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Heterologous expression and characterization of α-L-arabinofuranosidase 4 from *Penicillium purpurogenum* and comparison with the other isoenzymes produced by the fungus

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ABSTRACT

Penicillium purpurogenum secretes at least four arabinofuranosidases. In this work, the gene of α-L-arabinofuranosidase 4 (ABF4) has been sequenced and expressed in *Pichia pastoris*. The gene is 1521 pb long, has no introns and codes for a protein of 506 amino acid residues including a signal peptide of 26 residues. Mature protein has a calculated molecular mass of 55.4 kDa, shows 77% identity with α-L-arabinofuranosidase 1 from *P. purpurogenum* and belongs to family 54 of the glycosyl hydrolases. Purified enzyme has a molecular mass near 68 kDa, is active on p-nitrophenyl α-L-arabinofuranoside and p-nitrophenyl-β-D-galactofuranoside, and follows Michaelis-Menten kinetics with K_M of 1.58 ± 0.13 mM and 5.3 ± 1.18 mM, respectively. The pH optimum is 4.6 and optimal temperature is 50 °C. The enzyme is active on sugar beet arabinan and wheat flour arabinoxylan but does not act on short arabinooligosaccharides or debranched arabinan. It shows synergistic effect on arabinose liberation from wheat arabinoxylan when combined with endoxylanase from *P. purpurogenum*. The properties of ABF4 have been compared with those of the other arabinofuranosidases produced by the fungus. *P. purpurogenum* is the first fungus possessing four biochemically characterized arabinofuranosidases. The availability of four different ABFs may be valuable for biotechnological applications.

Introduction

Xylan, the major component of plant hemicelluloses is composed of a linear chain of D-xylopyranoses linked by β (1 → 4) and it is substituted by several types of residues: methyl glucuronate, L-arabinofuranose, and acetate. The arabinoses may in turn be substituted at position 5 by hydroxycinnamic acids (Joseleau *et al.* 1992). The hydrolysis of xylan is a complex process with the participation of a set of esterase and glycanases, among them α-L arabinofuranosidases. The α-L arabinofuranosidases (ABFs) (E.C. 3.2.1.55) hydrolyze arabinose residues in the alpha configuration linked at the 2 and/or 3 position of the xyloses.

Xylan is not the only substrate for ABFs hydrolytic activity. Other substrates are pectin components such as

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rhamnogalacturonan I, which contains arabinan and arabinogalactan chains. The arabinans may be substituted at positions O_2 and O_3 by arabinose, while arabinogalactans, with a main chain of galactose, can be in turn be decorated with arabinans (Scheller *et al.* 2007).

A classification of the glycosyl hydrolases, based mainly on amino acid sequence similarities has been proposed (Henrissat & Davies 1997): in the CAZy database (http://www.cazy.org), 133 enzyme families have been recognized, and α-L arabinofuranosidases are found in families 2, 3, 10, 43, 51, 54, and 62.

ABFs have been actively studied in recent years due to their biotechnological applications, such as hydrolysis of monoterpenes in wine making to increase aroma production, clarification of juices, bioethanol production, kraft pulp bleaching, baking processes, and improvement of feed digestibility (Günata *et al.* 1988; Ravanal *et al.* 2012; Saha 2000; Bezalel *et al.* 1993; Gobbetti *et al.* 1999; Mathlouthi *et al.* 2002).

The great variety of potential natural substrates may explain the production of multiple ABFs by some fungi. Three isoenzymes have been described from *Aspergillus terreus* (Luonteri *et al.* 1995; Luonteri *et al.* 1998) and *Penicillium funiculosum* (De la Mare *et al.* 2013) and two from *Penicillium capsulatum* (Filho *et al.* 1996), *Penicillium canescens* (Sinitsyna *et al.* 2003), *Penicillium chrysogenum* (Sakamoto & Kawasaki 2003), *Aspergillus awamori* (Kaneko *et al.* 1998), and *Aspergillus niger* (Rombouts *et al.* 1988; Flipphi *et al.* 1993, 1993a).

Penicillium purpurogenum is the first fungus for which four ABFs have been described and characterized. The properties of three of them have been reported in previous publications (ABF1, De Ioannes *et al.* 2000; ABF2, Fritz *et al.* 2008; ABF3, Ravanal *et al.* 2010). This work focuses on the heterologous expression of α-L arabinofuranosidase 4 (ABF4), its biochemical characterization and its comparison with the other ABF isoenzymes from the fungus.

Materials and methods

Microbial strains utilized

Penicillium purpurogenum ATCC strain MYA-38 was the source for DNA and RNA. *Escherichia coli* TOP10 F′ was used for cloning of the ABF4 encoding gene. Heterologous expression was performed in *Pichia pastoris* GS115, supplied in the EasySelect™ *Pichia* Expression Kit (Invitrogen, CA, USA).

DNA and RNA preparation

The fungus was grown in Mandels medium as described previously (Hidalgo *et al.* 1992) with carbon sources at 1 %. Genomic DNA was extracted with the GeneJET Genomic DNA Purification kit (Fermentas) from fungus grown on glucose at 1 % for 2 d. Total RNA was isolated from the fungus grown for 3 d on wheat straw, rice straw, sugar beet pulp, almond shell or gum Arabic. The mycelium was washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and RNA was extracted by means of the RNeasy Plant Mini Kit (Qiagen, CA, USA). mRNA was obtained from the total RNA using the Absolutely mRNA purification kit (Agilent Technologies, CA, USA). cDNA was prepared from mRNA by means of the First Choice RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) kit (Thermo Fisher Scientific, MA, USA). The instructions from the manufacturers were followed in all procedures utilizing kits in this work.

Cloning and expression of abf4 in Pichia pastoris

A putative α-L-arabinofuranosidase gene (*abf4*) was found in the *Penicillium purpurogenum* genome database (unpublished). Specific primers were designed based on this sequence. The primers are: ABF4-F1 (ATGTTTCCTGAACCGAATCGCAACTACGCT, forward) and ABF4-R1 (TTAAGCAAAGCCAGCACTAATCACCCAGCTAG, reverse). These primers were designed from the translation initiation and termination sequences and were used to amplify by PCR the genomic DNA and cDNA. The PCR program used was: 30 cycles of: 94 °C 1 min, 60 °C 1 min, 72 °C 2 min, and a final extension at 72 °C for 10 min.

The primers ABF4-FW (TCA**GAATTC**ATGTTTCCTGAACCGAATCGCAA, forward) and ABF4-RV (GCA**TCTAGA**TAAGCAAAGCCAGCACTAATCAC, reverse) were used to amplify by PCR the *abf4* gene including restriction sites for EcoRI and XbaI (in bold) respectively, needed for cloning (see below). The reverse primer was designed without a stop codon to include a polyhistidine tail coding sequence present in the vector. The PCR products (program performed as above) were cloned in the expression vector pPICZB (Invitrogen). *Escherichia coli* TOP10 F′ cells were transformed with the resulting plasmid pPICZB-*abf4*. Transformed cells were selected in low-salt LB agar plates (pH 7.5) containing 25 μg ml^{-1} Zeocin. The PCR product was also cloned in pGEMT easy (Promega, WI, USA) and sequenced in both strands by Macrogen Inc. (Seoul, Korea). pPICZB-*abf4* was linearized by digestion with *Sac*I and transformed into competent *Pichia pastoris* GS115. Approximately 2 μg of linearized DNA was used. Transformed *Pichia* clones were selected from YPDS plates (Yeast extract Peptone Dextrose Sorbitol Medium) grown for 3 d which contained 100 μg ml^{-1} Zeocin.

Seven Zeocin resistant clones of *P. pastoris* GS115/pPICZB-*abf4* were grown each in 20 ml YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose) medium for 24 h and 28 °C in a rotary shaker (200 rpm). An aliquot of 200 μl of each culture was added to 20 ml of BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % yeast nitrogen base, 4×10^{-5} % biotin, and 1 % glycerol) and incubated for 24 h (28 °C, 200 rpm). The cultures were centrifuged under sterile conditions and the cells were resuspended in 20 ml of BMMY medium (Buffered Methanol-complex medium: 1 % yeast extract, 2 % peptone, 1 M potassium phosphate, pH 6.0, 13.4 % yeast nitrogen base with ammonium sulphate without amino acids, 0.02 % biotin and 1 % methanol). To induce gen expression by methanol, the cultures were

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