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Penicillium purpurogenum produces a novel endo-1,5-arabinanase, active on debranched arabinan, short arabinooligosaccharides and on the artificial substrate p-nitrophenyl arabinofuranoside

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ABSTRACT

Penicillium purpurogenum secretes numerous lignocellulose-degrading enzymes, including four arabinofuranosidases and an exo-arabinanase. In this work, the biochemical properties of an endo-arabinanase (ABN1) are presented. A gene, coding for a potential ABN was mined from the genome. It includes three introns. The cDNA is 975 bp long and codes for a mature protein of 324 residues. The cDNA was expressed in *Pichia pastoris*. The enzyme is active on debranched arabinan and arabinooligosaccharides. In contrast to other characterized ABNs, inactive on p-nitrophenyl-α-L-arabinofuranoside (pNPAra), ABN1 is active on this substrate. The enzyme has an optimal pH of 4.5 and an optimal temperature of 30–35 °C. Calcium does not activate ABN1. ABN1 belongs to GH family 43 sub-family 6, and a Clustal alignment with sequences of characterized fungal ABNs shows highest identity (54.6%) with an ABN from *Aspergillus aculeatus*. A three-dimensional model of ABN1 was constructed and the docking with pNPAra was compared with similar models of an enzyme very active on this substrate and another lacking activity, both from GH family 43. Differences in the number of hydrogen bonds between enzyme and substrate, and distance between the substrate and the catalytic residues may explain the differences in activity shown by these enzymes.

1. Introduction

Along with D-xylose and D-ribose, L-arabinose is one of the most abundant pentoses in Nature. It is found as a component in the structure of arabinoxylan and pectin. Arabinoxylan, one of the hemicelluloses, is a constituent of plant lignocellulose, and L-arabinose participates in its structure as a substituent of the xylan backbone [1]. Pectin, another component of lignocellulose, is present mainly in the primary cell-wall of plants. One of the pectin components, rhamnogalacturonan 1 (RG1) is rich in arabinose. In RG1, arabinose is present as arabinan and arabinogalactan, which are linked to the rhamnoses of the main chain of RG1 [2].

Arabinose is finding increased attention in biomedical applications. This sugar has been found to inhibit intestinal invertase, and in this way, it reduces the glycemic response after sucrose ingestion [3]. In addition, arabinooligosaccharides are considered potential prebiotics [4]. As a result, the production of L-arabinose and its oligosaccharides from natural sources containing polysaccharides rich in this sugar is of growing interest.

Polysaccharides can be hydrolyzed by means of acid or enzymes. The former has the disadvantage of producing side-products such as furfurals, and requires the disposal of the acid, which contaminates the environment. Enzymes, on the other hand, are very specific and can operate under milder conditions. Three groups of enzymes have been found to participate in the liberation of arabinose from arabinose-containing polysaccharides: 1) Endo-1,5-

Abbreviations: pNPAra, p-nitrophenyl-α-L-arabinofuranoside; pNPXyl, p-nitrophenyl- β-D-xylopyranoside; pNPGlu, p-nitrophenyl β-D-glucopyranoside; pNPGal, p-nitrophenyl- β-D-galactopyranoside; pNPRham, p-nitrophenyl α-L-rhamnopyranoside; pNPFuc, p-nitrophenyl-α-L-fucopyranoside; pNPMan, p-nitrophenyl- β-D-mannopyranoside; GH, glycoside hydrolases; ABN, endo-arabinanase; RG1, rhamnogalacturonan 1; DNS, dinitrosalicylate.

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arabinanases (E.C. 3.2.1.99); they belong to CAZy family 43 of the glycoside hydrolases (GH), and liberate mainly short arabinooligosaccharides and arabinose from debranched arabinan. 2) Exo-1,5-arabinanases (E.C. 3.2.1.-) hydrolyze arabinobiose from debranched arabinan and belong to CAZy family GH 93, and 3) arabinofuranosidases (ABFs) (E.C. 3.2.1. 55). ABFs are “exo” acting enzymes, which hydrolyze L-arabinose residues from non-reducing ends of arabinan, arabinooligosaccharides and arabinoxylan. They are found in GH families 2, 3, 10, 43, 51, 54 and 62 [5,6].

Arabinose-hydrolyzing enzymes are produced by fungi and bacteria [7]. Fungi are preferred because of their high enzyme secretory capacity. Our laboratory has characterized four arabinofuranosidases [8] and an exo-arabinanase [9] from the soft-rot fungus *Penicillium purpurogenum*. In this work, we have studied a new endo-arabinanase from this fungus, which we call ABN1, belonging to family GH 43; it acts on debranched arabinan, short arabinooligosaccharides, and it shows weak activity on pNPAra, in contrast to other characterized ABNs which are inactive on this substrate. Its physiological function may be complementary to that of the other enzymes described.

2. Results and discussion

2.1. Gene, cDNA sequence and structure of ABN1

A mining of the genome of *P. purpurogenum* (unpublished) allowed the finding of a sequence of the gene of a potential endo-arabinanase. Based on this sequence, primers E-FW and E-RV were designed (Table 1). The PCR products obtained using genomic DNA and cDNA as templates and the said primers were sequenced, and the sequence results matched the sequences deduced from the genome and cDNA. The gene is 1277bp long, and the length of the cDNA is 975 bp. A comparison of both sequences shows the presence of 3 introns in the gene. The introns are 120 pb, 90 pb and 92 pb long, respectively. The translation of the cDNA gives a protein of 324 residues (Fig. 1), with a signal peptide of 15 residues as predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/).

The deduced protein sequence was subjected to a BLAST search, and the closest two hits were with hypothetical proteins from *Penicillium subrubescens* and *Penicillium brasilianum* (97% and 91% identity, respectively) and the next 50 hits were also hypothetical proteins. When a Clustal omega analysis (http://www.ebi.ac.uk/Tools/msa/clustalo/) was performed with sequences of characterized GH family 43 fungal ABNs, the highest identity (54.6%) is found with an ABN from *Aspergillus aculeatus* [10] (Supplementary Fig. 1). This last enzyme has been assigned by CAZy to sub-family 6 of GH 43 according to the proposal of Mewis et al. [11]. Therefore, *P. purpurogenum* ABN1 may be allocated also to this sub-family.

Table 1
Primers used in this work.

Name	Sense	Sequence	Tm (°C)
E-FW	Sense	5′-ATGCTGGCTATATTCTTCTGCTTG-3′	55.5
E-RV	Antisense	5′-TTAAACGGCCTTCACGACAG-3′	55.8
EE-FW	Sense	5′-AAC**GAATTC**TACCCAGCGCGG-3′	60.1
EE-RV	Antisense	5′-CGC**TCTAGA**TTAACGGCCTTCACGA-3′	60.3

Sequences in bold indicate restriction sites for *Eco*RI (EE-FW) and *Xba*I (EE-RV).

The three-dimensional structure of fungal ABNs is not available. However, this structure has been established for several bacterial enzymes: *Geobacillus thermodenitrificans* (PDB ID: 1WLT) [12], *Bacillus subtilis* (PDB ID: 1UV4) [13] and *Cellvibrio japonicas* (PDB ID: 3QED) [14]. The structure consists of a five-bladed β-propeller fold, and 3 catalytic residues have been identified in the *C. japonicus* enzyme: GLU221 (catalytic acid), ASP38 (catalytic base) and ASP158 (a possible pK modulator). Supplementary Fig. 2 shows a Clustal omega alignment of these sequences along with that of ABN1. Although the percent identity of ABN1 to the other sequences is about 30%, the catalytic residues are conserved in the four enzymes and, in the ABN1 sequence, they correspond to ASP30, ASP145 and GLU196.

2.2. Heterologous expression of ABN1 in P. pastoris

Two *P. pastoris* transformants which secreted ABN1 were isolated. The most active clone (0.0938 nmol/min mg protein using the pNPAra assay) was used for larger scale production and purification of the enzyme. Fig. 2 shows an SDS PAGE of the purified protein. The enzyme shows a molecular weight of about 38 000, compared to 33 478 calculated from the amino acid sequence of the mature protein. This difference may be attributed to glycosylation.

2.3. Properties of the purified ABN1

Fig. 3 shows the optimal pH (4.5) and temperature (30–35 °C) of ABN1. Fig. 4 shows the thermostability and stability at different pHs. The enzyme shows good stability up to 40 °C for 1 h, and is most stable at pH 4. These assays were performed with pNPAra.

Activity was assayed with a set of pNP derivatives: pNPAra, pNPXyl, pNPGlu, pNPGal, pNPRham, pNPFuc and pNPMan. The enzyme showed activity only against pNPAra, pNPXyl and pNPGlu, with the following specific activities: 5.37, 0.26 and 0.47 nmol/min mg, respectively. The low activity observed precluded an accurate determination of the kinetic constants for these substrates.

ABN1 was active on arabinooligosaccharides. Fig. 5 shows a TLC of the reaction products. The main product of hydrolysis is arabinose, but the TLC shows also the formation of intermediates. The enzyme was also found to be active on xylooligosaccharides (Supplementary Fig. 3). However, the activity is much lower than against the arabinooligosaccharides.

Substrate specificity was also analyzed using the following substrates: wheat arabinoxylan, rye arabinoxylan, arabinan, debranched arabinan, and AZCN-debranched arabinan. Activity was found only with sugar beet arabinan, debranched arabinan and ACZN-debranched arabinan, when arabinose liberation was quantified (Table 2). The results obtained with AZCN-debranched arabinan are shown in Fig. 6. Arabinose is the main product, but a set of

MLAIFFCLFIALASAYPARGPCTGDCWTHDPAMIQRESDGTYFRFSTGTGVNTMKSPSLKGPWTDVGPAL

PNGSKITLDGVDSSDIWAPDVHYQDGTYYMYYVLSKLGTQTSQIGVATSTTMEPGSWTDHGIIGVPANSA

YNRIDPNWITIGGKQYLQFGSYWQDIYQVALESPLQVGSNTPHQIAYNASSNHRVEGSFLYQHGSFYYLF

FSGGVAGSYTATYPAQGEEYRVHVCRSSSGTGGFVDQAGISCLESGGTIILASHDQVYAPGGQGVLTDKD

LGPVLYYHYYSLAAKEAGGTGIKGYLYGWNELDFSTGWPVVKAV

Fig. 1. Amino acid sequence of ABN1. The signal peptide is underlined.