Biotechnol Lett (2010) 32:67–72
DOI 10.1007/s10529-009-0129-8

ORIGINAL RESEARCH PAPER

Expression of *Trichoderma reesei* endo-β-glucanase II in silkworm, *Bombyx mori* L. by using BmNPV/Bac-to-Bac expression system and its bioactivity assay

Lie Zhou · Xiaofeng Wu · Lipan Lan · Jianxin Liu

Received: 27 May 2009 / Revised: 31 August 2009 / Accepted: 7 September 2009 / Published online: 17 October 2009

Abstract The silkworm, *Bombyx mori*, was used to produce recombinant endo-β-glucanase II (rEGII). The EGII gene (*egl2*) was cloned from the cellulolytic fungus *Trichoderma reesei* and inserted into *B. mori* nucleopolyhedrovirus (BmNPV) genome using BmNPV/Bac-to-Bac expression vector. For expression of rEGII, both the BmN cells and *B. mori* larvae were infected with the recombinant virus. The putative rEGII yield was about 386 μg per larva and the enzyme activity of the purified rEGII was approx 352 U/mg of rEGII. The optimal activity of this purified protein was observed at 55°C and pH 4, respectively.

Keywords BmNPV/Bac-to-Bac · Expression system · Endoglucanase · Feed additive · Silkworm · *Trichoderma reesei*

L. Zhou · J. Liu (✉)
Institute of Dairy Science, Ministry of Education Key Laboratory of Molecular Animal Nutrition, Zhejiang University, Hangzhou 310029, People's Republic of China
e-mail: liujx@zju.edu.cn

X. Wu · L. Lan
Institute of Sericulture and Apiculture, Zhejiang University, Hangzhou 310029, People's Republic of China

Introduction

Cellulose is composed of repeating glucose units linked by β-1,4-glycosidic bonds. The importance of cellulose hydrolysis in the conversion of plant biomass to fuels and chemicals is widely recognized (Himmel et al. 1999), and cellulose hydrolysis also represents one of the largest material flows in the global carbon cycle (Falkowski et al. 2000). The filamentous fungus, *Trichoderma reesei*, is one of the most effective producers of cellulases and has served as a model for fungal cellulose degradation (Saloheimo et al. 1997). For the conversion of cellulose into glucose, three types of cellulolytic enzymes are produced by *T. reesei*: endoglucanases (E.G, EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.1.91) and β-glucosidases (BGL, EC 3.2.1.21). Members of each of these enzyme groups have been characterized from *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 system was used to produce large quantities of

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

Materials and methods

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 Bailey and Nevalainen (1981) except that 2% (w/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* DH5α (Novagen, CA, USA) was used as a host for plasmid preparation.

Cell lines and silkworm

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 (FBS) and 0.26% Bacto-tryptose. A hybrid strain of silkworm (*Baiyu* × *Qiufeng*) was used in this experiment. The larvae were reared with mulberry leaves at 23–25°C. The BmNPV/Bac-to-Bac expression system was constructed and maintained by Prof. Wu Xiaofeng (Cao et al. 2006).

Cloning of *egl2* gene

Total RNA was extracted from the mycelia with Trizol reagent and the first-strand cDNA was synthesized by using the RevertAid First Strand cDNA Synthesis Kit (MBI). The *egl2* gene extending from Gln22 to the TAG stop codon of the gene was amplified with *Ex Taq* DNA polymerase by using the sense primer 5′-CAG CAG ACT GTC TGG GGC CA-3′, antisense primer 5′-GCT ACT TTC TTG CGA GAC ACG AG-3′, with the first-strand cDNA as the template. Amplification was performed under the following conditions: pre-denaturation for 5 min at 94°C, then 35 cycles consisting of denaturation for 50 s at 94°C, annealing for 50 s at 68°C, extension for 2 min at 72°C, followed by a final extension for 7 min at 72°C. The PCR product was confirmed by electrophoresis on a 1% (w/v) agarose gel and subsequently cloned into pGEM-T Easy vector. The resulting plasmid was named as pGEM-*egl2*. The nucleotide sequence of the cloned *egl2* gene was determined by using the dideoxy-chain termination method with a nucleotide sequencer.

Generation of recombinant bacmid

The *egl2* fragment was cut from pGEM-*egl2* digested with *Eco*RI, and inserted into the cut pFastBacHTc donor plasmid using the same restriction site. The resultant recombinant plasmid was designated pFastBacHTc-*egl2*. The BmNPV/Bac-to-Bac expression system was applied as follows to generate the recombinant bacmid: about 1 ng purified pFastBacHTc-*egl2* was transformed into 100 µl DH10BmBac competent cells. The mixture was incubated at 37°C for 4 h for transposition of the *egl2* gene into the BmNPV Bacmid (BmBacmid). The cells were serially diluted with SOC (Super Optimal broth with Catabolite repression) medium, and then 100 µl of each dilution was distributed evenly on Luria–Bertani (LB) agar plates containing 50 µg kanamycin/ml, 7 µg gentamicin/ml, 10 µg tetracycline/ml, 100 µg X-gal/ml, and 40 µg IPTG/ml. After 48 h incubation at 37°C, the largest and most isolated white colonies were selected and inoculated in liquid culture containing 50 µg kanamycin/ml. The recombinant bacmid DNA was then extracted according to the protocol for isolating large plasmids (>100 kb) (Invitrogen Instruction Manual of Bac-to-Bac systems), and analyzed to verify successful gene transposition to the bacmid by PCR with the M13 primers.

Construction of recombinant baculovirus

The recombinant baculovirus harboring *egl2* gene was constructed by transfection of recombinant bacmid DNA into the BmN cells for homologous recombination. About 1 µg recombinant bacmid DNA and 6 µl Cellfectin reagent were separately diluted into 100 µl unsupplemented TC 100 medium in 12 × 75 mm

sterile tubes. Both the diluted solution and incubate were 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 were washed with 2 ml unsupplemented medium. Then, 0.8 ml unsupplemented medium was added to the tube containing DNA:lipid complexes, and the DNA:lipid complexes were transferred to the washed cells. After 5 h incubation in unsupplemented TC-100 medium, the culture medium was replaced by fresh medium containing 10% FBS. The cells were allowed to grow at 27°C for 5 days and the medium was 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 silkworm larvae

Firstly, the stock virus was used to infect BmN cells. The culture media and cells at 24, 48, 72, 96 and 120 h post-infection were 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 were lysed to release rEGII by repeat freezing and thawing before SDS-PAGE.

The recombinant virus was also used to infect newly molted fifth-instar silkworm larvae. The larvae were anesthetized on ice for 10 min until they did not move actively. About 10 μl recombinant viruses were injected subcutaneously into each larva and 30 min after injection, the larvae were fed with mulberry leaves and then reared at 25–27°C. The fat body of infected larvae was 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 silkworm larvae were collected before this period.

Purification of recombinant EGII

For large-scale purification of rEGII, no more feed was given to the silkworms at 96 h post-infection to clean their guts and, finally, 100 infected larvae at 110 h post-infection were selected. Their whole bodies except for silk gland were homogenized in ice-cold citric acid buffer (50 mM, pH 5), and then the mixture was centrifuged at 12,000×*g* for 15 min at 4°C to remove large debris and lipids. The supernatant was filtered to remove the remaining lipids, and the filtrate was centrifuged again at 12,000×*g* for 20 min at 4°C. The supernatants were collected as the crude extracts of rEGII. To prevent melanization, 0.1 vol 10 mM dithioerythritol (DTE) was added but no protease inhibitors were added.

As the rEGII carries an *N*-terminal 6× His-tag, purification was done with the Ni-NTA affinity columns under native conditions. The crude extracts were 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 was 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 was 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 were prepared for Western blot analysis.

Bioassay of recombinant EGII

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

Enzyme activity for optimum temperature was determined by incubating the purified rEGII in citric acid buffer (0.05 M, pH 5) containing 1% (w/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 was treated in 0.05 M buffer at various pH ranges (pH 2–8) at 50°C for 30 min. The buffers used were acetate (pH 2 and 3), citrate (pH 4–6), phosphate (pH 7).

Results and discussion

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-Gly).

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 low-copy-number mini-F replicon, a kanamycin 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, 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 transpose 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.

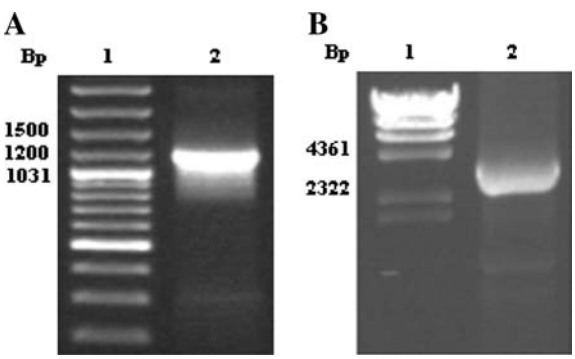

Fig. 1 **a** Cloning of the complete encoding sequence of *egl2* gene. *Lane 1*, Molecular marker, the size (bp) is listed on the *left*; *lane 2*, the complete encoding sequence of *egl2* gene. **b** Analysis of recombinant bacmid DNA by PCR to verify the presence of the *egl2* gene in the recombinant BmBacmid by using M13 forward (−40) and M13 reverse primers. The bacmid contained M13 priming sites flanking the mini-*att*Tn7 site within the *lacZα* 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

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 approx 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

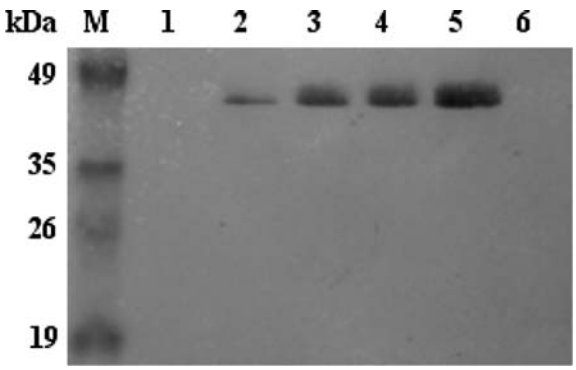

Fig. 2 Time course analysis of the recombinant EGII expressed in BmN cells. The protein was detected by Western blot analysis. *Lane M*, prestained protein molecular weight marker; *lane 1–5*, cell lysates of 24, 48, 72, 96 and 120 h post-infection; *lane 6*, uninfected cell lysate (control)

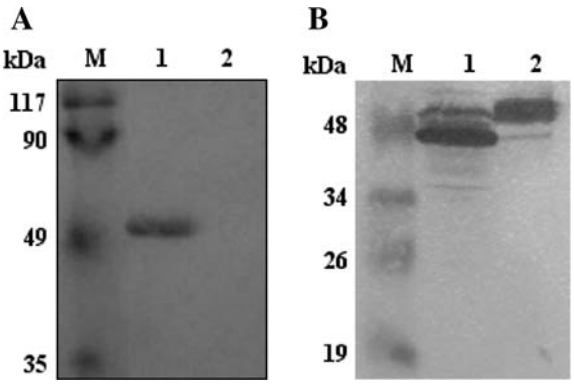

Fig. 3 **a** Western blot analysis of the purified recombinant EGII expressed in silkworm larvae at 110 h post-infection. *Lane M*, prestained protein molecular weight marker; *lane 1*, the purified rEGII protein band; *lane 2*, the control group without infection. **b** Deglycosylation of rEGII purified from the larvae by endoglycosidase F treatment. *Lane M*, prestained protein molecular weight marker; *lane 1*, the purified rEGII treated with endoglycosidase F; *lane 2*, the purified rEGII untreated with endoglycosidase F. The samples were assayed by Western blot analysis

activity of the purified recombinant EGII was approx 352 U/mg of recombinant EGII, indicated that expression of rEGII was high and that large-scale and successive production of rEGII in silkworms is feasible. Most foreign proteins expressed in silkworms 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 silkworm 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. Silkworms 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).

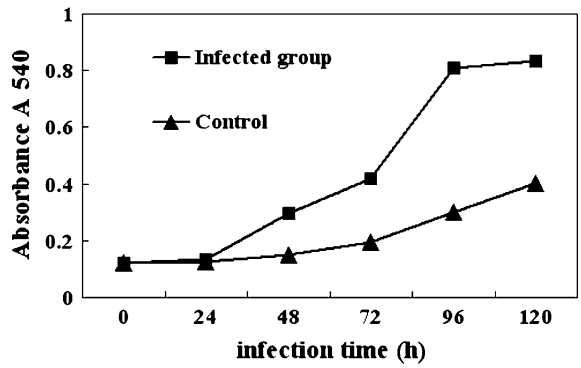

Fig. 4 Bioactivity assay of the recombinant EGII expressed in silkworm larvae at 24, 48, 72, 96 and 120 h post-infection. Every ten infected silkworms were homogenized at various infection times. All experiments were done in duplicate

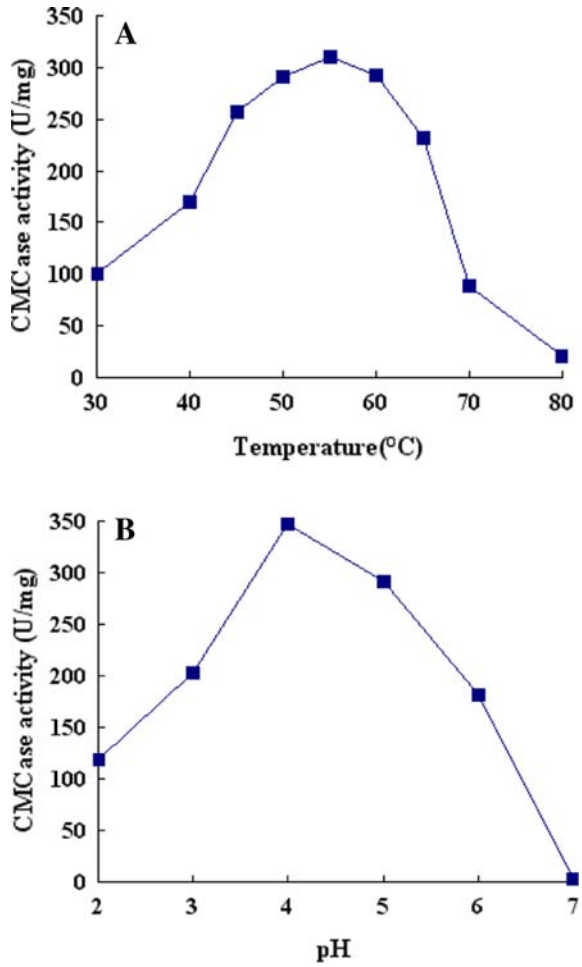

Fig. 5 Enzymatic properties of the purified recombinant EGII expressed in silkworm larvae. **a** Optimum temperature of the rEGII protein. **b** Optimum pH of the recombinant EGII. All experiments were done in duplicate

Silkworm is economically important for silk production in many countries. Since the BmNPV/baculovirus expression system was constructed in 1980, silkworm larvae have become an ideal bioreactor for producing recombinant proteins because of the following advantages: easy rearing, low cost, a large body, short lifecycle, well documented genetics and biology, and good biosafety. Silkworm 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 "biofactory" 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-alpha (Zhao et al. 2008). Silkworms 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.

In summary, the mass production of rEGII in silkworms 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.

Acknowledgements This work was supported partly by a grant from the National Natural Science Foundation of China (NSFC) (No.30530560). Thanks are due to Scholastica P. Doto for revision of the manuscript.

References

Bailey MJ, Nevalainen KMH (1981) Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enzyme Microb Technol 3:153–157

Cao C, Wu X, Zhao N, Yao H, Lu X, Tan Y (2006) Development of a rapid and efficient BmNPV baculovirus expression system for application in mulberry silkworm, *Bombyx mori*. Curr Sci 91(12):1692–1697

Falkowski P, Scholes RJ, Boyle E, Canadell J, Canfield D, Elser J, Gruber N, Hibbard K, Högberg P, Linder S, Mackenzie FT, Moore B III, Pedersen T, Rosenthal Y, Seitzinger S, Smetacek V, Steffen W (2000) The global carbon cycle: a test of our knowledge of earth as a system. Science 290(5490):291–296

Higashihashi N, Arai Y, Enjyo T, Horiuchi T, Saeki Y, Sakano K, Sato Y, Takeda K, Takashina S, Takahashi T (1991) High-level expression and characterization of hepatitis B virus surface antigen in silkworm using a baculovirus vector. J Virol Methods 35:159–167

Himmel ME, Ruth MF, Wyman CE (1999) Cellulase for commodity products from cellulosic biomass. Curr Opin Biotechnol 10(4):358–364

Ho WKK, Meng ZQ, Lin HR, Poon CT, Leung YK, Yan KT, Dias N, Che APK, Liu J, Zheng WM, Sun Y, Wong AOL (1998) Expression of grass carp growth hormone by baculovirus in silkworm larvae. Biochim Biophys Acta 1381:331–339

Lee KS, Je YH, Woo SD, Sohn HD, Jin BR (2006) Production of a cellulase in silkworm larvae using a recombinant *Bombyx mori* nucleopolyhedrovirus lacking the virus-encoded chitinase and cathepsin genes. Biotechnol Lett 28(9):645–650

Liu T, Zhang YZ, Wu XF (2005) High level expression of functionally active human lactoferrin in silkworm larvae. J Biotechnol 118(3):246–256

Nevalainen H, Penttilä M (1995) Molecular biology of cellulolytic fungi. In: Kück U (ed) Genetics and biotechnology. The Mycota II, Springer-Verlag, Berlin, pp 303–319

Penttilä ME, André L, Saloheimo M, Lehtovaara P, Knowles JK (1987) Expression of two *Trichoderma reesei* endoglucanases in the yeast *Saccharomyces cerevisiae*. Yeast 3(3):175–185

Qiao Y, Mao A, He Y, Liu W, Dong Z (2004) Secreted expression of *Trichoderma reesei* endo-β-glucanase II gene in *pichia pastoris* and analysis of enzymic properties. Mycosystema 23(3):388–396 (in chinese)

Saloheimo M, Lehtovaara P, Penttilä M, Teeri TT, Ståhlberg J, Johansson G, Pettersson G, Claeyssens M, Tomme P, Knowles JK (1988) EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. Gene 63(1):11–21

Saloheimo M, Nakari-Setälä T, Tenkanen M, Penttilä M (1997) cDNA cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity by expression in yeast. Eur J Biochem 249(2):584–591

Wu X, Yin Z, Cao C, Ling H, Lu X, Liu J, Cui W (2004) Expression of human VEGF165 in silkworm (*Bombyx mori* L.) by using a recombinant baculovirus and its bioactivity assay. J Biotechnol 111(3):253–261

Zhao N, Yao H, Lan L, Cao C, Umashankar ML, Lu X, Wu X, Wang B, Cui W, Cenis JL (2008) Efficient production of canine interferon-alpha in silkworm *Bombyx mori* by use of a BmNPV/Bac-to-Bac expression system. Appl Microbiol Biotechnol 78:221–226