

Trichoderma reesei *Bombyx mori*

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44 *T. reesei* both at the biochemical and genetic levels (see Nevalainen and Penttilä 1995).

Trichoderma reesei secretes at least five types of endoglucanases (EGI, EGII, EGIII, EGIV, and EGV) of which EGII (originally called EGIII, Saloheimo et al. 1988) accounts for most of the endoglucanase activity. The gene for EGII (*egl2*) has been cloned and sequenced (Saloheimo et al. 1988) and expressed in *Saccharomyces cerevisiae* (Penttilä et al. 1987) and *Pichia pastoris* (Qiao et al. 2004). However, the plasmid transformants may be unstable in yeast. In the present study, the BmNPV/Bac-to-Bac expression

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system was used to produce large quantities of



recombinant EGII in the silk form because of advantages in yield, cost, handling and safety (Lee et al. 2006). The intention is to obtain a silk form powder and add it to animal feed to improve cellulose degradation.

Strains, plasmids and culture conditions

Trichoderma reesei QM9414 was purchased from the China General Microbiological Culture Collection Center (CGMCC). Liquid cultures of *T. reesei* were started from conidiospores grown on the potato/dextrose/agar (PDA) slants. For induction of cellulase formation, mycelia were cultivated in shake-flasks for 2 days (28 °C, 200 rpm) as described by Baile and Nevalainen (1981) except that 2% (v/v) lactose was added. The cloning vector (pGEM-T Easy Vector System) was purchased from Promega (Madison, Wisconsin, USA). The Bac-to-Bac donor plasmid pFastBacHTc was from Invitrogen (San Diego, CA, USA). *E. coli* DH5 α (Novagen, CA, USA) was used as a host for plasmid preparation.

Cell lines and silk form

The *Bombyx mori*-derived cell line, BmN (conserved by our laboratory), was maintained at 27 °C with TC-100 medium containing 10% (v/v) fetal bovine serum

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sterile tubes. Both the diluted solution and incubate are combined at room temperature for 30 min. In a 35 mm tissue culture plate, 9×10^5 BmN cells pre-seeded 24 h before hand are washed with 2 ml unsupplemented medium. Then, 0.8 ml unsupplemented medium is added to the tube containing DNA:lipid complexes, and the DNA:lipid complexes are transferred to the washed cells. After 5 h incubation in unsupplemented TC-100 medium, the culture medium is replaced by fresh medium containing 10% FBS. The cells are allowed to grow at 27 °C for 5 days and the medium is collected as the primary viral stock. The recombinant stock virus can be used to infect both BmN cells and *B. mori* larvae.

Production of recombinant EGII in BmN cells and silk worm larvae

Firstly, the stock virus is used to infect BmN cells. The culture media and cells at 24, 48, 72, 96 and 120 h post-infection are collected separately by low-speed centrifugation for 5 min at 4 °C, and subsequently used for protein analysis to investigate its time-course gene expression. The infected cells are lysed to release rEGII by repeat freezing and thawing before SDS-PAGE.

The recombinant virus is also used to infect newly molted fifth-instar silk worm larvae. The larvae are anesthetized on ice for 10 min until they did not move actively. About 10 µl recombinant viruses are injected subcutaneously into each larva and 30 min after injection, the larvae are fed with mulberry leaves and then reared at 25–27 °C. The fat body of infected larvae is collected at 24, 48, 72, 96 and 120 h post-infection and stored at –20 °C until use. Most infected larvae died on the 5th or 6th day of post-infection. The silk worm larvae are collected before this period.

Purification of recombinant EGII

For large-scale purification of rEGII, no more feed is given to the silk worms at 96 h post-infection to clean their guts and, finally, 100 infected larvae at 110 h post-infection are selected. Their whole bodies except for silk glands are homogenized in ice-cold citric acid buffer (50 mM, pH 5), and then the mixture is centrifuged at $12,000 \times g$ for 15 min at 4 °C to

remove large debris and lipids. The supernatant is filtered to remove the remaining lipids, and the filtrate is centrifuged again at $12,000 \times g$ for 20 min at 4 °C. The supernatants are collected as the crude extracts of rEGII. To prevent melanization, 0.1 vol 10 mM dithioerythritol (DTE) is added but no protease inhibitors are added.

As the rEGII carries an *N*-terminal 6× His-tag, purification is done with the Ni-NTA affinity columns under native conditions. The crude extracts are diluted with native binding buffer (50 mM NaH_2PO_4 , 500 mM NaCl, pH 8) and then applied directly to Ni-NTA columns for binding. The rEGII is finally eluted with native elution buffer (binding buffer plus 250 mM imidazole).

Endoglycosidase F treatment

The recombinant EGII purified from 100 infected larvae at 110 h post-infection is treated with endoglycosidase F (Sigma) (0.05 U/µg rEGII, 200 mM Sodium acetate buffer, pH 5, and 20 mM EDTA). After incubation for 24 h at 37 °C, the endoglycosidase-treated and untreated samples are prepared for Western blot analysis.

Bioassay of recombinant EGII

At various times, ten recombinant silk worms are homogenized, centrifuged and the CMCase activity of supernatant is measured using 1% (v/v) CMC as a substrate in 50 mM citric acid buffer (pH 5) at 50 °C for 30 min. The reactions are terminated by adding DNS reagent and boiled for 5 min; A_{540} is measured after cooling. The specific activity of the purified recombinant EGII from 100 infected larvae at 110 h post-infection is also determined. One unit of the enzyme is defined as the activity producing 1 µmol of reducing sugars in glucose equivalents per min.

Enzyme activity for optimum temperature is determined by incubating the purified rEGII in citric acid buffer (0.05 M, pH 5) containing 1% (v/v) CMC at the temperature ranges from 30 °C to 80 °C with 5 °C or 10 °C intervals for 30 min. For the optimal pH, the purified rEGII is treated in 0.05 M buffer at various pH ranges (pH 2–8) at 50 °C for 30 min. The buffers used are acetate (pH 2 and 3), citrate (pH 4–6), phosphate (pH 7).

The *egl2* gene was cloned from *T. reesei* QM9414 and analyzed (Fig. 1a). Its predicted size was 1,194 bp. Comparison of this sequence with that registered in GenBank (accession no. M19373) showed only three nucleotide changes at positions 156 (A-G), 369 (G-C), and 1135 (A-G), that is two changes in the deduced amino acid sequence at positions 123 (Glu-Asp) and 379 (Ser-Gl).

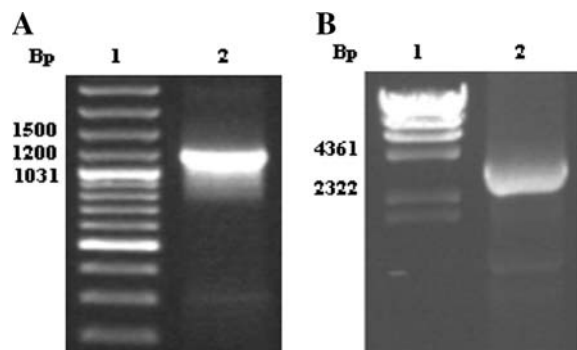
The BmNPV/Bac-to-Bac expression system applicable to silkworm was developed based on the working principle of AcMNPV Bac-to-Bac system. In our previous study (Cao et al. 2006), a large 8.6 kb fragment containing the *l*-cop⁺-number mini-F replicon, a kanamycin resistance marker, a segment of DNA encoding the *lacZ* α peptide and a targeting site for bacterial transposon Tn7 (mini-*att*Tn7) from the AcMNPV bacmid, was cloned into polyhedrin locus of BmNPV genome to replace the polyhedrin gene. This recombinant BmNPV DNA was transformed as a large plasmid, named bacmid, into *E. coli* DH10 β strain, in which a helper plasmid encoding the transposase was already transformed. The DH10 β strain containing BmBacmid and helper was designated as DH10BmBac. At the same time, the multiple cloning site in the donor plasmid is flanked by the left and right

bacterial transposon Tn7 sequences, which can carry the foreign gene transposon into the mini-*att*Tn7 site in the BmNPV genome. With this novel Bac-to-Bac system, the recombinant baculovirus can be rapidly and easily generated through gene transposition.

The correct insertion of *egl2* gene into the BmNPV bacmid was confirmed (Fig. 1b), indicating that the gene transposition was well performed. After transfection of recombinant bacmid DNA into BmN cells, the recombinant baculovirus containing *egl2* gene was thus successfully generated. In the traditional method, the procedure of construction and purification of a recombinant baculovirus using the plaque assay usually takes 2–3 months or longer. Compared with the traditional method, the Bac-to-Bac system for silkworm is time-saving and highly efficient and took only 10 days to construct the recombinant baculovirus. The primary virus stock with a titer of 10^6 p.f.u./ml was obtained.

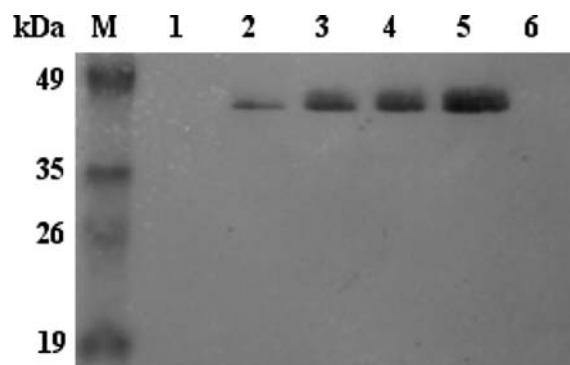
Based on the deduced amino acid sequence, the molecular weight of the rEGII was calculated to be 45.93 kDa (including six histidines and others from the multiple cloning site, 3.89 kDa). As shown in Fig. 2, a protein of approximately 46 kDa was identified by Western blot analysis, indicating that the size of this fusion protein is correct. These results suggested that the EGII protein was well expressed in BmN cells and reached maximum levels at 120 h post-infection. However, the expressed rEGII was only detected in the cells but not in the supernatant (data not shown).

In total, 38.6 mg biologically active EGII was obtained from 100 infected larvae and the enzymatic



Cloning of the complete encoding sequence of *egl2* gene. Lane 1, Molecular marker, the size (bp) is listed on the left; lane 2, the complete encoding sequence of *egl2* gene.

Analysis of recombinant bacmid DNA by PCR to verify the presence of the *egl2* gene in the recombinant BmBacmid by using M13 forward (–40) and M13 reverse primers. The bacmid contained M13 priming sites flanking the mini-*att*Tn7 site within the *lacZ* α complementation region to facilitate PCR analysis (detailed information available at P23, Bac-to-Bac manual by Invitrogen). Lane 1, Molecular marker, the size (bp) is listed on the left; lane 2, PCR product using the recombinant BmBacmid as a template. The predicted size was about 3.6 kb



Time course analysis of the recombinant EGII expressed in BmN cells. The protein was detected by Western blot analysis. Lane M, prestained protein molecular weight marker; lanes 1–5, cell lysates of 24, 48, 72, 96 and 120 h post-infection; lane 6, uninfected cell lysate (control)

activity of the purified recombinant EGII was approximately 352 U/mg of recombinant EGII, indicated that expression of rEGII was high and that large-scale and successive production of rEGII in silk worms is feasible. Most foreign proteins expressed in silk worms undergo normal post-translational modifications. For example, glycosylation occurs more easily and efficiently in larvae than in cell lines. In this study, Western blot analysis showed that the molecular size of the purified rEGII expressed in silk worm larvae was larger than that of rEGII expressed in BmN cells (Fig. 3a). This band in the purified rEGII was presumed to be the mature form, glycosylated at the putative *N*-glycosylation site Asn-Phe-Thr at the amino acid positions 103-105 (Saloheimo et al. 1988). Figure 3b shows the molecular weight in the purified rEGII after endoglycosidase F treatment was consistent with rEGII expressed in BmN cells, indicating that rEGII is apparently *N*-glycosylated when expressed in *B. mori* larvae.

Production of recombinant proteins was maximal at 96–120 h post-infection (Fig. 4), and CMCase activity of 120 h uninfected group was higher than that of the 48 h post-infection group. Silk worms possibly secrete their own cellulolytic enzymes because they feed on mulberry leaves. Optimal activity for rEGII was at 55 °C (Fig. 5a) and at pH 4 (Fig. 5b).

Silk worm is economically important for silk production in many countries. Since the BmNPV/baculovirus expression system was constructed in 1980, silk worm larvae have become an ideal bioreactor for producing recombinant proteins because of the following advantages: easy rearing, low cost, a large body, short life cycle, well documented genetics and biology, and good biosafety. Silk worm has four different stages in its life cycle: egg, larva, pupa and moth of which the larval stage is the only feeding stage and the larvae in their fifth-instar grow to almost 5 g. This “biofactor” has already been used for producing hepatitis B surface antigen (Higashihashi et al. 1991), grass carp growth hormone (Ho et al. 1998), human VEGF165 (Wu et al. 2004), human lactoferrin (Liu et al. 2005) and canine interferon- α (Zhao et al. 2008). Silk worms are also used as feed for other animals, it is nutritious and the recombinant baculovirus is non-infectious to animals. The approach established here is probably one of the most economical and efficient ways of producing the EGII protein. Therefore, the mass production of rEGII in silk worms by the BmNPV/Bac-to-Bac expression system is feasible and it paves the way for further utilization of this protein as a feed additive for animals.

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