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High-level expression and purification of a molluskan endoglucanase from *Ampullaria crossean* in *Pichia pastoris*

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ABSTRACT

EG27I is an endogenous glucanase belonging to glycoside hydrolase family (GHF) 45 from the mollusk *Ampullaria crossean*. In this study, the mature EG27I peptide gene fused to the HFBII secretion signal of *Trichoderma reesei* was expressed under the GAP promoter of *Pichia pastoris* in SMD1163 strain. A bioactive EG27I with a molecular weight of 27 kDa was successfully expressed and secreted into our culture medium. The respective final OD_{600} and hydrolytic activity were 333 and 1.28 U/mL when high-cell-density fermentation of the recombinant *P. pastoris* was performed in a 7.5 L fermenter through a fed-batch strategy for 132 h. The recombinant protein concentration of the fermentation supernatant was 47.7 mg/L. EG27I was consecutively purified from the fermentation supernatant through ultrafiltration, cation exchange, and hydrophobic interaction. The specific activity of the recombinant EG27I was 26.8 U/mg, and the optimal pH and temperature of the enzyme were 5 and 50 °C, respectively. The half-life of the enzyme activity at 100 °C could reach 40 min. The N-terminal amino acid sequence analysis of the purified recombinant protein confirmed that the amino terminal sequence was consistent with the natural structure. The high quantity and purity of the EG27I provide a basis for future structural and functional studies.

1. Introduction

Cellulose, a β-1,4-linked homopolymer of D-glucose, is the main structural material in natural environments [1]. This polymeric component of plant matter has been considered an important and sustainable source of mixed sugars for bioenergy production [2]. Cellulose is also the most abundant biomass in the biosphere, and its yield is approximately 10^{12} tons per year [3,4]. The production of bioethanol from lignocellulosic raw materials, such as wheat straw, cotton stalk, and corn stover, has been preferred as an alternative to energy conservation. Carbohydrate polymers should undergo hydrolysis to yield fermentable sugars, and this process is catalyzed by cellulolytic enzymes [5]. For example, cellulases, including exo-β-1,4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4), and β-1,4-glucosidases (EC 3.2.1.21), play key roles in the degradation of cellulosic materials [6].

Various organisms, such as fungi, bacteria, plants, protists, and invertebrate animals, can produce cellulases. According to the primary report of an endogenous cellulase gene in termites [7], endogenous cellulases have been found in numerous invertebrates, such as insects [8], crustaceans [9], mollusks [10], and nematodes [11]. EG27I is an endogenous cellulase of the mollusk *Ampullaria crossean*, and it belongs to glycoside hydrolase family 45 (GHF45) [6], which is characterized by a smaller molecular mass than that of other glucanases. In invertebrate animals, GHF45 cellulase genes have been isolated from pine wood nematode (*Bursaphelenchus xylophilus*) [11], mulberry *longicorn beetle* [12], and blue mussel (*Mytilus edulis*) [13]. Thus far, the heterologous expression of endoglucanase genes in *Escherichia coli*, *Pichia pastoris*, and insect-cultured cells has been reported [12,14,15], but the yield of enzyme is low and poorly described. Therefore, its low yield may impede

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further research on endogenous cellulases.

P. pastoris expression system has been widely known because of its great potential to secrete heterologous proteins, which is suitable for the production of proteins in functionally active forms [16]. As a single-cell eukaryote, *P. pastoris* provides many advantages of higher eukaryotic systems, such as protein folding and post-translational modification [17]. With the strong promoter of glyceraldehyde-3-phosphate dehydrogenase (GAP), *P. pastoris* extracellularly or intracellularly expresses high levels of recombinant proteins. As a microorganism, *P. pastoris* can grow well in a high-cell-density bioreactor, which has been used to achieve significantly higher biomass accumulation and higher recombinant protein production than that of traditional shake-flask cultures [18].

In this study, a new expressing vector pGAPH based on the commercial vector pGAPZαA (Invitrogen) was constructed to extracellularly produce a β-1,4-endoglucanase belonging to GHF45 from the mollusk *A. crossean* in *P. pastoris*. The EG27I gene was expressed under the GAP promoter in *P. pastoris* SMD1163 strain. Cellulase was then purified through three-step purification after high-density fermentation was completed.

2. Materials and methods

2.1. Plasmid construction

The dsDNA HFB (5′-ACTCAATTGAACAACTATTTC**GCCACC** ATGCAATTCTTCGCTGTTGCTTTGTTCGCTACTAGTGCTTTGGCTGAATT CGGTACCAAT-3′) was synthesized by Sunny Biotechnology Co., Ltd., Shanghai, China. The ds DNA contains the kozak sequence (shown in bold) and the gene sequence of HFB II secretion signal peptide [19] (in underline) whose codons were optimized according to the codon usage database of *P. pastoris* (http://www.kazusa.or.jp/codon/). The dsDNA HFB II was cleaved by *Mfe* I and *Kpn* I and inserted into pGAPZαA vector (Invitrogen) by using the same restriction enzyme. In this manner, the HFB II secretion signal peptide replaced the α-factor secretion signal peptide to form a new plasmid, which was named pGAPH.

The plasmid pMD18-*eg27I* containing *eg27I* gene (GenBank Accession No. EF471315) was obtained from Shanghai Institute for Biological Sciences in the Chinese Academy of Sciences [6]. The amplification of cDNA fragments encoding the mature EG27I lacking the native signal peptide sequence was conducted by using High-fidelity PCR Master (New England Biolabs) and the following primers: (EG27-F,5′-GGCACTAGTGCTTTGGCTGCACAGTTGTGTCAG-3′; EG27-R,5′-CGCTCTAGATTAGCCCGAATTGTGGCATTCAC-3′). The PCR product was digested by *Spe* I and *Xba* I and cloned into pGAPH by utilizing the same restriction enzyme to produce the vector pGAPH-*eg27I*. The plasmid was transformed into *E. coli* DH5α strain. The plasmid DNA was amplified, purified, linearized with the restriction enzyme *Bln* I, and integrated into the chromosomal DNA of *P. pastoris* SMD1163 strain through electroporation (Bio-Rad, Hercules, USA). The transformed cells were screened on the YPD agar plate with 100 mg/L Zeocin at 30 °C for 3–5 days of incubation. The selected integrants were confirmed through PCR by using gene-specific primers.

Twenty colonies were cultured on a YPD agar plate with different concentrations of Zeocin (100, 200, 300, 400, μg/mL) to obtain the multi-copy transformants. The copy number of the transformants growing on heavy Zeocin concentrations were determined through quantitative real-time PCR [20]. These transformants were inoculated into 100 mL of YPD in a flask for 72 h (30 °C, 200 rpm), and the enzyme activity in the supernatant was identified. All of the samples were analyzed in triplicate, and average values were considered for data analysis.

2.2. Fermentation conditions and metabolite determination for constitutive expression

The potential multicopy transformant was inoculated in 1 L Erlenmeyer flasks with 250 mL of YPD medium at 30 °C in a shaking incubator until OD_{600} reached 2 to 5. Afterward, the culture was used to inoculate a 7.5 L jacket fermenter (NBS BioFlo115) containing 2 L of basal salt (40.00 g/L glycerol, 4.13 g/L KOH, 26.70 g/L H_3PO_4, 0.93 g/L $CaSO_4$, 14.90 g/L $MgSO_4 \cdot 7H_2O$, 18.20 g/L K_2SO_4, and 4.00 mL/L PTM1 trace salt solution, which consisted of 6.00 g/L $CuSO_4 \cdot 5H_2O$, 3.00 g/L $MnSO_4 \cdot H_2O$, 0.08 g/L NaI, 0.20 g/L Na_2MoO_4, 65.00 g/L $FeSO_4 \cdot 7H_2O$, 0.02 g/L H_3BO_3, 0.50 g/L $CoCl_2$, 20.00 g/L $ZnCl_2$, 0.20 g/L biotin, and 5.00 mL/L H_2SO_4) [17]. The cultivation temperature was 30 °C and pH was maintained at 5.0 by an automatic pH controller to add 28% NH_4OH or 1 mol/L HCl. The air was supplied at a rate of 1.0 vvm and the dissolved oxygen (DO) was kept above 30% throughout the whole process by auto-adjusting the agitation rate of 600 rpm–1200 rpm. When glycerol in the initial medium was completely consumed (18–24 h), as indicated by a sudden increase in DO, started to the glycerol fed-batch phase by feeding 50% (w/v) glycerol containing 12 mL/L PTM1 trace salt solution at a rate of 18.15 mL/h/L initial fermentation volume for a period of 5 h. The DO level was maintained between 25% and 30% by intermittent feeding. The samples were collected every 12 h to measure the OD_{600} and enzyme activity of the cellulase EG27I during fermentation. Cultivation was stopped when cell density remained significantly unchanged. The fermentation liquid was then centrifugated at 4 °C with 10,000×*g* for 15 min to remove the cells, and the supernatant containing EG27I was subsequently frozen at −20 °C.

2.3. Purification of the recombinant EG27I

After the cells were removed through centrifugation, the supernatant of the fermentation liquid was concentrated and desalted by ultrafiltration through a membrane with a molecular weight cut-off of 10 kDa (GE Healthcare). Afterward, the solution, including the recombinant, was loaded onto a column containing SP-Sepharose FF resin previously equilibrated with NaAc-HAc buffer (20 mM, pH 5.0). The SP-Sepharose FF column was washed with 5 × column volumes of equilibrium buffer. The bound proteins were eluted successively with a linear gradient from 0 M to 1 M NaCl. The fractions containing endo-β-1,4-glucanase activity were collected and loaded onto a phenyl Sepharose HP column previously equilibrated with Tris-HCl buffer [20 mM, pH7.0, 0.75 M $(NH_4)_2SO_4$]. The column was washed with 5 × column volumes of equilibrium buffer. The bound proteins were eluted with Tris-HCl buffer (20 mM, pH7.0) without $(NH_4)_2SO_4$. The presence of recombinant EG27I was identified through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and special activity analysis.

2.4. Protein concentration measurement and SDS–PAGE analysis

SDS–PAGE analysis was performed using 12% gel in accordance with previously described methods [21], and proteins were detected with Coomassie Brilliant Blue R250. The protein concentration was determined by Bradford's assay [22], and bovine serum was used as standard.

2.5. Enzyme assays

For enzyme activity determination, 20 μL of the enzyme solution was added to 180 μL of 1% carboxymethyl cellulose (CMC) solution in 100 mM sodium citrate buffers (pH 5.0). The mixed liquor was