ORIGINAL RESEARCH PAPER

Trichoderma reesei
Bombyx mori

recombinant EGII in the silk orm because of advantages in ield, cost, handling and safet (Lee et al. 2006). The intention is to obtain a silk orm po der and add it to animal feed to improve cellulose degradation.

Strains, plasmids and culture conditions

Trichoderma reesei QM9414 as purchased from the China General Microbiological Culture Collection Center (CGMCC). Liquid cultures of *T. reesei* ere started from conidiospores gro n on the potato/de trose/agar (PDA) slants. For induction of cellulase formation, m celia ere cultivated in shake- asks for 2 da s (28 C, 200 rpm) as described b Baile and Nevalainen (1981) e cept that 2% (/v) lactose as added. The cloning vector (pGEM-T Eas Vector S stem) as purchased from Promega (Madison, Wisconsin, USA). The Bac-to-Bac donor plasmid pFastBacHTc as from Invitrogen (San Diego, CA, USA). *E. coli* DH5α (Novagen, CA, USA) as used as a host for plasmid preparation.

Cell lines and silk orm

The *Bombyx mori*-derived cell line, BmN (conserved b our laborator), as maintained at 27 C ith TC-100 medium containing 10% (v/v) fetal bovine serum

sterile tubes. Both the diluted solution and incubate ere 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 ere ashed ith 2 ml unsupplemented medium. Then, 0.8 ml unsupplemented medium as added to the tube containing DNA:lipid comple es, and the DNA:lipid comple es ere transferred to the ashed cells. After 5 h incubation in unsupplemented TC-100 medium, the culture medium as replaced b fresh medium containing 10% FBS. The cells ere allo ed to gro at 27 C for 5 da s and the medium as collected as the primar 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 orm larvae

Firstl , the stock virus as used to infect BmN cells. The culture media and cells at 24, 48, 72, 96 and 120 h post-infection ere collected separatel b lo -speed centrifugation for 5 min at 4 C, and subsequentl used for protein anal sis to investigate its time-course gene e pression. The infected cells ere l sed to release rEGII b repeat free ing and tha ing before SDS-PAGE.

The recombinant virus as also used to infect ne l molted fth-instar silk orm larvae. The larvae ere anestheti ed on ice for 10 min until the did not move activel . About 10 µl recombinant viruses ere injected subcutaneousl into each larva and 30 min after injection, the larvae ere fed ith mulberr leaves and then reared at 25 27 C. The fat bod of infected larvae as 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 da of post-infection. The silk orm larvae ere collected before this period.

Puri cation of recombinant EGII

For large-scale puri cation of rEGII, no more feed as given to the silk orms at 96 h post-infection to clean their guts and, nall , 100 infected larvae at 110 h post-infection ere selected. Their hole bodies e cept for silk gland ere homogeni ed in ice-cold citric acid buffer (50 mM, pH 5), and then the mi ture as centrifuged at 12,000×*g* for 15 min at 4 C to remove large debris and lipids. The supernatant as ltered to remove the remaining lipids, and the ltrate as centrifuged again at 12,000×*g* for 20 min at 4 C. The supernatants ere collected as the crude e tracts of rEGII. To prevent melani ation, 0.1 vol 10 mM dithioer thritol (DTE) as added but no protease inhibitors ere added.

As the rEGII carries an *N*-terminal 6× His-tag, puri cation as done ith the Ni-NTA af nit columns under native conditions. The crude e tracts ere diluted ith native binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8) and then applied directl to Ni-NTA columns for binding. The rEGII as nall eluted ith native elution buffer (binding buffer plus 250 mM imida ole).

Endogl cosidase F treatment

The recombinant EGII puri ed from 100 infected larvae at 110 h post-infection as treated ith endogl cosidase 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 endogl cosidase-treated and untreated samples ere prepared for Western blot anal sis.

Bioassa of recombinant EGII

At various times, ten recombinant silk orms ere homogeni ed, centrifuged and the CMCase activit of supernatant as measured using 1% (/v) CMC as a substrate in 50 mM citric acid buffer (pH 5) at 50 C for 30 min. The reactions ere terminated b adding DNS reagent and boiled for 5 min; A_{540} as measured after cooling. The speci c activit of the puri ed recombinant EGII from 100 infected larvae at 110 h post-infection as also determined. One unit of the en me as de ned as the activit producing 1 µmol of reducing sugars in glucose equivalents per min.

En me activit for optimum temperature as determined b incubating the puri ed rEGII in citric acid buffer (0.05 M, pH 5) containing 1% (/v) CMC at the temperature ranges from 30 C to 80 C ith 5 C or 10 C intervals for 30 min. For the optimal pH, the puri ed rEGII as treated in 0.05 M buffer at various pH ranges (pH 2 8) at 50 C for 30 min. The buffers used ere acetate (pH 2 and 3), citrate (pH 4 6), phosphate (pH 7).

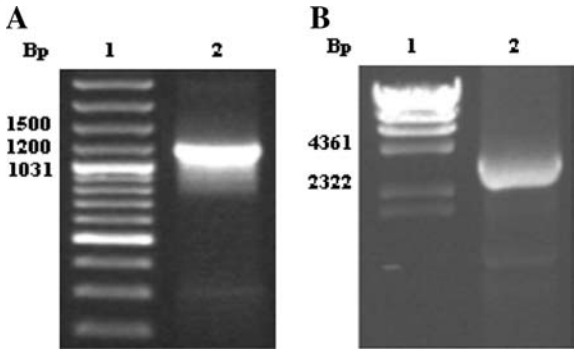
The *egl2* gene as cloned from *T. reesei* QM9414 and anal ed (Fig. 1a). Its predicted si e as 1,194 bp. Comparison of this sequence ith that registered in GenBank (accession no. M19373) sho ed onl three nucleotide changes at positions 156 (A-G), 369 (G-C), and 1135 (A-G), that is t o changes in the deduced amino acid sequence at positions 123 (Glu-Asp) and 379 (Ser-Gl).

The BmNPV/Bac-to-Bac e pression s stem applicable to silk orm as developed based on the orking principle of AcMNPV Bac-to-Bac s stem. In our previous stud (Cao et al. 2006), a large 8.6 kb fragment containing the lo -cop -number mini-F replicon, a kanam cin resistance marker, a segment of DNA encoding the *lacZα* peptide and a targeting site for bacterial transposon Tn7 (mini-*att*Tn7) from the AcMNPV bacmid, as cloned into pol hedrin locus of BmNPV genome to replace the pol hedrin gene. This recombinant BmNPV DNA as transformed as a large plasmid, named bacmid, into *E. coli* DH10*β* strain, in hich a helper plasmid encoding the transposase as alread transformed. The DH10*β* strain containing BmBacmid and helper as designated as DH10BmBac. At the same time, the multiple cloning site in the donor plasmid is anked b the left and right bacterial transposon Tn7 sequences, hich can carr the foreign gene transpose into the mini-*att*Tn7 site in the BmNPV genome. With this novel Bac-to-Bac s stem, the recombinant baculovirus can be rapidl and easil generated through gene transposition.

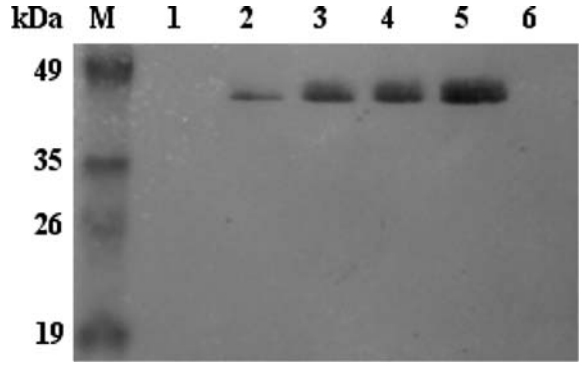
The correct insertion of *egl2* gene into the BmNPV bacmid as con rmed (Fig. 1b), indicating that the gene transposition as ell performed. After transfection of recombinant bacmid DNA into BmN cells, the recombinant baculovirus containing *egl2* gene as thus successfull generated. In the traditional method, the procedure of construction and puri cation of a recombinant baculovirus using the plaque assa usuall takes 2 3 months or longer. Compared ith the traditional method, the Bac-to-Bac s stem for silk orm is time-saving and highl ef cient and took onl 10 da s to construct the recombinant baculovirus. The primar virus stock ith a titer of 10^6 p.f.u./ml as obtained.

Based on the deduced amino acid sequence, the molecular eight of the rEGII as calculated to be 45.93 kDa (including si histidines and others from the multiple cloning site, 3.89 kDa). As sho n in Fig. 2, a protein of appro 46 kDa as identi ed b Western blot anal sis, indicating that the si e of this fusion protein is correct. These results suggested that the EGII protein as ell e pressed in BmN cells and reached ma imum levels at 120 h post-infection. Ho ever, the e pressed rEGII as onl detected in the cells but not in the supernatant (data not sho n).

In total, 38.6 mg biologicall active EGII as obtained from 100 infected larvae and the en matic



. Cloning of the complete encoding sequence of *egl2* gene. *Lane 1*, Molecular marker, the si e (bp) is listed on the *left*; *lane 2*, the complete encoding sequence of *egl2* gene. Anal sis of recombinant bacmid DNA b PCR to verif the presence of the *egl2* gene in the recombinant BmBacmid b using M13 for ard (−40) and M13 reverse primers. The bacmid contained M13 priming sites anking the mini-*att*Tn7 site ithin the *lacZα* complementation region to facilitate PCR anal sis (detailed information available at P23, Bac-to-Bac manual b *Invitrogen*). *Lane 1*, Molecular marker, the si e (bp) is listed on the *left*; *lane 2*, PCR product using the recombinant BmBacmid as a template. The predicted si e as about 3.6 kb



. 2 Time course anal sis of the recombinant EGII e pressed in BmN cells. The protein as detected b Western blot anal sis. *Lane M*, prestained protein molecular eight marker; *lane 1–5*, cell l sates of 24, 48, 72, 96 and 120 h post-infection; *lane 6*, uninfected cell l sate (control)

activit of the puri ed recombinant EGII as appro 352 U/mg of recombinant EGII, indicated that e pression of rEGII as high and that large-scale and successive production of rEGII in silk orms is feasible. Most foreign proteins e pressed in silk orms undergo normal post-translational modi cations. For e ample, gl cos lation occurs more easil and ef cientl in larvae than in cell lines. In this stud , Western blot anal sis sho ed that the molecular si e of the puri ed rEGII e pressed in silk orm larvae as larger than that of rEGII e pressed in BmN cells (Fig. 3a). This band in the puri ed rEGII as presumed to be the mature form, gl cos lated at the putative *N*-gl cos lation site Asn-Phe-Thr at the amino acid positions 103-105 (Saloheimo et al. 1988). Figure 3b sho s the molecular eight in the puri ed rEGII after endogl cosidase F treatment as consistent ith rEGII e pressed in BmN cells, indicating that rEGII is apparentl N-gl cos lated hen e pressed in *B. mori* larvae.

Production of recombinant proteins as ma imal at 96 120 h post-infection (Fig. 4), and CMCase activit of 120 h uninfected group as higher than that of the 48 h post-infection group. Silk orms possibl secrete their o n cellulol tic en mes because the feed on mulberr leaves. Optimal activit for rEGII as at 55 C (Fig. 5a) and at pH 4 (Fig. 5b).

Silk orm is economicall important for silk production in man countries. Since the BmNPV/baculovirus e pression s stem as constructed in 1980, silk orm larvae have become an ideal bioreactor for producing recombinant proteins because of the follo ing advantages: eas rearing, lo cost, a large bod , short lifec cle, ell documented genetics and biolog , and good biosafet . Silk orm has four different stages in its life c cle: egg, larva, pupa and moth of hich the larval stage is the onl feeding stage and the larvae in their fth-instar gro to almost 5 g. This "biofactor " has alread been used for producing hepatitis B surface antigen (Higashihashi et al. 1991), grass carp gro th hormone (Ho et al. 1998), human VEGF165 (Wu et al. 2004), human lactoferrin (Liu et al. 2005) and canine interferon-alpha (Zhao et al. 2008). Silk orms 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 probabl one of the most economical and ef cient a s of producing the EGII protein. , the mass production of rEGII in silk orms b the BmNPV/Bac-to-Bac e pression s stem is feasible and it paves the a for further utili ation of this protein as a feed additive for animals.

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