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Expression, purification and characterization of two thermostable endoglucanases cloned from a lignocellulosic decomposing fungi *Aspergillus fumigatus* Z5 isolated from compost

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ABSTRACT

Two genes encoding endoglucanase, designated as egl2 and egl3, were cloned from a lignocellulosic decomposing fungus *Aspergillus fumigatus* Z5 and were successfully expressed in *Pichia pastoris* X33. The deduced amino acid sequences encoded by egl2 and egl3 showed strong similarity with the sequence of glycoside hydrolase family 5. SDS–PAGE and western blot assays indicated that the recombinant enzymes were secreted into the culture medium and the zymogram analysis confirmed that both recombinant enzymes had endoglucanase activity. Several biochemical properties of the two recombinant enzymes were studied: Egl2 and Egl3 showed optimal activity at pH 5.0 and 4.0, respectively, and at 50 and 60 °C, respectively. Egl2 and Egl3 showed good pH stability in the range of 4–7, and both enzymes demonstrated good thermostability ranging from 30 to 60 °C. The K_m and V_{max} values using carboxymethyl cellulose (CMC, soluble cellulose, polymerized by β-1, 4-linked glucose residues) as the substrate at optimal conditions were determined. The activities of the enzymes on a variety of cello-oligosaccharide substrates were investigated, and Egl2 can hydrolyze cellotetraose and cellopentaose but not cellobiose and cellotriose, whereas Egl3 can hydrolyze all cello-oligosaccharides, except cellobiose.

Introduction

Cellulose, a linear polymer of D-glucose units linked by 1, 4-ß-D-glucosidic bonds, is found in nature almost exclusively in plant cell walls and is the most abundant renewable biomass available on earth [1]. However, without appropriate treatment, a mass of cellulosic wastes may accumulate and be deposited outdoors, resulting in environmental pollution [2]. Sulfuric acid is generally used in the hydrolysis of the cellulose chain into glucose, but this process imposes a burden on the environment by producing furfural and 5-hydroxymethyl-2 furfural [3]. In consideration of these issues, cellulolytic enzymes have been the subject of intense research in attempt to develop technologies to bioconvert the abundant quantities of cellulose-rich wastes [4]. In enzyme systems, microorganisms produce multiple enzymes to degrade cellulose materials [5], and these systems are mainly composed of endoglucanases (EGs),[1] cellobiohydrolases (CBHs) and β-glucosidases, which work synergistically to degrade the cellulose fraction [6]. Endoglucanase, an important type of cellulase, plays a crucial role in the cellulose degradation process by catalyzing the initial attack on the polymer; it hydrolyzes the β-1, 4 glucosidic bonds within amorphous regions of cellulose chains to yield long-chain oligosaccharides [7]. Endoglucanase-degraded cellulose biomass has many commercial applications, such as in the fabric, bioenergy, paper, or food industries [8,9]. The demand for endoglucanase is increasing rapidly and has become the driving force for obtaining a wide range of endoglucanases that have varying optimal pH and temperature values [10,11].

Enzyme thermostability is essential during the saccharification step, which converts lignocellulosic biomass to reducing sugars, because steam is used to make the biomass more suitable for enzymatic hydrolysis. Thus, the thermostable enzymes can be used directly after the heating step without a pre-cooling process, thereby decreasing the processing time, saving energy, reducing the risk of contamination, and improving fermentation yields and qualities. Furthermore, the saccharification step can be combined with fermentation to reduce the inhibition of hydrolysis by glucose or other monosaccharides [12]. Therefore, it is preferable to increase enzyme thermostability.

Endoglucanase secreted by fungi has been reported to be stable and efficiently functional at high temperatures [13,14], but few papers have examined the endoglucanase secreted by *Aspergillus fumigatus*, except Ximenes et al. [15] and Grigorevski-Lima et al.

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[1] Abbreviations used: CMC, carboxymethyl cellulose; EGs, endoglucanases; CBHs, cellobiohydrolases; PDA, potato glucose agar; RT, reverse transcriptase; PCR, polymerase chain reaction; ORF, open reading frames; NC, nitrocellulose; BSA, bovine serum albumin; TLC, thin-layer chromatography; GH5, glycoside hydrolase family 5; CBDs, cellulose-binding domains; CD, catalytic domain.

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Table 1
Primers used in the process of hiTAIL-PCR.

AD and reactions	Names	Sequences (5′–3′)
Arbitrary degenerate primer	AD1	**ACGATGGACTCCAGAG***VNVNNNGGAA*
	AD2	**ACGATGGACTCCAGAG***BNBNNNGGTT*
	AD3	**ACGATGGACTCCAGAG***VVNVNNNCCAA*
	AD4	**ACGATGGACTCCAGAG***BDNBNNNCGGT*
	AC1	**ACGATGGACTCCAGAG**
The upstream hiTAIL-PCR	egl2F-1	CATGTCATGGTACTCGTTGTCTATCT
	egl2F-2	**ACGATGGACTCCAGTC**TGACATGGTCATTGGACGC
	egl2F-3	CACTGTCTCGCATCAGCAGCA
	egl3F-1	CGTGGTATTCATTGTCTGGGAA
	egl3F-2	**ACGATGGACTCCAGTC**CGAAGATGACCTTGTCATTGGAG
	egl3F-3	TGTACGGCGGAGTTGAGTTAGTTAC
The downstream hiTAIL-PCR	egl2R-1	CCTCAACCAGGCTGCCATCAAC
	egl2R-2	**ACGATGGACTCCAGTC**CGTCGAGGGCAACTCGTG
	egl2R-3	CGCCACACAGTGGTTGAAGA
	egl3R-1	GCCATCAATGGTATCCGTGCTG
	egl3R-2	**ACGATGGACTCCAGTC**ACAATGACAACCTTAGGGGTCTGA
	egl3R-3	AGGAGCGTGTGGAGTCTGCCAC

V (G/C/A), B (G/C/T), D (G/A/T), N (A/T/C/G).

[16]. We isolated a lignocellulosic decomposing fungus *A. fumigatus* Z5 (GenBank Accession No. GQ337429.1) from compost, and our preliminary studies showed that the strain can secrete thermostable endoglucanase [17]. However, the high viscosity of the induction media, such as rice straw and bagasse, caused difficulties during fermentation and limited the scale-up for the production of this enzyme at an industrial scale [12]. The rapid developments of molecular biology make it possible to express and purify active endoglucanase and to produce a large amount of enzymes by large-scale fermentation.

This study describes the cloning of two endoglucanase genes from the strain *A. fumigatus* Z5 and the successful expression of two enzymes in *Pichia pastoris* X33. Moreover, several biochemical properties of the two recombinant enzymes were studied.

Material and methods

Microorganism and culture

The lignocellulosic decomposing strain, *A. fumigatus* Z5, was isolated from the compost of a fertilizer factory (Nanjing Mingzhu Fertilizer Co., Ltd. Nanjing, China). Potato glucose agar (PDA) was used for the cultivation of the strain *A. fumigatus* Z5. To induce the producing of cellulase, the strain was cultivated in a liquid medium [18] containing 10 g purely ball-milled cellulose powder (Sigma, USA), 1 g KH_2PO_4, 0.5 g Urea, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 7.5 mg $FeSO_4{\cdot}7H_2O$, 2.5 mg $MnSO_4{\cdot}H_2O$, 3.6 mg $ZnSO_4{\cdot}7H_2O$, 3.7 mg $CoC1_2{\cdot}6H_2O$, and 0.5 g $CaC1_2$, in 1000 ml of water. Subsequently, the cultivation was stored at 50 °C for the appropriate period.

P. pastoris X33 (Invitrogen, USA) was used to express egl2 and egl3, and *Escherichia coli* Top10 (stored in our lab) was used for the plasmid construction. YPD medium (1% yeast extract, 2% peptone, and 2% glucose, pH 6.0), which was prepared according to the Pichia expression system manual from Invitrogen, was used for propagation. YPM medium (1% yeast extract, 2% peptone, and 0.5% methanol, pH 6.0) was used as the induction medium.

Isolation of genomic DNA, mRNA and synthesis of cDNA

Genomic DNA of *A. fumigatus* Z5 was extracted as described by Moller et al. [19]. After induction by cellulose for approximately 4 days, the mycelium was collected for the mRNA extraction. The mRNA isolation was carried out by the E.Z.N.A.™ Fungal RNA Kit (Omega Bio-tek, Inc. R6840-01). Synthesis of cDNA and reverse transcriptase (RT) reactions were performed using the RevertAid™ First Strand cDNA synthesis Kit (Fermentas, #K1621).

Cloning of the endoglucanase gene egl2 and egl3

Two conserved fragments (DPHNYGRY and GEFAGG) were obtained by aligning various endoglucanase gene sequences of different filamentous fungi using ClustalX 1.83 [20]. The partial sequence of *A. fumigatus* Z5 endoglucanase was amplified using polymerase chain reaction (PCR), and the PCR primers eglF (GATCCYCATAACTATGGAAGNTAC) and eglR (GCCKCCNGCGAACTCKCC) were designed based on the conserved fragments. PCR was performed as follows: 2.5 μL 10 × PRC buffer, 2.5 μL Mg^{2+}, 2 μL of 10 mM dNTPs, 10 pmol/μL of each primer, and 0.5 U of Taq DNA polymerase in 25 μL. Amplification was performed in a thermal cycler (Bio-Rad S1000, USA) with 1 cycle of 95 °C for 5 min followed by 30 cycles of denaturation (60 s at 94 °C), annealing (60 s at 56 °C), and extension (90 s at 72 °C), with a final extension at 72 °C for 10 min. For analysis, 10 μL of the reaction mixture was electrophoresed on a 1% agarose gel and stained with ethidium bromide solution (5 μg ml^{-1}).

After DNA sequencing, a partial DNA sequence was identified. To obtain the 5′-end and 3′-end of the endoglucanase fragments, hiTAIL-PCR was applied according to Liu et al. [21]. The primers used in hiTAIL-PCR are shown in Table 1, and Genomic DNA was used as the template. The PCR product was purified and cloned into the PMD19-T vector (TaKaRa, Dalian, China), and subsequently, its nucleotide sequence was determined. By aligning the sequences of the 5′-end and 3′-end PCR products, the full-length cDNA sequences of endoglucanase were deduced and obtained through RT-PCR using the following specific primers: egl2-5′ (ATGAAATTCGGTAGCATTG TGCTCA), egl2-3′ (CTACAGGCATTGAGAGTAGTAGTCGT), egl3-5′ (ATGAGAATCAGCAGCTTGATCATG) and egl3-3′ (TTAAACACACTG GTGGTAGTAAGGGT). The 5'-fragments, 3′-fragments, and full-length products were purified, ligated into the PMD-19 T vector, and transformed into *E. coli* Top10 (stored in our lab). The full-length endoglucanase sequences were confirmed by restriction digestion and DNA sequencing and were named PMD-egl2 and PMD-egl3.

Construction of the expression plasmid and transformation of P. pastoris X33

The open reading frames (ORF) of the two endoglucanase genes, excluding the native signal sequence (amino acids 1–16 for egl2