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RESEARCH ARTICLE

Cloning of *endo-β-glucanase I* gene and expression in *Pichia pastoris*

Yu BAI, Runfang GUO (✉), Hongwei YU, Long JIAO, Shuli DING, Yingmin JIA

College of Food Science and Technology, Agricultural University of Hebei, Baoding 071001, China

Abstract **Total RNA of *Thermoascus aurantiacus* was isolated from its mycelium and acted as template for RT-PCR. The full-length cDNA encoding an endo-*β*-glucanase I was cloned via RACE-PCR method and the cDNA contained an ORF of 1005 bp encoding 305 amino acids. A recombinant plasmid, pPIC9k-*egI*, was constructed by inserting the ORF sequence of *endo-β-glucanase I* gene (*egI*) into the yeast expression vector pPIC9k and transformed to *Pichia pastoris* GS115. The results showed that the recombinant endo-*β*-glucanase I was excreted into the fermentation medium. The highest activity of endo-*β*-glucanase I and the protein content were up to 45.42 U/mL and 788.26 μg/mL at incubation time of 144 h. The optimal temperature and pH for the recombinant endo-*β*-glucanase I were found to be 70°C and 3.5, respectively.**

Keywords *Thermoascus aurantiacus*, endo-1, 4-*β*-glucanase, RACE, *Pichia pastoris*, gene expression

Introduction

Cellulose, a linear polysaccharide formed by *β*-1,4-linked D-glucose residues, found in wood, cotton, and plant cell walls, is one of the most abundant biopolymers in nature (Gonzalez and Weiner, 2000; Goedegebuur et al., 2002). Cellulase plays an important role in the conversion of renewable cellulosic biomass to simple sugar, which can be further applied to produce bioethanol through fermentation. Bioethanol is believed to be one of the options for helping the world to settle the issue of energy security (Zaldivar et al., 2001; Fujita et al., 2004; Liu and Wang, 2008). Cellulase, a complex cellulolytic enzymes system, is composed of endo-*β*-glucanase (endoglucanase, EGs), cellobiohydrolases (CBHs), and *β*-glucosidase (Chen et al., 2006; Ibrahim and El-diwany, 2007; Zhang et al., 2007). Synergistic action of all these classes is required for complete hydrolysis of crystalline cellulose to smaller oligosaccharides and finally to glucose (Henrissat and Davies, 2000). Endo-*β*-glucanase (EC 3.2.1.4), the major component of the cellulolytic enzyme system, first attacks on the cellulosic polymer by breaking the internal *β*-1,4 glucosidic linkages within amorphous regions of cellulose chains. Subsequently, CBHs hydrolyze cellulose at reducing or nonreducing terminals to produce cellobiose and a large amount of glucose (Medve et al., 1998; Cohen et al., 2005; Luo and Chang, 2010). Therefore, the endo-*β*-glucanase is very important in hydrolyzing the whole crystalline cellulose.

Although a number of cellulases have been investigated, some cellulase application problems still exist, such as low production and high product costs, low activity, and thermal instability. The thermostable cellulases have received increasing attention due to their stability and low enzyme product costs (Wood and Ingram, 1992).

However, the information of endo-*β*-glucanase from thermophilic fungi has yet been limited. In this study, we selected the strain *Thermoascus aurantiacus* isolated from the soil sample (collected from Yunnan Province, China) to isolate the full-length cDNA of *endo-β-glucanase I* gene (*egI*) and expressed successfully as a secreted recombinant endo-*β*-glucanase I in *Pichia pastoris*.

Materials and methods

Materials

Strains, culture condition, and plasmids

T. aurantiacus was gifted by the Laboratory of Environmental

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Correspondence: Runfang GUO
E-mail: runfangg@163.com

Biology of Shandong Agricultural University. *T. aurantiacus* was grown at 50°C in a minimal liquid medium with 1% (w/v) crystalline cellulose (Sigma). *Escherichia coli* JM109 (Shanghai Sangon, China) was used as the recipient. *E. coli* was grown at 37°C in Luria-Bertani broth (LB) or on LB agar (Sigma) plate. The pMD-18T vector was used for cloning and DNA sequencing. *P. pastoris* expression vector pPIC9k and strain GS115 (Invitrogen) were used for the expression study. Yeast transformation was performed according to the method described in the manual, version 3.0, of the pPIC9k (Invitrogen).

Medium

Basic Salt Medium (BSM) consisted of 0.93 g/L $CaSO_4 \cdot 2H_2O$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4 \cdot 7H_2O$, 1.47 g/L sodium citrate, 40 g/L glycerin, 10 g/L $(NH_4)_2SO_4$, and 0.1 M PBS (pH 6.0), which was autoclaved for 20 min and added with 2 mL/L PTM1 before using.

PTM1 consisted of 6.0 g/L $CuSO_4 \cdot 5H_2O$, 3.0 g/L $MnSO_4 \cdot H_2O$, 0.08 g/L KI, 65.0 g/L $FeSO_4 \cdot 7H_2O$, 20.0 g/L $ZnCl_2$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 0.2 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5.0 mL H_2SO_4, and 0.2 g/L Biotin, which was added with water to 1 L total volume, sterilized by 0.2 μm membrane filtration, and stored at 4ºC.

PCR primers

Primers used for cDNA cloning and heterologous expression of *egI* were presented as follows:

PEG-S1: 5′-GCAGACAGAAAGCAGGAGACC-3′

PEG-A: 5′-CTGGTTGAGATTGAGGACTAAG-3′

PEG-S2: 5′-GGTCGCCTCACAGTTTGCTTC-3′

GSP-EG1: 5′-TCCATCATAAAGGGGACACGAAAGA-3′

Ps: 5′-CGTACGTAGCGAAAGTATTCCAAT-GGTTCGGT-3′ (*Sna*BI)

Pa: 5′-TAGCGGCCGCTCAGTCAAAGATACGGAGT-CAA-3′ (*Not*I)

Methods

Isolation of egI

After *T. aurantiacus* was cultured for 3 days, mycelium was harvested and ground to fine powder under liquid nitrogen. Total RNA isolated from this material using Trizol reagents (Invitrogen) was used as a template for RT-PCR. The RT-PCR was carried out according the methods described in the manual of the RT-PCR kit (Takara). The bi-directional oligonucleotide primers PEG-S1 and PEG-A were synthesized based on the conserved amino acid sequence corresponding to the active site region of other fungal endo-*β*-glucanases.

RT-PCR amplification of the cDNA fragment encoding a portion of *egI* was performed by using a Thermal Cycler 100 (USA) in 50 μL reaction volumes. The cycle profile was as follows: predenaturation at 94ºC for 45 s followed by 30 cycles of denaturation at 94ºC for 45 s, annealing at 49ºC for 45 s, and extension at 72ºC for 1 min. The PCR product of about 600 bp was purified by agarose gel electrophoresis and ligated into the pMD-18T vector and then sequenced. RACE was performed to obtain full-length cDNA clones using the SMART RACE cDNA Amplification Kit (Clontech). The specific primers for RACE-PCR were designed on the basis of the gene fragment sequence obtained above. Primer GSP-EG1 was for 5′-RACE and primer PEG-S2 was for 3′-RACE. The full-length cDNA of *egI* was cloned by PCR amplification with primers Ps and Pa and sequenced following the protocol described above. Sequence data were submitted to GenBank and analyzed with DNAMAN 6.0.

Expression of egI in yeast

After digestion with *Sna*BI/*Not*I, the DNA fragment containing the full-length *egI* gene was inserted into the expression vector pPIC9k digested with the same restriction endonucleases to construct the recombinant plasmid pPIC9k/*egI*. To confirm *egI* under the transcriptional control of the AOX1 promotor, the recombinant plasmid pPIC9k-*egI* was sequenced. The recombinant vector pPIC9k-*egI* was linearized with *Sal*I and then transformed *P. pastoris* GS115 by electroporation (1500 V, 25 μF, 200 Ω). The transformants were screened at the MD/MM plate and YPD-Geneticin plates contained Geneticin at the following concentrations of 250, 300, 400, 500, and 600 μg/mL. The different Geneticin resistance transformants were picked and inoculated into 25 mL BSM growth medium, growing at 28ºC in a shaking incubator (200 r/min) until OD_{600} of the culture increased up to 2 to 6. Cells were harvested by centrifugation at 1500 g for 5 min at room temperature and the cell pellet was resuspended to an OD_{600} of 1.0 in BSM induction medium to induce expression followed by adding 100% methanol to a final concentration of 1% methanol every 24 h to maintain induction. Then, 1 mL expression culture was transferred to a 1.5 mL microcentrifuge tube every 24 h and centrifuged at $10000 \times g$ for 2 to 3 min. The resultant sample was used to analyze the expression level and determine the enzyme activity.

SDS-PAGE analysis

SDS-PAGE was performed in a gel containing 12% (w/v) acrylamide and 0.1% SDS (w/v) using a Tris/glycine buffer system. The protein was stained with 0.1% Coomassie brilliant blue R250 in 7% (v/v) acetic acid and 50% (v/v) methanol solution. Destaining was carried out with 7% acetic acid in 50% methanol.

Protein content and endo-β-glucanase I activity assays

Protein content was determined by the method of Bradford (1976). Endo-*β*-glucanase I activity was analyzed with CM-cellulose as substrate by measuring the amount of reducing sugar in the supernatant with the dinitrosalicylic acid method

of Miller (1959). One unit of endo-*β*-glucanase I activity was defined as the amount of enzyme that releases 1 μmol reducing sugar per min under the assay conditions.

Characterization of recombinant endo-β-glucanase I

The optimal pH for endo-*β*-glucanase I activity was determined by performing the standard activity assay at 65ºC and over a wide pH range between 2.5 and 8.5. The following buffers Na_2HPO_4-citric acid (pH 2.5–8.0) and Tris-HCl (pH 8.0–8.5) were used for measuring the effect of pH value on endo-*β*-glucanase I stability at 65ºC. The effect of temperature on the activity of endo-*β*-glucanase I was also determined at different temperatures from 40ºC to 85ºC. The enzyme was incubated at various temperatures for 30 min, and the residual activity was measured using standard assay for evaluating the thermal stability.

Results

Cloning of a cDNA of egI encoding the endo-*β*-glucanase I

A 480-bp putative RT-PCR product was obtained by PCR amplification using a pair of specific primers PEG-S1 and PEG-S2. About 300-bp 5′-RACE fragment and 800-bp 3′-RACE fragment were then amplified by SMART RACE. The full-length sequence of the recombination cDNA was deduced by alignment of the three DNA fragments above. Also, 97 and 165 bp overlap domains were found (Fig. 1). To verify if these fragments come from the same gene, a PCR was carried out by using a pair of primer designed from the sequence of the 5′ and 3′ ends of the recombinant cDNA. The PCR product was sequenced and the sequence results demonstrated that the full-length recombination cDNA was correct. The nucleotide sequence had been deposited in the DDBI, EMBL, and GenBank nucleotide sequence databases under the Accession No. AY847014.

The cDNA contained a predicted ORF of 1005 bp encoding for 305 amino acids. The calculated molecular weight and pI of the predicted protein was 33.6 kDa and 5.0 (Compute pI-Mw software), respectively. Alignment of the deduced amino acid sequence of *egI* with that of AY791845 showed 98.5% homology.

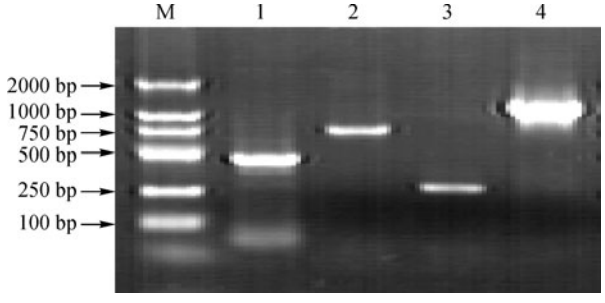

Figure 1 An agarose gel electrophoresis of product from PCR. Lane M is marker-DL2000. Lanes 1–4 represent product from RT-PCR, product from 3′-RACE, product from 5′-RACE, and full-length cDNA.

Expression and characterization of endo-*β*-glucanase I in *P. pastoris*

The endo-*β*-glucanase I was successfully produced in *P. pastoris* GS115. The endo-*β*-glucanase I activity of the recombinant strain named GS-EG2 was determined to be the highest one among others. The endo-*β*-glucanase I of GS-EG2 was secreted into the medium at the level of 788.26 μg/mL. Its activity in the supernatant was up to 45.42 U/mL after induction for 144 h with methanol (Fig. 2). No endo-*β*-glucanase I activity was detected in control cultures of *P. pastoris* GS115 transformed with vector only.

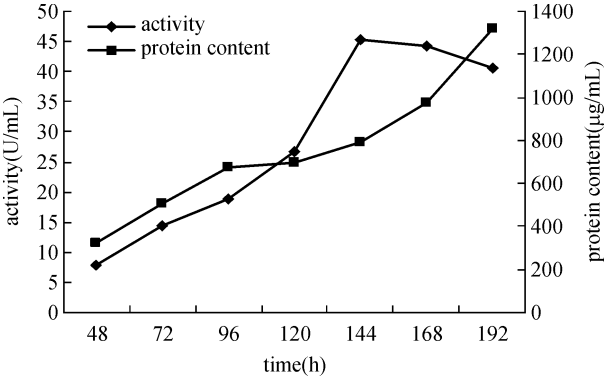

Figure 2 The curves of the endo-*β*-glucanase I activities and the protein content in the culture medium of GS-EG2 after induction at different times.

SDS-PAGE analysis

The endo-*β*-glucanase I expression of GS-EG2 in the culture media collected from 24 to 216 h at a 24-h-interval was analyzed by SDS-PAGE. The large quantities of target endo-*β*-glucanase I were gradually accumulated in the culture medium by methanol induction. No corresponding one was detected in control cultures of *P. pastoris* GS115 transformed with vector only. The molecular size of the expression product was estimated by SDS-PAGE to be 34.1 kDa (Fig. 3), which is similar to the putative molecular weight (33.6 kDa).

Characterization of the endo-*β*-glucanase I

The optimal temperature and pH for the endo-*β*-glucanase I were found to be 70ºC and 3.5, respectively (Fig. 4A and B). The endo-*β*-glucanase activity was relatively stable and retained above 99% after preincubation at 50ºC and 55ºC for 30 min. The enzyme activity retained was 60% while the enzyme was incubation at 65ºC. Although the highest activity

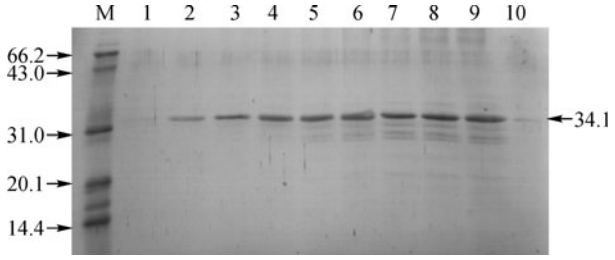

Figure 3 SDS-PAGE analysis of the expression of endo-*β*-glucanase I from GS-EG2 strain. Lane M is low-molecular-mass protein standard (from top to bottom: 97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa). Lanes 1–9 are products after induction from 24 to 216 h. Lane 10 is negative control (GS115/pPIC9K).

was measured at 70ºC, the enzyme was lost at this temperature (only 36% activity was retained after 30 min incubation). There was hardly any activity at 75ºC and 80ºC. The stabilization of pH was determined by preincubating the enzyme over a pH range of 2.5 to 8.5 at normal temperature for 24 h before measuring its activity. The enzyme of recombinant protein was rather stable. The activity retained above was nearly 80% from 2.5 to 8.5, so it is an acid cellulose enzyme (Figs. 4C and D).

Discussion

Biomass is the most attractive alternative on the earth among fuel sources and most sustainable energy resource. Today, ethanol produced from biomass is the most widely used biofuel when it is blended with gasoline. Cellulase production is found to be the most expensive step during ethanol production from cellulosic biomass and accounted for approximately 40% of the total cost (Huang and He, 2008). It is obvious that the successful utilization of cellulose is dependent on the development of technologies for the production of cellulase. To reduce the enzyme product costs, the isolation of new cellulase with high activity is very important. Thus, many researchers have been tried to develop an efficient and inexpensive process by using recombinant bacteria and yeast (Zhou and Ingram, 2001; Guedon et al., 2002; Wang et al., 2008).

In this study, the *egI* was isolated from a thermophilic fungal and introduced into *P. pastoris* GS115. Yeasts normally do not produce cellulases, but recombinant yeast cells containing the endo-*β*-glucanase I gene secreted successfully endo-*β*-glucanase I into the culture medium. The endo-*β*-glucanase I production capacity of the recombinant yeast strain was up to 778.26 μg/mL with a specific activity of 45.42 U/mL. In this experiment, the endo-*β*-glucanase I production represents the fermentation level under the flask culture condition. DING Shaojun ever reported the production of a neutral endoglucanase I with 543.36 IU/mL CMC activity obtained in *P. pastoris* by high cell density fermentation (Ding et al., 2006). Another report

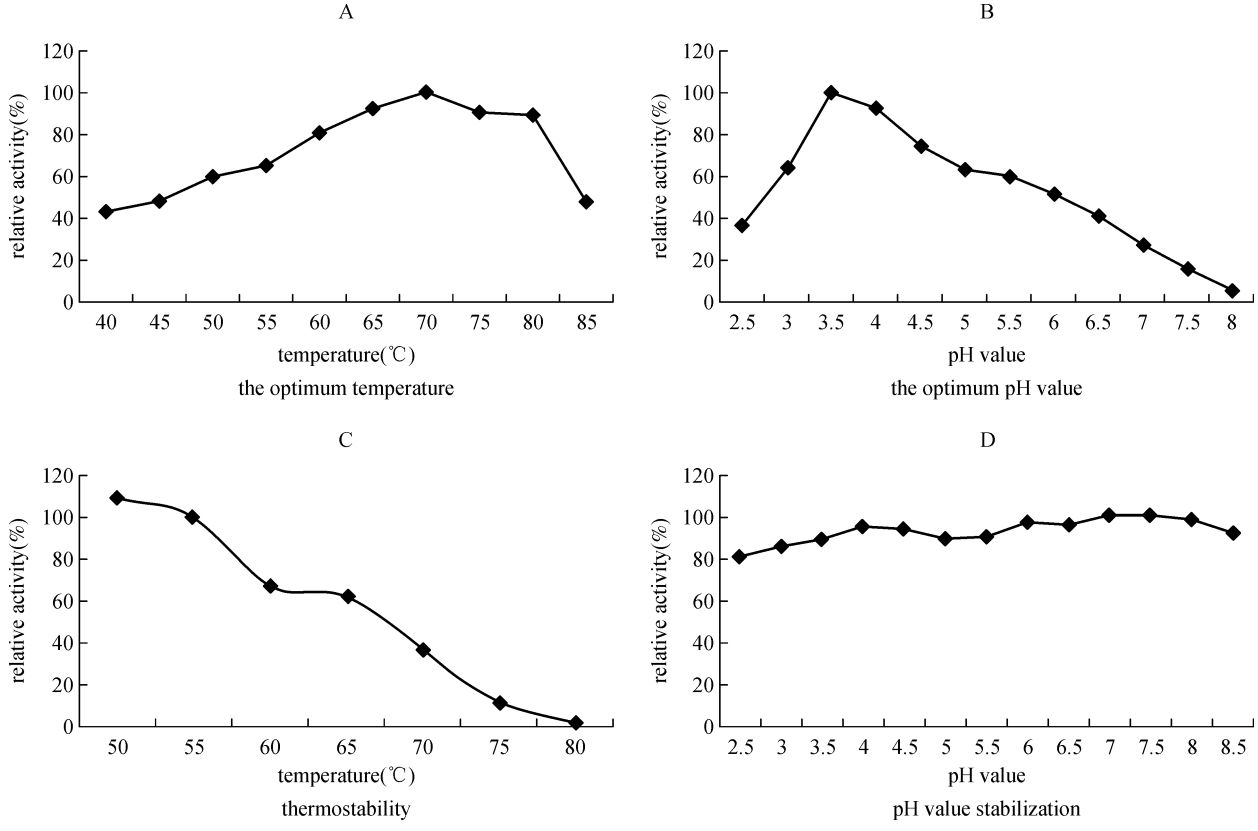

Figure 4 Effects of temperature and pH on the activity of the recombinant endo-*β*-glucanase I.

was that the *endo-β-cellulase* gene from *Clostridium thermocellu* was expressed in *E. coli* BL21, and the highest endocellulase activity was 90 U/mL. However, the enzyme located in the intracellular and *Clostridium thermocellu* can survive only in the absence of OXYGen. Therefore, it is hard to apply in the industry (Lü et al., 2009). The optimum pH and temperature of the enzyme were 70ºC and 3.5, respectively, which make it act in the acid-thermal environment. Due to its merit, the enzyme has wide application prospects and practicality value in production of cellulosic ethanol. Many researches found that the thermal stability could be improved by Directed Molecular Evolution (Hao and Berry, 2004; Suen et al., 2004). Further studies on directed evolution of endo-*β*-glucanase are in progress in order to obtain the perfect characteristics of industrial applications.

In conclusion, the *egI* was cloned from *T. aurantiacus*, and the recombinant endo-*β*-glucanase I was secreted into the culture medium at high expression level. The high activity of endo-*β*-glucanase I at high temperatures is very useful for the degradation of cellulose at high temperature. In addition, BSM is cheap, which can reduce the cost. It will play an important role in the fields of food, feedstuff, environmental protection, and energy development.

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