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Heterologous Expression Characteristics of *Trichoderma viride* Endoglucanase V in the Silkworm, *Bombyx mori* L.

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Abstract Efficient degradation of cellulose needs a synergistic reaction of the cellulolytic enzymes, which include exoglucanases, endoglucanases, and β-1,4-glucosidase. In this study, we used an improved Bac-to-Bac/BmNPV baculovirus expression system, which lacks the virus-encoded chitinase cathepsin (*v-cath*) genes of *Bombyx mori* nucleopolyhedrovirus (BmNPV), to express the endoglucanase V (*EG V*) gene from *Trichoderma viride* in silkworm BmN cells and silkworm larvae, and analyzed the characteristics of the recombinant enzyme in silkworm larvae. The result showed that an around 36-kDa protein was visualized in BmN cells at 48 h after the second-generation recombinant mBacmid/BmNPV/EG V baculovirus infection. The crude enzyme extract from the recombinant baculoviruses-infected silkworms exhibited a significant maximum activity at the environmental condition of pH 5.0 and a temperature of 50 °C, and increased 39.86% and 37.76% compared with that from blank mBacmid/BmNPV baculovirus-infected silkworms and normal silkworms, respectively. It was stable at pH range from 5.0 to 10.0 and at temperature range from 40 to 60 °C. The availability of large quantities of EG V that the silkworm provides might greatly facilitate the future research and the potential application in industries.

Keywords *Trichoderma viride* · Cellulase · *Bombyx mori*

Introduction

Given the imminent declines of fossil fuels and the large seriousness of environmental issues such as global warming and acid rain, utilization of plant biomass, especially

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cellulosic materials, as a renewable source has recently become an attractive option. It is plentiful, cheap, and friendly to the environment [1]. Therefore, extensive research efforts to exploit lignocellulosic materials for potential bioethanol production with an efficient and cost-effective method are required.

Efficient degradation of cellulose requires a synergistic reaction of the cellulolytic enzymes, which include exo-β-1,4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4), and β-1,4-glucosidase (EC 3.2.1.21) [2]. They act in a synergistic manner to facilitate complete cleavage of the cellulose β-1,4-glycosidic bonds to form glucose [3].

Cellulase can be produced in a wide variety of organisms, including fungi, bacteria, plants and protists, as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, molluscs and nematodes [4, 5]. It was recognized that filamentous fungi *Trichoderma* can degrade cellulose very effectively and its cellulase has been widely investigated [6–8]. The *Trichoderma* cellulolytic system is at least composed of five endoglucanases (EG I, EG II, EG III, EG IV, and EG V), two cellobiohydrolases (CBH I and CBH II) and two β-1,4-glucosidases (BGL I and BGL II). Among those, endoglucanases are known to be key enzymes in biostoning and biofinishing [9].

Recently, the silkworm has been used as an important bioreactor to produce recombinant proteins through baculovirus expression system [10], and an improved Bac-to-Bac/BmNPV baculovirus expression system has been developed, which lacks the virus-encoded chitinase (*chiA*) and cathepsin (*v-cath*) genes for the purpose of increasing the expression of foreign proteins [11].

In this study, we used the improved Bac-to-Bac/BmNPV baculovirus expression system to construct an expression vector encoding EG V mature peptide gene by integrating this gene from *T. viride* into the transposition site of host genome, and then constructed the EG V-producing recombinant baculovirus by transfecting the expression vector into the silkworm BmN cells. After infecting the silkworm larvae with the recombinant baculovirus, we confirmed the capability of the recombinant baculovirus to produce bioactive recombinant EG V protein and analyzed its enzyme activity characteristics.

Materials and Methods

Materials

Hybrid silkworm larvae (commercial name: Qingsong×Haoyue) were reared in the standard condition for recombinant protein expression. The fungus *Trichoderma viride* strain AS 3.3711 purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing) was cultured in the wheat bran medium. The *Escherichia coli* TG1 cultured in the LB medium was used for the replication of donor plasmid. The DH10Bac™ *E. coli* mutant strain also cultured in the LB medium was used as the host of the donor plasmid, which contained a mutant baculovirus shuttle vector (mBacmid), lacking the virus-encoded chitinase (*chi*A) and cathepsin (*v-cath*) genes of *Bombyx mori* nucleopolyhedrovirus (BmNPV).

Cloning of *EG V* Gene from *T. viride*

Based on the sequence alignment between a *T. viride* strain AS 3.3711 endoglucanase V mRNA gene (GenBank no. AY343989) and *Trichoderma reesei* (QM9414) endo-1,4-beta-

glucanase gene (GenBank no. Z33381), the *EG V* gene of *T. viride* strain AS 3.3711 was synthesized from its genome by PCR-based exon splicing methods with the primers listed in Table 1. The processes of PCR with primer pairs EG V P1–EG V P2, EG V P3–EG V P4, and EG V P5–EG V P6 were under the following conditions: 94 °C, 2 min; 32 cycles of 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 45 s; followed by 72 °C for 10 min. The purified products were used as templates in the next PCR reaction to generate EG V exon splicing DNA, that is EG V cDNA sequence (744 bp in size), with primer pair EG V P1–EG V P6 under 94 °C, 2 min; 32 cycles of 94 °C, 1 min; 60 °C, 1 min; and 72°C, 48 s; 1 cycle of 72°C, 10 min, followed by cloning into pMD18-T vector (Takara), designated pMD18-T/EG V.

The EG V mature peptide gene sequence was amplified using the primer pair (5′-TTA**GAATTC**TACAAGGCCACCACCACGC-3′) and (5′-TGG**AAGCTT**CTATCTTGGGCCTTCCCAAGG-3′) with restriction enzymes *Eco*RI and *Hin*dIII, respectively, under 94 °C, 2 min; 32 cycles of 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 45 s; followed 72 °C, 10 min. The product with the expected size (711 bp) was purified, enzyme hydrolyzed and then cloned into the corresponding site of pFastBac™ HT A, yielding the recombinant donor plasmid named pFast A/EG V.

Construction of Recombinant Baculovirus

The pFast A/EG V was then transformed into the DH10Bac^{TM} *E. coli* mutant strain competent cells to generate recombinant mBacmid baculoviruses DNA mBacmid/BmNPV/EG V, which was confirmed by PCR analysis with M13 primer pair and was then transfected into BmN cells to generate recombinant mBacmid/BmNPV/EG V baculovirus. The high-titer second-generation viral solution was used for expression of recombinant protein.

Expression of *EG V* Gene in Silkworm

BmN cells (2×10^6 cells/flask) were infected with the second-generation recombinant virus and collected 48 h postinfection, followed by Western blotting analysis. For the expression of the *EG V* gene in silkworm larvae, the recombinant baculovirus was injected into the first day larvae of the fifth instar at about 4×10^5 particles per worm in a volume of 10 μl before feeding. Fifty silkworms were randomly sampled at 84 h for the enzyme activity analysis.

Western Blotting Analysis

The cell lysate was subjected to a 10% separating gel, and the proteins were transferred onto an NC membrane under 110 mA for 1.5 h. After blocking for 2 h at room temperature,

Table 1 Primers used for exon PCR amplifications and splicings of *T. viride* EG V genomic DNA

Primers	Sequence (5′ to 3′)	Location on EG V genomic DNA
EG V P1	ATGAAGGCAACTCTGGTTCTCG	1–22
EG V P2	GTAGTAGCGCGTGGTGGTGGCCTTGTACGC	49–68, 129–138
EG V P3	CCACCACCACGCGCTACTACGATGGGCAGG	59–68, 129–148
EG V P4	GCCGAGCTGCCACGGGAATGCGCCGGAGC	173–191, 254–263
EG V P5	CATTCCCGTGGCAGCTCGGCATCGGCAACG	182–191, 254–273
EG V P6	CTATCTTGGGCCTTCCCAAG	847–866

the membrane was incubated with first 6× His antibody and HRP-labeled second antibody (both from GenScript, USA), successively. Finally, a TMB solution (Promega) was used to detect the protein band.

Enzyme Activity Analysis

Fifty silkworms randomly sampled at 84 h were homogenized in 100 ml of pH 5.0 buffer, followed by centrifugation at 10,000 rpm and 4 °C for 10 min. The clarified supernatant used as the source of enzymes was analyzed by DNS method with CMC-Na as a substrate. When the reaction product of the enzyme and CMC-Na encounters DNS at 100 °C, the solution color changes quickly and it can be analyzed by a spectrophotometer. The international unit of activity is defined as the amount of enzyme, which liberates 1 μmol of glucose per minute in a standard assay.

Characteristic Analysis of Enzyme

The enzyme characteristics were analyzed in the conditions of different pHs (pH 3–10) and different temperatures (40–90 °C).

Statistical Analysis

An analysis of variance (ANOVA) and a Tukey honest significant difference (HSD) test were used. All experiments were performed in triplicate.

Results

Synthesis and Analysis of *EG V* Gene from *T. viride*

After exon splicing illustrated in Fig. 1, the *EG V* gene (744 bp in size) was gained. It coded 247 amino acids totally, and 6 of them were strongly basic amino acids, 13 strongly acidic amino acids, 67 hydrophobic amino acids, and 98 polar amino acids. The *EG V* gene translated protein, 17 amino acids in its N-terminal serving as a signal peptide, was predicted 25 kDa in molecular weight and 4.112 in isoelectric point.

Construction of Recombinant mBacmid and Recombinant Baculovirus

The 693-bp EG V mature peptide gene fragment (Fig. 2a) was successfully cloned into pFastBac^{TM}HT A vector. After the transformation of the purified recombinant donor plasmid pFast A/EG V into the DH10Bac™ *E. coli* competent cells, in which the transposition occurred at the rate of about 10% in the presence of the helper plasmid, the large recombinant DNA mBacmid/BmNPV/EG V was isolated from white (Lac^{-}) colonies followed by PCR analysis. The PCR result showed that an ~3,123-bp bands were generated from the recombinant mBacmid/BmNPV/EG V (white colonies) (Fig. 2b), which was theoretically concordant with ~2,430 bp plus target *EG V* gene (693 bp), while just an ~300 bp band appeared from the nontransposed mBacmid/BmNPV (blank mBacmid/BmNPV, blue colonies) (Fig. 2c), indicating the successful construction of recombinant DNA mBacmid/BmNPV/EG V.

The recombinant baculovirus mBacmid/BmNPV/EG V was generated in the cell cultures by transfection. Compared with the uninfected cells, the transfected cells typically

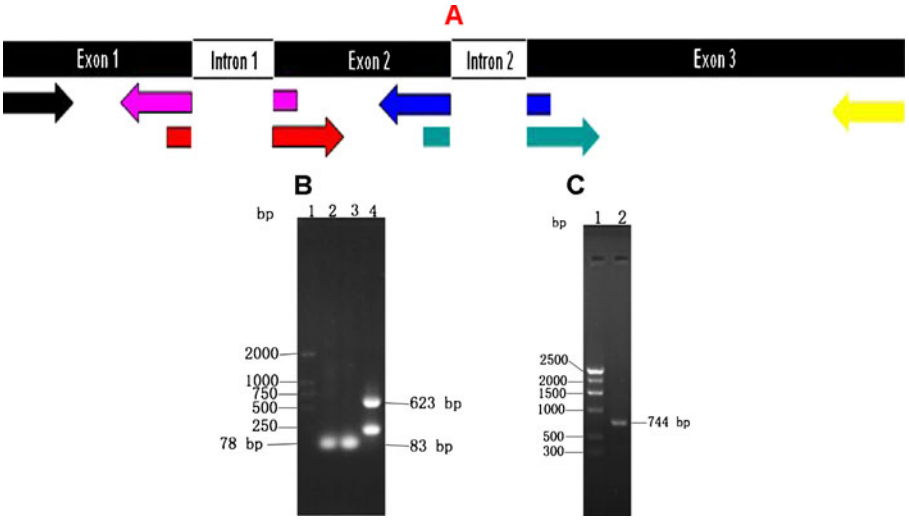

Fig. 1 The schematic diagram and result of EG V exon splicing. **a** The *black arrow*, *orange arrow*, *red arrow*, *blue arrow*, *green arrow*, and *yellow arrow* represent primer EG V P1, EG V P2, EG V P3, EG V P4, EG V P5, and EG V P6, respectively. The primer pairs EG V P1–EG V P2, EG V P3–EG V P4, and EG V P5-EG V P6 were used for exon 1 amplification, exon 2 amplification, and exon 3 amplification, respectively. Lastly, the primer pair EG V P1–EG V P6 was used to synthesize the EG V exon splicing DNA. **b** PCR products of exons in EG V from genomic DNA. *Lane 1*, DL2000 DNA Marker (TaKaRa); *lanes 2*, *3*, and *4* are the first, second, and third exons, respectively. **c** *Lane 1*, DNA marker G (BBI); *lane 2*, EG V exon splicing product

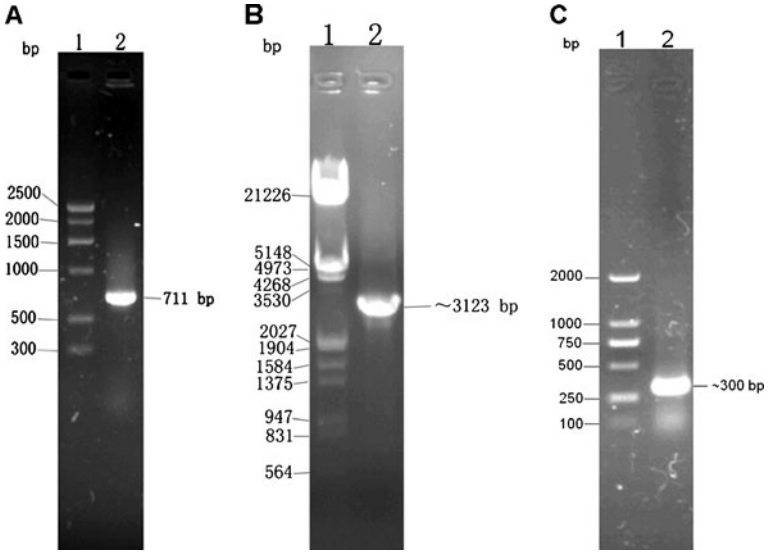

Fig. 2 The EG V mature gene fragment synthesized by PCR. **a** *Lane1*, DNA marker G (BBI); *lane 2*, PCR product of EG V mature gene fragment containing 18 bp of restriction enzymes. **b** *Lane 1*, λDNA/*Eco*RI+ *Hin*dIII marker, three (MBI); *lane 2*, PCR product of a white colony. **c** *Lane 1*, DL2000 DNA Marker (TaKaRa); *lane 2*, PCR product of a blue colony

increased their cell diameter and nuclei, stopped growing and suspended at about 120 h posttransfection. The second-generation recombinant baculovirus was collected to infect the silkworm larvae later.

Expression of *EG V* Gene in Silkworm

The *EG V* gene was expressed in the BmN cells along with the recombinant baculovirus mBacmid/BmNPV/EG V propagated in vivo. After 48 h postinfection, the suspending cells were collected and analyzed by Western blotting. The result showed that an ~36-kDa protein was found (Fig. 3), indicating the successful expression of EG V in the silkworm BmN cells. As the experimental molecular weight (MW) of the recombinant protein (~36 kDa) was larger than theoretical MW (~27 kDa), it was possible that the recombinant EG V protein underwent posttranslation modification in the silkworm BmN cells.

For the expression of the *EG V* gene in silkworm larvae, the larvae were subcutaneous injected with the recombinant baculovirus. They displayed no obvious differences from blank baculovirus-infected larvae and normal larvae during the first 3 days, but the baculovirus-infected larvae presented typical symptoms from 72 h postinjection. They had lost appetite, had elevated intersegmental membranes, swelled body segments, and finally died at about 120 h postinfection.

In our previous study, the recombinant EG I protein was highest level expressed at 84 h postinfection [12], so empirically this time, 50 silkworms were randomly sampled at this time point for recombinant EG V enzyme activity assay later.

Analysis of Enzyme Activity

The ANOVA showed that there were significant differences in the crude enzyme activities between different pH and different temperature, respectively (ANOVA, $p<0.01$), and the crude enzyme activity from recombinant baculoviruses-infected silkworms was significantly higher than those from blank baculoviruses-infected silkworms and normal silkworms (ANOVA, $p<0.01$). It exhibited significant maximum enzyme activity (Tukey HSD test, $p<0.01$) at the environmental condition of pH 5.0 and temperature of 50°C (Fig. 4a, b), and was stable at pH range from 5.0 to 10.0 (Fig. 4a) and at a temperature range of 40 to 60 °C (Fig. 4b). It increased 39.86% and 37.76% compared with that from blank baculoviruses-infected silkworms and normal silkworms, respectively (Fig. 4c).

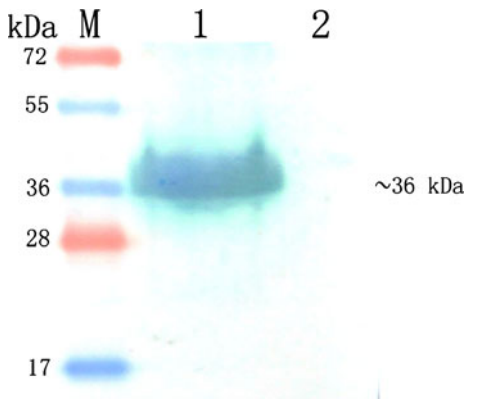

Fig. 3 Western blotting analysis of silkworm BmN cells infected with recombinant mBacmid/BmNPV/EG V baculovirus. *M*, Pageruler™ Plus Prestained Protein Ladder (Fermentas); *1*, recombinant EG V protein expressed in silkworm BmN cells. *2* negative control infected by blank BmNPV

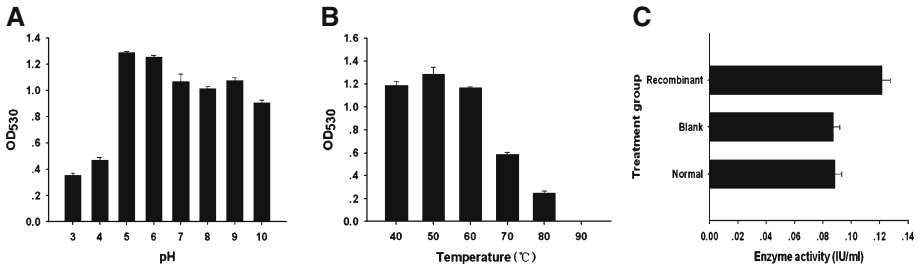

Fig. 4 Analysis of enzyme activity. **a** Optimum pH on the enzyme activity. The pH profile was determined in the varying pHs (pH 3.0–10.0). **b** Optimum temperature on the enzyme activity. Temperature profile was determined in the varying reaction temperature (reaction temperature 40–90 °C). **c** Comparison of enzyme activities between enzyme from recombinant baculoviruses-infected silkworms and those from blank baculoviruses-infected silkworms and normal silkworms. Data were shown as mean±SD (n=6)

Discussion

Currently, it is very important to produce bioethanol as a fuel from recycling of biomass resources, for the purpose of relieving the problems of energy and environment, which are large obstacles to the development of human civilization. So far, nearly all fuel ethanol is produced by fermentation of corn glucose in America or sucrose in Brazil, and any countries advanced in agriculture can use current technology for fuel ethanol fermentation [13], but this technology consumes a lot of food materials and costs too much, thus there will be even a serious competence between human food and fuel ethanol. A better way to solve this problem is to exploit large quantities of cellulase with high bioactive efficiency, which can hydrolyze cellulose in biomass with high efficiency.

In the present study, to avoid hnRNA contamination, which also has poly A structures and introns, and to easily synthesize the *EG V* gene from *T. viride*, we used a PCR-based exon splicing method, other than the traditional method of total RNA isolation followed by RT-PCR. Further, it was expressed in silkworms using an improved Bac-to-Bac/BmNPV baculovirus expression system, which lacks the virus-encoded chitinase (*chiA*) and cathepsin (*v-cath*) genes for the purpose of increasing the expression of foreign proteins. As a result, the recombinant baculovirus with bioactive endoglucanase V of *T. viride* was successfully constructed by integrating this gene into the transposition site of host genome and then transfecting the recombinant DNA into the silkworm BmN cells. This was confirmed by Western blotting and enzyme activity analysis. Because the silkworm can be easily reared in a large scale as we know, the availability of large quantities of EG V that the silkworm provides might greatly facilitate the future research and testing of this protein for potential application in industries.

The Bac-to-Bac/BmNPV baculovirus expression system, which is based on the silkworm as a bioreactor, has shown its advantages such as simple technique, short experimental period, and high expression efficiency. Furthermore, the silkworm is abundant and friendly to both man and the environment [14, 15], therefore, this technique is expected to be applied for large-scale protein production [16]. It has been successfully used for the heterologous expression of proteins including human butyrylcholinesterase [17], human insulin-like growth factor-I [18], orange fluorescent protein [19], and so on.

However, compared with other microorganism expression systems, such as *E. coli* and yeast expression systems, which also can express cellulase with high bioactivity [20, 21],

the silkworm expression system seems more costly. Therefore, improvements must be made to maintain the silkworm culture system at a low cost in the future. Furthermore, the silkworm does not have any cellulase gene in its genome, therefore, it would be very useful to generate transgenic silkworms expressing bioactive cellulase, in which cellulase is expressed especially in the digestive tract, thus the transgenic silkworm can catabolize dietary fibers more efficiently, and it might be of great significance for sericulture industry.

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