. SDS-PAGE and Western blotting analysis. M, Protein marker; Lane 1, BmN cells infected with recombinant baculoviruses Bm-BacPAK8-Cocoonase-EGFP; Lane 2, BmN cells infected with Bm-BacPAK8-EGFP; Lane 3, Western blot results of Cocoonase-EGFP fusion protein; Lane 4, Western blot results of EGFP protein. The fusion protein bands were indicated by arrows.

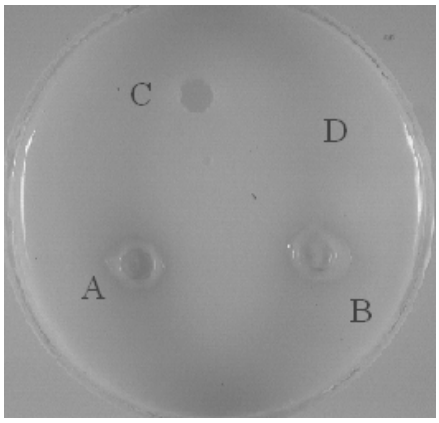


Figure 6. Assay for cellulolytic activity of expression product in vitro. (A) 20 μl of expression product of BmN cells infected with Bm-BacPAK8-Cocoonase-EGFP. (B) 40 μl of expression product of BmN cells infected with Bm-BacPAK8-Cocoonase-EGFP. (C) 40 μl of expression product of BmN cells infected with Bm-BacPAK6. (D) Non-treatment.