RESEARCH ARTICLE

Expression and Enzyme Activity Determination of Apripona Germari Cellulase in Bmnpv/Bac-to-Bac Baculovirus Expression Systems

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Abstract: To Search for a more efficient way of degrading cellulose. METHODS: Apripona germari Cellulase genes were cloned and expressed in Bombyx mori derived cell line, BmN cell line by BmNPV/Bac-to-Bac baculovirus expression systems. Infected BmN cells were harvested 72 h post-infection (hpi). Expressd recombinant cellulose was analyzed in 12.5% SDS– PAGE and western blot. The activity of purified recombinant cellulose was assessed by 3, 5-nitro salicylic acid (DNS) colorimetric method. RESULTS: Western blot analysis showed that Recombinant cellulase was expressed in BmN insect cells as a 29 kDa protein. Enzyme activity assay demonstrated that recombinant cellulose has the activity of decomposing cellulose. CONCLUSION: Apripona germari Cellulase was successfully expressed in BmN insect cell line. The method established in this study provides an efficient way to produce a large amount of cellulase and paves the way for further utilization of cellulose.
Keywords: Apripona Germari; Cellulase; Bac-to-Bac Baculovirus Expressio; Enzyme Activity

1. Introduction

Cellulose is composed of repeating glucose units linked by β-1, 4-glycosidic bonds[1]. Cellulose can be biologically degraded by cellulases and then converted into smaller simple sugars. But a few types of animals only use Cellulose. Lower organisms such as fungi and bacteria can produce mixtures of synergistically acting cellulases and digest cellulose[2]. Some higher multicellular organisms such as insect, horse and sheep can rely on symbiotic bacteria that produce cellulolytic enzymes to hydrolyze cellulose[3]. As a promising source of carbohydrates, cellulose has attracted widly attention. Because cellulase cannot be synthesized, the ability of most animals to get glucose from cellulose is limited.

The larvae of the Apripona germari make hosts of mulberry by tunneling inside the stem and ingesting the living wood[4]. Fresh plant or wooden tissues are then used for bioenergy conversion by A. germari. To date, three genes encoding cellulase have been found on Apripona germari[5-7]. The unique property of cellulolytic enzymes from A. germari for decomposing tough fresh tissues attracted much attention of sericultural scholar. Silkworms feed on mulberry leaves, but they do not eat veins and stems. The veins and stems of mulberry leaves are rich in cellulose. The cellulase from A. germari could serve as a target for silkworm larve to exploit the energy and carbon available from cellulose and improve the utilization rate of silkworm to mulberry leaves.

The baculovirus expression system has been proven to be one of the most effective expression tools[8]. The expression system of B. mori nuclear polyhedrosis virus (BmNPV) can produce heterologous proteins at high level in silkworm cells. In this paper, we report the construction of recombinant baculovirus containing cellulose from A. germari. Recombinant proteins were expressed functionally in baculovirus-infected insect BmN cells. Recombinant cellulase was assayed for enzymatic properties.

2. Materials and methods

2.1 Cell culture and expression system

A B. mori-derived cell line, BmN was obtained from Insitute of Life Science, Jiangsu University. BmN cells were cultured in monolayer at 28℃ in Grace's supplemented medium with 10 % heat inactivated fetal bovine serum. The BmNPV/ Bac-to-Bac expression system consists of the transposing vector pFastBac, and high Efficiency DH10BmBac competent cells that contain bacmid (baculorivus shuttle vector plasmid) and helper plasmid to be used to generate recombinant baculovirus.

2.2 Cloning and characterization of Apripona germari Cellulase

Total RNA was extracted from the midgut tissues of Apripona germari using TRIzol (Invitrogen, Inc). The RNA (5 μg) was reverse transcribed into cDNA by using M-MLV reverse transcriptase (Promega, Inc). The specific primers of

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cellulase were designed for PCR according to the sequence information available in GenBank (accession no. AY162317). The sense primer was 5'-GGGAATTCAAATGAAGGTGTTCGTAGCAATC-3' (EcoRI), and the antisense primer was 5'-CCCTCGAGTTAATAATTGCATCCAGTAATGG-3' (XhoI). PCR was performed with Pyrobest TM DNA polymerase (Takara), using 30 cycles of 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1min, with a final 5 mins extension step at 72℃. The products were purified and subcloned into pMD 19-T Vector (Takara) and sequenced.

2.3 Construction of recombinant baculovirus DNA

The cellulase gene was first cloned into the sites of EcoR I/Xho I in the donor vector pFastBacHTb. The donor contains an expression cassette within the left and right arms of the Tn7 transposon. The recombinant pFast-BacHTb–cellulase was subsequently transformed into DH10Bac cells containing a BmNPV baculovirus genome. The cells were incubated at 37℃ for 4 h to achieve transposition. Then, the cells were spread evenly on the agar plates containing kanamycin (50 μg/ml), tetracycline (7 μg/ml), X-gal (100 μg/ml), and isopropylthiogalactoside (40 μg/ml). The white colonies were selected and cultured overnight in a medium containing kanamycin. Recombinant bacmid DNA was isolated and transfected into BmN cells using 10μl of Lipofection Reagent (Invitrogen, USA). After 4 days incubation, the supernatant containing recombinant virus was harvested.

2.4 Production of recombinant proteins

BmN cells were cultured on a 75-cm2-cell culture flask. When the cell density reached 1 × 106 cells/ml, and infected with the recombinant viral stock (MOI of 10). Following incubation for 1 h at room temperature, medium was added and the cells were cultured at 27 ℃. For 3 days at 27℃, the cells were harvested by centrifugation at 1000 g for 15 min at 4℃. The supernatants were discarded, and the cell pellets were stored at 20℃ until used.

2.5 SDS-PAGE and Western blot analysis

BmN cells infected with wild-type BmNPV, and recombinant BmNPV viruses expressing cellulase, were analyzed in 12.5% SDS– PAGE. The cell pellets were resuspended with the lysis buffer [25 mM HEPES, pH 7.4; 1% Triton X-100; 0.1% SDS; 150 mM KCl; 1 mM EDTA; 1 mM DTT, and protease inhibitor cocktail (Roche)]. Protein concentrations were determined by BCA Protein Assay Kit (Pierce). Whole cell lysates were mixed with SDS sample buffer and heated to 100 ºC for 5 min. Samples, containing 20 μg protein, were electrophoresed in 10 % SDS-polyacrylamidegels and transferred onto nitrocellulose membranes. Subsequently, the membrane was incubated at RT for 1 h with mouse anti-6xHis monoclonal antibody (Novagen) at a 1:1000 dilution in a 3% BSA-TBS buffer. The appropriate secondary horseradish.peroxidase-conjugated antibody (DAKO) was added and complexes were detected.

2.6 Enzyme activity determination

Cellulase-His protein was purified from the supernatant of BmN cell cultures using the Insect RoboPopTM Ni-NTA His Bind Purification Kit (Novagen). The 3, 5- nitro salicylic acid (DNS) colorimetric method was used to determine the activity of purified Cellulase[9]. 1% carboxymethyl cellulose (CMC) solution was prepared with pH value of 4.6 HAc-NaAc buffer（1.5M）, and 1.5ml was added to the 25 ml test tube, then placed in 40℃ water bath for 5 min. Add 0.5 ml of appropriate diluted enzyme solution, react 30 min at 40℃, add DNS reagent, color in boiling water bath, and then volume to 25 ml. Under the same conditions, the enzyme solution was inactivated for 5mins in boiling water bath, and was used as a blank control. The absorbance value is measured at 540 nm wavelengths. Then the amount of glucose produced was calculated on the corresponding glucose standard curve. The definition of enzyme activity unit: under the above reaction conditions, within 1 min, the amount of enzyme needed to hydrolyze CMC to produce 1μg glucose is defined as 1 enzyme units. It is expressed in U. Results are expressed as means ± SD.

Results

2.7 Cloning and characterization of Apripona germari Cellulase

The fragments of gene cellulase were amplified by PCR using specific primers from Apripona germari midgut cDNA. The PCR products were sequenced to ensure the correctness of the open reading frame (ORF). The gel electrophoresis in 1 % agarose showed 711bp of cellulose. Restriction sites at both ends ensure that gene can be inserted into pFasBac plasmid correctly (Figure 1).

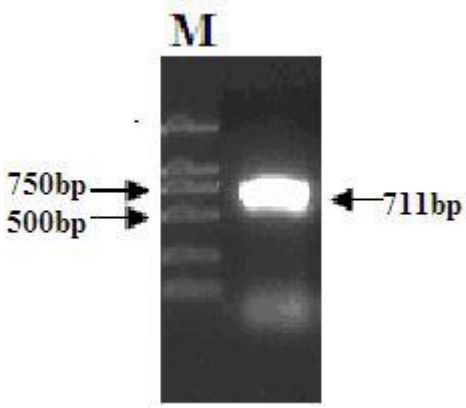

Figure 1; Electrophoresis of PCR product of Apripona germari Cellulase**.**

2.8 Construction of recombinant baculovirus DNA

Restriction endonuclease digestion was performed to verify the correct insertion of the gene cellulase in the donor pFasBac plasmid. Recombinant plasmids pFasBac-cellulase were transfected into DH10Bac cells. The recombinant baculovirus containing the cellulose gene was quickly generated by our BmNPV/Bac-to-Bac expression system (Figure 2). pUC/M13 amplification primers were used to comfirm recombinant bacmid-cellulase. Amplification of the recombinant Bacmid plasmid generated a 3100bp band, while amplification of the non-recombinant Bacmid plasmid generated a 300bp band (Figure 3).

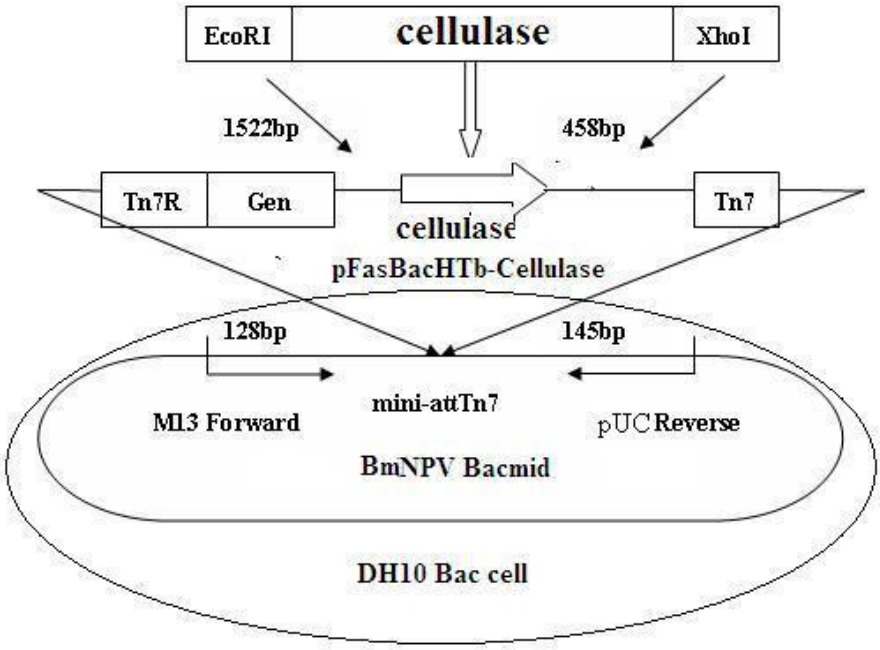

Figure 2; Construction of recombinant Bacmid-cellulase DNA**.**

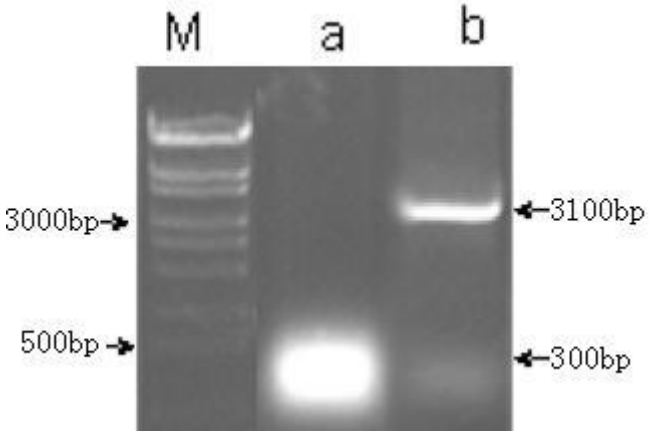

Figure 3; Electrophoresis of PCR product of recombinant baculovirus DNA extracted from infected BmN cells using M13/pUC primer. a non-recombinant Bacmid DNA; b. recombinant Bacmid DNA**.**

Expression of cellulase recombinant proteinCells infected by recombinant virus were collected and subjected to SDS-PAGE and Western blotting. As shown in Figure 3, an interesting band of approximately 29 kDa was observed. In western blots, cellulase recombinant protein was detected by the anti-6xHis monoclonal antibody and showed specific binding. (Figure 4).

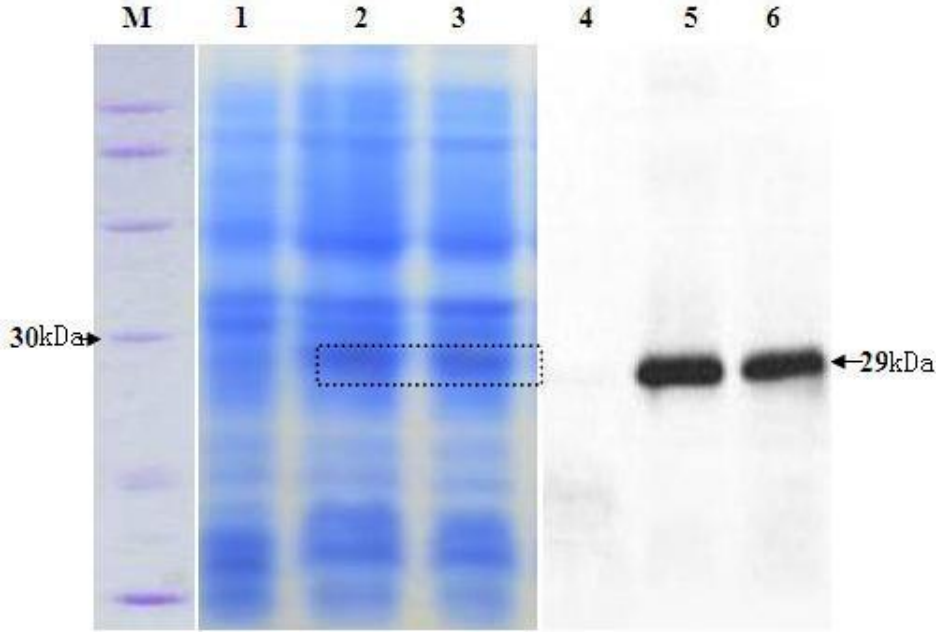

Figure 4; Baculovirus expression of cellulase in BmN cells infected with recombinant viruses for 3 days. Lane 1-3: ie brilliant blue stained 12.5% SDS–PAGE gel. Lane1: mock infection; Lane 2, 3: infected with BmNPV-cellulase; Lane 4-6: Western blot of a duplicate gel probed with anti-6His monoclonal antibody and developed by an ECL chemiluminescence system using goat anti-mouse IgG coupled to horseradish peroxidase as secondary antibody. Lane4: mock infection; Lane 5, 6: infected with BmNPV-cellulase**.**

2.9 Enzyme activity determination

The recombinant cellulase extracted from BmN cells infected by recombinant virus showed the activity of CMC decomposition. The activity is 2100 ±134U. The extract from BmN cell infected by wild type virus only showed a small amount of cellulase activity. The activity of cellulase expressed by recombinant virus is significantly higher than that of wild type virus infected cells ($P<0.01$). The results showed that recombinant cellulase had the ability to decompose CMC (Figure 5).

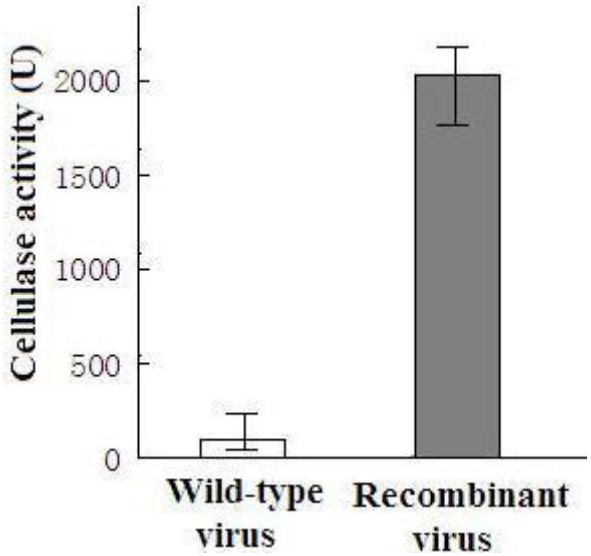

Figure 5; Determination of the activity of recombinant protein cellulase**.**

3. Discussion

Cellulose is the most abundant biomass resource on earth, and it is also a natural renewable resource. Cellulose is difficult to be naturally decomposed into materials that can be used, but cellulase can hydrolyze cellulose to the simple structure of glucose[10]. Cellulase degrading cellulose has the advantages of high specificity, mild reaction conditions

and little environmental pollution[11]. So, biodegradation is a highly efficient and environmentally friendly cellulose digesting method.

The larvae of the mulberry beetle are parasitic in the trunk of a mulberry tree. In previous reports,the results showed that 29kDa cellulase polypeptide existed in the midgut of A. germari larvae. Southern blot hybridization clearly showed that cellulase gene was located in the genome of A. germari. The expression analysis of cellulase at transcriptional level suggested that the cellulase gene was tissue-specifically expressed in the midgut[12]. Cellulases can be produced from A. germari itself. High level of cellulase activity in the larval midgut showed that the midgut is a likely site where large quantities of cellulase are synthesized for the degradation of the absorbed cellulose from the diet. The presence of cellulolytic enzymes may be advantageous to A. germari by increasing available energy/nutriments being obtained from food sources.

Silkworm larvas like to eat mulberry leaves. The silkworm midgut epithelial cells can directly absorb water, vitamins and inorganic salt in the mulberry leaves. Protein, fat, carbohydrate and other nutrients can be absorbed and utilized after digestion of the digestive system. There is no cellulase gene in the genome of the silkworm[13]. At present, the symbiotic bacteria that can produce cellulase have not been isolated from the intestine of Bombyx mori[14]. Therefore, cellulose contained in mulberry leaves cannot be digested and absorbed. In this study, a cellulase gene was cloned from the larvae of the mulberry longicorn beetle, A. germari. cDNA was successfully expressed in baculovirus-infected insect BmN cells. SDS-PAGE and Western blot analysis showed bands of about 29 kDa from cell lysates. Enzyme activity result showed that recombinant protein has high catalytic activity of cellulose as well as an optimal temperature and pH of 40 ℃ and 4.6, respectively. This result is similar to that found from A. germari. This result indicates that silkworm can express heterologous cellulase.

There are always problems in sericulture production process. Over 50% of mulberry leaf cost is wasted, and mulberry leaf feed conversion rate is low[15]. If the silkworm juice containing cellulase, the silkworm can use cellulose, so as to improve the feed efficiency of silkworm. Improving the feed efficiency of silkworm can reduce the cost of raising and increase the economic benefits of silkworm rearing. It can greatly improve the competitiveness of sericulture in the international market.

In conclusion, our results strengthen the understanding of enzyme activity of A. germari cellulases. Cellulose can be used as a candidate gene for transgenic silkworm research to improve mulberry leaves efficiency.

Acknowledgments

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References

1. XH Liao, Y Liu, *et al*. Effective Cleavage of β-1,4-Glycosidic Bond by Functional Micelle with l -Histidine Residue. Catalysis Letters 2016; 146 (7): 1249-1255.
2. KMD Gunathilake , RR Ratnayake, *et al*. Evaluation of cellulosedegrading efficiency of some fungi and bacteria and their biofilms. J Natl Sci Found 2013; 41 (2): 155-163
3. EW Schmidt , MS Donia, *et al*. Life in cellulose houses: symbioticbacterial biosynthesis of ascidian drugs and drug leads. CurrentOpinion in Biotechnology 2010; 21 (6): 827.
4. L Wang , L Qu, *et al*. Metabolic Responses of Poplar to Apripona germari(Hope) as Revealed by Metabolite Profiling.Int J Mol Sci 2016; 17 (6): 923.
5. Lee, S.J., Kim, S.R., *et al*. cDNA cloning, expression, and enzymatic activity of a cellulase from the mulberry longicorn beetle, Apriona germari. Comp. Biochem. Physiol. B Biochem. Mol. Biol 2004; 139, 107–116.
6. Lee, S.J., Lee, K.S., *et al*. A novel cellulase gene from the mul- berry longicorn beetle, Apriona germari: gene structure, expression, and enzymatic activity. Comp. Biochem. Physiol. B Biochem. Mol. Biol 2005; 140, 551–560.
7. Wei, Y.D., Lee, K.S., *et al*. Molecular cloning, expression, and enzymatic activity of a novel endogenous cellulase from the mulberry longicorn beetle, Apriona germari. Comp. Biochem. Physiol. B Biochem. Mol. Biol 2006; 145, 220–229.
8. Q Wang , Y Zhou, *et al*. Suppression of Bm-Caspase-1 Expression in BmNCells Enhances Recombinant Protein Production in a BaculovirusExpression Vector System. Molecular Biotechnology 2016; 58 (5): 319-327.
9. DM SUN, Q YANG, *et al*. Studies on Cellulase Production by Immobilized Cells and Characteristics of Cellulase. Chemistry and Industry of Forest Products 2006; 26(2): 79-82.
10. Levin, R.J. Digestion and absorption of carbohydrates—from molecules and membranes to humans. Am. J. Clin. Nutr 1994; 59: 690S–698S.
11. FitzPatrick, M., Champagne, P. *et al*. A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol 2010; 101: 8915–8922.
12. AV Jiménez, H Wang, *et al*. Expression and Characterization of a Recombinant Endoglucanase From Western Corn Rootworm, in Pichia pastoris. Journal of Insect Science 2014; 14 (1): 242-242.
13. XH Li, MX Wang, *et al*. Heterologous expression characteristics of Trichoderma viride endoglucanase V in the

silkworm, Bombyx mori L.Applied Biochemistry & Biotechnology 2011; 165 (2): 728-736.
14. JY Kim, SM Yoon, *et al*. Cellulase Activity of Symbiotic Bacteria from Snails, Achatina fulica. Journal of the Korean Wood Science & Technology 2015; 43 (5): 628-640.
15. VK Rahmathulla, P Das, *et al*. Effect of folic acid supplementation on nutritional and feed conversion efficiency of mulberry silkworm (Bombyx mori Linnaeus 1758). Tropical Zoology 2011; 24 (2): 145-157.