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Functional characterization of GH7 endo-1,4-β-glucanase from *Aspergillus fumigatus* and its potential industrial application

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ABSTRACT

A gene encoding an endo-1,4-β-glucanase (Afu6g01800) from *A. fumigatus* was cloned into the vector pET-28a (+) and expressed in the *E. coli* strain RosettaTM (DE3) pLysS. Sequence analysis indicated that the enzyme Af-EGL7 belonged to the GH7 family. The gene *Af-egl7* encoded a protein comprising 460 amino acids, with a CBM1 domain at residues 424–460 and molecular mass of 52 kDa, as estimated by SDS-PAGE. This enzyme was optimally active at pH and temperatures ranging from 4.5 to 5.5 and from 40 to 60 °C, respectively. Mn^{2+} addition significantly enhanced the Af-EGL7 cellulase activity by 233%, whereas SDS addition fully inhibited this activity. Higher activity was observed toward β-glucan than toward xyloglucan and CM-Cellulose, suggesting that the enzyme corresponds to a β-1,3-1,4-glucanase. qRT-PCR in different culture media helped to establish the time-course expression profile. Different polysaccharides induced the gene *Af-egl7* in a time-dependent manner; in the particular case of the substrate sugarcane exploded bagasse (SEB), *Af-egl7* was induced 2500-fold. Upon addition to a commercial cellulase cocktail, Af-EGL7 significantly improved SEB saccharification, which suggested that the enzyme Af-EGL7 had great potential to hydrolyze complex biomass. From a biotechnological point of view, *A. fumigatus* Af-EGL7 is a promising candidate to enhance enzyme cocktails used in biorefineries such as consolidated bioprocessing.

1. Introduction

Recently, there has been great economic and environmental concern about energy sources. To prevent issues regarding fossil fuel scarcity and/or limited fossil fuel use for environmental reasons, searching for alternative renewable and sustainable energy sources has become an urgent matter. Employing agricultural residues as lignocellulosic materials is one of the most relevant strategies to produce second-generation (2G) ethanol. 2G ethanol production comprises at least three steps: pre-treatment, hydrolysis, and fermentation. The main bottleneck of this process is that enzymes used during the biomass hydrolysis step are expensive. Obtaining low-cost cellulases remains a great challenge when it comes to developing a sustainable process to produce 2G ethanol [1,2].

Cellulose is the major plant biomass component, and it is the most abundant polysaccharide on Earth [1,3]. Cellulose is a linear polymer comprising 7000–15000 glucose monomers linked by β-1,4-glycosidic bonds and intramolecular hydrogen bonds [3,4]. In nature, cellulose occurs as microfibrils with diameters of 2–10 nm, and it consists of 30–36 cellulosic fibers laterally aggregated by intermolecular hydrogen bonds and van der Waals forces [5]. Classic native cellulose degradation requires that three types of hydrolytic enzymes—endo-1,4-β-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.9), and β-glucosidases (EC 3.2.1.21)—act synergistically [6]. Endoglucanases catalyze initial attack on the polymer and account for random cleavage of the β-1,4-glycosidic bonds present in the cellulose chain amorphous regions [7]. This action releases smaller fragments that facilitate subsequent hydrolysis by the other two mentioned cellulases [8].

In recent years, endoglucanases have gained special attention due to their industrial and biotechnological applications; e.g., in the textile, animal food, food, and biofuel industries [8]. During 2G ethanol production, cellulose depolymerization into glucose monomers is essential

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for efficient fermentation; enzymatic hydrolysis is preferable in this process [9].

Filamentous fungi such as *Trichoderma reesei, Aspergillus niger*, and *Aspergillus fumigatus* play an important role in the secretion of endo-1,4-β-glucanases and other carbohydrate-active enzymes (CAZymes), which can act synergistically to break down biomass [10–15].

A. fumigatus is a pathogenic and opportunistic fungus that can cause a variety of allergic reactions. However, this fungus can produce many CAZymes that act synergistically to increase the enzymatic activity during lignocellulosic biomass breakdown [15,16]. When this fungus is cultivated in the presence of sugarcane bagasse, it secretes several important CAZymes involved in lignocellulose degradation. Such CAZymes include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.9), β-glucosidases (EC 3.2.1.21), endoxylanases (EC 3.2.1.32) and xylosidases (EC 3.2.1.37), which can withstand elevated temperatures [17–20]. Among these CAZymes, a GH7 endo-1,4-β-glucanase Afu6g01800 (GenBank access number XM_742,804.1) was significantly up-regulated in RNAseq data ($\log_2$FC = 9.575) [15]. This enzyme can cleave internal glucosidic bonds in the cellulose microfibril crystalline structure: thanks to the presence of a CBM1 domain in its structure, it acts on β-1,3- and β-1,4-glycosidic bonds through a retaining catalytic mechanism [16,21,22].

Endoglucanases secreted by fungi are stable at high temperatures [23,24], but few papers have described and characterized endoglucanases secreted by *A. fumigatus* [7,16,22,25].

Here, we purified and characterized a novel GH7 endoglucanase from *Aspergillus fumigatus* Af293 that can be further used to supplement enzyme cocktails.

2. Materials and methods

2.1. Strains, culture conditions, and vectors

The *A. fumigatus* strain Af293, gently donated by the Prof. Dr. Sérgio Akira Uyemura (University of São Paulo, BR), was cultivated in YAG medium [2.0% *(w/v)* dextrose, 2.0% *(w/v)* agar, 0.5% *(w/v)* yeast extract, and 0.1% *(v/v)* trace elements at 37 °C for two days to obtain a fresh conidium suspension. For qRT-PCR, the conidia were inoculated to a final concentration of 1×10^8 per 50 mL of YNB medium containing 1% *(w/v)* fructose and incubated at 37 °C and under shaking at 200 rpm for 16 h. Next, the mycelia were harvested, washed, and transferred to YNB medium containing 1% *(w/v)* fructose, 1% *(w/v)* sugarcane exploded bagasse (SEB) (47.5% cellulose; 9.0% hemicellulose and 34.3% lignin), 1% *(w/v)* CM-Cellulose, 1% *(w/v)* β-glucan, 1% *(w/v)* xyloglucan, and 1% *(w/v)* Avicel at 37 °C and 200 rpm for 3, 6, 12, and 24 h. Then, the mycelia were harvested by centrifugation for RNA purification.

The vector pGEM®-T Easy (Promega, Madison, WI, USA) was used for gene cloning and sequencing. The recombinant vector was transformed into *E. coli* DH10β. The vector pET-28a(+) (Novagen, Darmstadt, HE, Germany) was constructed for recombinant protein expression in *E. coli* Rosetta™ (DE3) pLysS.

2.2. Sequence and structural analysis

The signal peptide in Af-EGL7 was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). The potential N-glycosylation sites were predicted with the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequence alignment was carried out with the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Homology modeling was conducted with the software Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The three-dimensional structure of the protein modeled with Phyre2 was analyzed with the aid of the Swiss PDB viewer.

2.3. RNA isolation, cDNA synthesis, and gene amplification and cloning

Total RNA was isolated from *A. fumigatus* mycelia by using the kit Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The gene *Af-egl7* full-length cDNA was amplified by PCR; specific primer sequences were employed (F: 5′–**GAATTC**ATGGACTCCAAAAGAGGCGT–3′ and R: 5′– **AAGCTT**CTACAGACACTGAGAGTACC–3'; restriction sites in bold). The temperatures used during the amplification reactions performed with Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 68 °C for 2 min; and 68 °C for 10 min. The PCR-amplified fragments were purified with the aid of the kit QIAquick Gel Extraction (Qiagen, Hilden, Germany).

The amplified product was cloned into the vector pGEM®-T Easy and transformed into *E. coli* DH10β. The recombinant vector *Af-egl7*/pGEM-T Easy was digested with the restriction enzymes *Eco*RI and *Hind*III, and fragment *Af-egl7* was cloned into the vector pET-28a(+) containing an N-terminal His_6-tag. The protein was expressed by transformation into the *E. coli* strain Rosetta™ (DE3) pLysS.

2.4. qRT-PCR analysis

RNA was extracted, and cDNA was synthetized as described previously. Quantitative PCR (qPCR) analyses were accomplished according to Semighini et al. [26]. The endoglucanase mRNA abundance was normalized with β-tubulin probes. Table S1 lists the primer sequences. The reactions were carried out as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each experiment was performed in triplicate, and three different biological experiments were conducted.

2.5. Recombinant Af-EGL7 heterologous expression in E. coli

The *E. coli* strain Rosetta™ (DE3) pLysS harboring the recombinant vector *Af-egl7*/pET-28a(+) was inoculated into LB medium [1% *(w/v)* tryptone, 0.5% *(w/v)* yeast extract, and 1% *(w/v)* NaCl] added with 1% *(w/v)* glucose, kanamycin 50 μg mL^{-1}, and chloramphenicol 25 μg mL^{-1} and incubated at 37 °C for 16 h. After this period, the culture was transferred to TB medium [2.4% *(w/v)* yeast extract, 1.2% *(w/v)* tryptone, 0.4% *(v/v)* glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4] added with 1% *(w/v)* glucose, 50 μg mL^{-1} kanamycin, and 25 μg mL^{-1} chloramphenicol and incubated at 37 °C and 200 rpm until $O.D._{600nm}$ 0.4–0.6 was achieved. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated under the same conditions for 4 h.

2.6. Protein purification and refolding

After protein expression, the cell pellets were harvested by centrifugation at 4 °C and 18,514 g for 10 min. The inclusion bodies were purified and denatured according to the protocols described in the kit "iFOLD™ Protein Refolding System I" (Novagen, Darmstadt, HE, Germany). After dialysis against 1x iFOLD dialysis buffer [Tris 10 mM, TCEP 0.1 mM, 0.05 mM EDTA, and 0.06% *(v/v)* N-Lauroylsarcosine; pH 8.0], protein refolding was performed in C9 buffer [50 mM Tris, 12.5 mM Cyclodextrin, and 1 mM EDTA; pH 7.5]. The protein was loaded onto a dialysis membrane (Sigma-Aldrich, St. Louis, MO, USA), which was soaked in C9 buffer and incubated at 4 °C for 48 h, under stirring.

2.7. SDS-PAGE and zymogram analysis

The Af-EGL7 purity and molecular weight were analyzed by 10% *(w/v)* SDS-PAGE, stained with Comassie Brilliant Blue R-250. For the