Fungal Genetics and Biology 48 (2011) 185–191

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Characterization of the GPI-anchored endo β-1,3-glucanase Eng2 of *Aspergillus fumigatus*

Lukas Hartl, Amandine Gastebois, Vishukumar Aimanianda, Jean-Paul Latgé *

Unité des Aspergillus, Département de Parasitologie et Mycologie, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France

ARTICLE INFO

Article history:
Received 28 May 2010
Accepted 18 June 2010
Available online 7 July 2010

Keywords:
Aspergillus fumigatus
Cell wall
β-1,3-Glucan
Endo β-1,3-glucanase
GPI-anchor
ENG2

ABSTRACT

A GPI-anchored endo β-1,3-glucanase of *Aspergillus fumigatus* was characterized. The enzyme encoded by *ENG2* (AFUA_2g14360) belongs to the glycoside hydrolase family 16 (GH16). The activity was characterized using a recombinant protein produced by *Pichia pastoris*. The recombinant enzyme preferentially acts on soluble β-1,3-glucans. Enzymatic analysis of the endoglucanase activity using Carboxymethyl-Curdlan-Remazol Brilliant Blue (CM-Curdlan-RBB) as a substrate revealed a wide temperature optimum of 24–40 °C, a pH optimum of 5.0–6.5 and a K_m of 0.8 mg ml^{-1}. HPAEC analysis of the products formed by Eng2 when acting on different oligo-β-1,3-glucans confirmed the predicted endoglucanase activity and also revealed a transferase activity for oligosaccharides of a low degree of polymerization. The growth phenotype of the *Afeng2* mutant was identical to that of the wt strain.

1. Introduction

β-1,3-Glucan is the most abundant polysaccharide of the fungal cell wall and together with chitin it is responsible for cell wall rigidity (Gastebois et al., 2009). When facing morphological changes, for example during germination and branching in filamentous fungi or the separation of mother and daughter cells in yeasts, the cell wall needs to undergo partial lysis in order to obtain the required plasticity. Because of their mode of action endo β-1,3-glucanases should play an essential role during such morphogenetic events. Indeed the endoglucanase Eng1p has been shown to be essential for cell separation in yeasts (Baladron et al., 2002; Martin-Cuadrado et al., 2003; Esteban et al., 2005). A role of endo β-1,3-glucanases in cell wall remodeling of filamentous fungi on the other hand has not been shown to date. For example the deletion of the respective homolog *ENG1* in *Aspergillus fumigatus* did not lead to a phenotype different from the parental strain (Mouyna et al., 2002).

Glycosylphosphatidylinositol (GPI)-anchored proteins have been repeatedly shown to play a role in cell wall polysaccharide remodeling. For example it was found that GPI-anchored proteins belonging to the Gas (Gel) and Crh (Crf) families are able to modify cell wall β-glucans (Mouyna et al., 2000; Cabib et al., 2007). This makes GPI-anchored carbohydrate-active enzymes a promising target when studying the formation of the cell wall of *A. fumigatus*. Here we report the characterization of Eng2 (XP_755769) which was selected because it is a new endo β-1,3-glucanase belonging to the glycoside hydrolase family 16 (GH16), it features a GPI-anchor and it is unique to filamentous fungi.

2. Materials and methods

2.1. Strains and growth conditions

The *Afeng2* mutant and its parental strain CEA17*ku80Δ* (da Silva Ferreira et al., 2006) were maintained on 2% malt agar slants at room temperature. In the case of the *Afeng2* strain the medium was supplemented with 150 μg ml^{-1} hygromycin B (Sigma). For DNA extraction cultures were grown in Sabouraud's liquid medium (2% glucose + 1% mycopeptone). Transformations were performed on minimal medium (10 g l^{-1} glucose, 0.92 g l^{-1} ammonium tartrate, 0.52 g l^{-1} KCl, 0.52 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.52 g l^{-1} KH_2PO_4, 1 ml l^{-1} trace element solution (Cove, 1966), pH adjusted to 7.0). The *Pichia pastoris* strain GS115 was used to produce recombinant Eng2. All media were prepared according to the supplier's manual (Invitrogen). The *Escherichia coli* strain One Shot TOP10 (Invitrogen) was used for plasmid propagation following the manufacturer's protocol.

Abbreviations: AI, alkali insoluble cell wall fraction; BSA, bovine serum albumin; CD, conserved domain; CM, carboxymethyl; DP, degree of polymerization; GC, gas liquid chromatography; GH, glycoside hydrolase; Gn, laminarioligosaccharide containing *n* glucose residues; GPI, glycosylphosphatidylinositol; HPAEC, high performance anion exchange chromatography; RBB, Remazol Brilliant Blue; RBV, Remazol Brilliant Violet; rGn, borohydride reduced laminarioligosaccharide containing *n* glucose residues.

* Corresponding author. Fax: +33 1 40 61 34 19.
E-mail address: jplatge@pasteur.fr (J.-P. Latgé).

1087-1845/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2010.06.011

2.2. Nucleic acid extraction and RT-PCR

DNA was isolated as described by Girardin et al. (1993). RNA was isolated using phenol–chloroform extraction followed by ethanol precipitation. After an additional precipitation step using 5 M lithium chloride, RNA samples were loaded on RNeasy columns (QIAGEN) and subjected to on column DNase treatment using recombinant RNase-free DNase I from Roche. After elution from the RNeasy columns, a second DNase treatment was performed using the Turbo DNA-free kit from Ambion. RT-PCRs were performed using SuperScript II Reverse Transcriptase from Invitrogen following the manufacturer's instructions.

2.3. Production of recombinant Eng2 using P. pastoris

The *Pichia* Expression Kit from Invitrogen was used for recombinant protein production. A 1.9 kb cDNA fragment of *ENG2* was amplified using the primers Eng2p-FW and Eng2p-RV (Table 2) introducing an *Xho* I site at the 5′-end and a 6×His-tag followed by a stop-codon and an *Eco* RI site at the 3′-end. This fragment, coding for a truncated protein not featuring the 21 N-terminal (signal peptide) and 23 C-terminal (GPI anchoring sequence) amino acids, was subcloned into pCR-Blunt (Invitrogen) and sent for sequencing. After verification of the sequence, the two inserted restriction sites were used to clone the fragment into the expression vector pHIL-S1 resulting in pEng2. Correct integration was verified by digesting with *Sal* I (resulting in a 3.6 and a 6.5 kb fragment) and *Xho* I + *Eco* RI (resulting in a 1.9 and an 8.2 kb fragment). The plasmid was linearized using *Pst* I and used to transform the *P. pastoris* strain GS115 using the lithium chloride method according to the manufacturer. Transformants were selected on a histidin-depleted medium. The clones carrying integration at the alcohol oxidase gene (*AOX1*) were selected for their poor growth on methanol medium. The protein was produced following the manufacturer's instructions (Invitrogen) using an induction time of 56 h. Eng2 was concentrated and purified from culture supernatants using ProBond Resin (Invitrogen), eluted from the resin using 50 mM histidine and dialyzed against deionized water to remove histidine. The purity of the protein was checked by SDS–PAGE using silver nitrate staining. N-terminal peptide sequencing was performed as described previously (Beauvais et al., 1997).

2.4. SDS–PAGE and Western blot

Protein concentrations were measured using the Bradford Protein Assay (Bio-Rad) with BSA as a standard. Protein samples were mixed 3:1 with 4× protein loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), boiled for 5 min and analyzed by SDS–PAGE using 10–15% acrylamide separating gels (Laemmli, 1970). Protein bands were visualized by silver nitrate or Coomassie blue staining. Recombinant Eng2 was detected by western blot with a monoclonal anti-polyhistidine antibody (peroxidase conjugated monoclonal anti-polyhistidine clone HIS-1, Sigma) using the Pierce ECL Western Blotting Substrate (Thermo Scientific). To test the functionality of recombinant Eng2 as an antigen, sera from a previous study were used (Sarfati et al., 2006). Sera from four aspergilloma patients and from four healthy persons as a negative control were pooled and detected with an anti-human IgG secondary antibody (peroxidase conjugated anti-human IgG antibody developed in goat, Sigma) using the same ECL substrate as mentioned above.

2.5. Enzymatic characterization

The recombinant protein Eng2 was characterized using the substrate Carboxymethyl-Curdlan-Remazol Brilliant Blue (CM-Curdlan-RBB, Loewe Biochemica GmbH) (Brisset et al., 2000). Enzyme assays comprised 0.1 ml of substrate, 0.2 ml 0.1 M sodium acetate buffer pH 5.5 and 0.1 ml enzyme solution containing 0.8 μg of protein (Bradford Protein Assay, Bio-Rad). Reactions were performed at 37 °C for 2 h and stopped by adding 0.1 ml 0.5 M HCl following incubation on ice for 10 min and centrifugation at 10,000*g* for 5 min. OD of the supernatants was measured at 600 nm.

To test Eng2 activity with different oligoglucan substrates, the amount of reducing sugars formed was estimated as described previously (Fontaine et al., 1997). Curdlan (insoluble β-1,3-glucan) was purchased from Serva, laminarin (rarely branched β-1,3-glucan), lichenan (β-1,3-1,4-glucan) and mannan were from Sigma, pustulan (linear β-1,6-glucan) from Chalbiochem, schizophyllan (highly β-1,6-branched β-1,3-glucan) from Kaken (Japan), and mutan (insoluble β-1,3-glucan) from Novozymes. The AI cell wall fraction and galactomannan were prepared in the lab as described previously (Beauvais et al., 2005; Costachel et al., 2005). Reactions were performed in 50 mM acetate buffer pH 6.2 containing 2 mg ml^{-1} of the respective substrate and 8 μg ml^{-1} of recombinant protein. After incubation at 37 °C for 15–120 min, 20 μl aliquots of the assays were added to 980 μl of a 50 mM sodium sulfite, 250 mM NaOH, 25 mM sodium citrate, 10 mM $CaCl_2$ solution containing 10 mg ml^{-1} ρ-amino-hydroxybenzoic acid. After boiling for 10 min, OD was taken at 405 nm using glucose as a standard. Hydrolysis of chitin was tested using the substrate Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-Chitin-RBV, Loewe Biochemica GmbH) in an assay similar to that of CM-Curdlan-RBB.

2.6. High performance anion exchange chromatography (HPAEC, Dionex, model ISC3000) of oligosaccharides

To characterize Eng2 glucanase and transferase activity reaction mixtures were prepared as follows: 500 μg ml^{-1} substrate and 6 μg ml^{-1} protein (Bradford Protein Assay, Bio-Rad) in 50 mM sodium acetate buffer (pH 6.2). Borohydride reduced laminarioligos prepared in the lab and reducing laminarioligos (G2–G7) purchased from Seikagaku Biobusiness (Japan) served as substrates. After incubation at 37 °C for the desired time, 10 μl aliquots were used for analysis. To test for branching activity, 25 μl aliquots of the reaction mixtures described above were boiled for 10 min to deactivate Eng2 and incubated over night at 37 °C with 0.2 U (μmol of reducing equivalents released h^{-1} mg^{-1} of protein) of recombinant LamA of *Thermotoga neapolitana* expressed in *E. coli* (a kind gift from Dr. Vladimir V. Zverlov, Institute of Molecular Genetics, Russian Academy of Science, Moscow, Russia; Zverlov et al., 1997). Again, 10 μl aliquots were used for analysis. The resulting oligosaccharides were analyzed by HPAEC on a CarboPAC-PA200 column (3.2 × 250 mm, Dionex) using NaOH (50 mM) and NaOAc (500 mM) in 50 mM NaOH as eluent A and B respectively. The column was pre-equilibrated for 20 min in 98% A + 2% B. Following sample injection, a gradient run (flow rate 0.35 ml/min) was performed as follows: 0–2 min, isocratic step (95% A + 2% B), 2–15 min 98% A + 2% B – 65% A + 35% B, 15–35 min 65% A + 35% B – 40% A + 60% B, 35–37 min 40% A + 60% B – 100% B, and 37–40 min 100% B. Samples were detected on a pulsed electrochemical detector.

2.7. Analysis of the carbohydrate moiety of Eng2 using GC

Protein concentration was measured using the BCA protein assay (Pierce). Aliquots of the protein were mixed with meso-inositol serving as an internal standard. Hexoses were hydrolyzed (4 N trifluoroacetic acid, 100 °C, 2.5 h), reduced and peracetylated. The resulting alditol acetates were analyzed by gas–liquid chromatography using a Perichrom PR2100 Instrument equipped with flame