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

Characterization of a recombinant α-glucuronidase from *Aspergillus fumigatus*

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ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form
5 March 2013
Accepted 7 April 2013
Available online 17 April 2013
Corresponding Editor:
Steven Harris

Keywords:
α-Glucuronidase
Aspergillus fumigatus
GH family 67
Heterologous expression
Pichia pastoris

ABSTRACT

The degradation of xylan requires the action of glycanases and esterases which hydrolyse, in a synergistic fashion, the main chain and the different substituents which decorate its structure. Among the xylanolytic enzymes acting on side-chains are the α-glucuronidases (AguA) (E.C. 3.2.1.139) which release methyl glucuronic acid residues. These are the least studies among the xylanolytic enzymes. In this work, the gene and cDNA of an α-glucuronidase from a newly isolated strain of *Aspergillus fumigatus* have been sequenced, and the gene has been expressed in *Pichia pastoris*. The gene is 2523 bp long, has no introns and codes for a protein of 840 amino acid residues including a putative signal peptide of 19 residues. The mature protein has a calculated molecular weight of 91 725 and shows 99 % identity with a putative α-glucuronidase from *A. fumigatus* A1163. The recombinant enzyme was expressed with a histidine tag and was purified to near homogeneity with a nickel nitriloacetic acid (Ni-NTA) column. The purified enzyme has a molecular weight near 100 000. It is inactive using birchwood glucuronoxylan as substrate. Activity is observed in the presence of xylooligosaccharides generated from this substrate by a family 10 endoxylanase and when a mixture of aldouronic acids are used as substrates. If, instead, family 11 endoxylanase is used to generate oligosaccharides, no activity is detected, indicating a different specificity in the cleavage of xylan by family 10 and 11 endoxylanases. Enzyme activity is optimal at 37 °C and pH 4.5–5. The enzyme binds cellulose, thus it likely possesses a carbohydrate binding module. Based on its properties and sequence similarities the catalytic module of the newly described α-glucuronidase can be classified in family 67 of the glycosyl hydrolases. The recombinant enzyme may be useful for biotechnological applications of α-glucuronidases.

Introduction

Lignocellulose, the most abundant biological material on earth, is composed mainly of lignin (a polyphenol) and several polysaccharides: cellulose, hemicelluloses, and pectin. Being a renewable store, it is a potential primary source for different applications (Ragauskas *et al.* 2006). The degradation (or saccharification) of the lignocellulose polysaccharides (either biologically or chemically) gives rise to a mixture of monosaccharides which can be used to generate biofuels and numerous products of biotechnological and pharmaceutical interest (Ragauskas *et al.* 2006). The use of enzymes in the saccharification of these polysaccharides is preferred, since it can be performed under mild conditions, avoiding the accumulation of

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1878-6146/$ – see front matter
http://dx.doi.org/10.1016/j.funbio.2013.04.002

undesired by-products and reducing possible environmental contamination.

The hemicelluloses are an important component of lignocellulose (around 25–35 % of its dry weight). They are defined operationally as the alkali-extractable portion of lignocellulose, and chemically they are a mixture of polysaccharides, mainly xylan, and mannan (Joseleau *et al.* 1992). Xylan is the main hemicellulose of annual plants and hardwoods, and it is composed of a linear chain of xylopyranose residues joined by β-(1 → 4) glycosidic linkages. The main chain is substituted by a variety of compounds, such as arabinofuranose, methyl glucuronate, and acetate; the arabinoses can be linked to hydroxycinnamic acids (Joseleau *et al.* 1992).

The biodegradation of xylan is a complex process, involving a number of glycanases and esterases. The main chain is hydrolysed by the action of endoxylanases (E.C. 3.2.1.8) which liberate xylooligosaccharides of different length and are eventually hydrolysed to xylose by β-xylosidases (E.C. 3.2.1.37). Acetyl xylan esterases (E.C. 3.1.1.72), arabinofuranosidases (E.C. 3.2.1.55), cinnamoyl esterases (E.C. 3.1.1.73), and α-glucuronidases (AguA) (E.C. 3.2.1.139), acting in synergy with the endoxylanases, hydrolyse the side-chains (Biely 1985). These enzymes are produced by fungi and bacteria and are mainly extracellular (Biely 1985).

Among the xylanases, the α-glucuronidases (AguA) have received relatively less attention. These enzymes hydrolyse the α-1,2-linkage between 4-O-methyl glucuronic/glucuronic acid and xylose (Heneghan *et al.* 2007). Enzymes with these general properties have been identified and isolated from both bacteria (Bronnenmeier *et al.* 1995; Choi *et al.* 2000) and fungi (Siika-aho *et al.* 1994; Mierzwa *et al.* 2005). A classification based on amino acid sequence places AguAs in families 67 and 115 of the glycosyl hydrolases (GHs) (Cantarel *et al.* 2009). An example of a family 67 AguA is the enzyme from *Aspergillus tubingensis* (Biely *et al.* 2000). This enzyme liberates 4-O-methyl-D-glucuronic acid (MeGlcA) only from glucuronoxylan fragments in which the acid is attached to the non-reducing terminal xylopyranosyl residue and it acts with inversion of configuration. On the other hand, AguAs from family 115 (Ryabova *et al.* 2009), which utilize also an inverting mechanism, are active on polymeric xylan as well. AguAs from family 67 are inactive towards the synthetic substrate p-nitrophenyl (pNP) alpha-D-glucuronopyranoside (Kiryu *et al.* 2005). An additional type of AguA, belonging to family 4, but active on the pNP derivative only has also been described (Suresh *et al.* 2003). Finally, a non-classified AguA from *Aspergillus niger* with a specificity for trehalose dicarboxylate as only substrate has been studied (Kiryu *et al.* 2005).

In this work, an AguA produced by a newly isolated strain of *Aspergillus fumigatus* is described. The gene has been sequenced, expressed in *Pichia pastoris* and the enzyme has been characterized, showing similarity with AguAs from GH family 67. The availability of a heterologously expressed enzyme may be useful for biotechnological applications of AguA.

Materials and methods

Fungal strain and culture conditions

A strain of *Aspergillus* (initially identified morphologically) was isolated from a contaminated potato dextrose agar plate. It was later identified as *Aspergillus fumigatus* by molecular characterization as described below. Liquid cultures of the fungus were performed in Mandels medium (Mandels & Weber 1969) with a carbon source at 1 % for 3 d with agitation (200 rpm) at 28 °C.

Identification of the fungus as Aspergillus fumigatus

For the species identification, the internal transcribed spacer (ITS)1–5.8S–ITS2 rDNA region of the fungus was amplified by PCR using primer set ITS1 (5′-TCCGTAGGTGAACCTG CCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990). The 550 bp amplicon thus obtained was cloned and sequenced. This sequence was submitted to GenBank with N° JQ950749.

DNA and RNA preparations

Genomic DNA of *Aspergillus* sp. was extracted with the GeneJET™ Genomic DNA Purification kit (Fermentas) from fungus grown on 1 % glucose. Fungus grown on birchwood xylan (Sigma) and washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was utilized for total RNA extraction by means of the RNeasy Plant Mini Kit (Qiagen). mRNA was extracted from the total RNA using the Absolutely mRNA kit (Stratagene). cDNA was prepared from mRNA by means of the First Choice RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion). The instructions from the manufacturers were followed in all procedures utilizing kits in this work.

Gene and cDNA sequencing strategy

Degenerate primers were designed from conserved nucleotide sequences coding for hypothetical α-glucuronidases from different fungi, aligned using ClustalW (http://www.genome.jp/tools/clustalw/). All primers are listed in Table 1. Primers JE-

Table 1 – Primers used for gene sequencing and for heterologous expression of AguA.

Primer name	Sequence	Theoretical tm	
JE-1LR	TAYGCYCGYCT**K**TTGGC	55.7 °C	Sense
JE-3LR	AAGTAY**G**G**V**CCY**A**TY**G**ATTTCCA	56.5 °C	Sense
JE-4LR	GTCAG**Y**GT**Y**TGGAT**R**CC**S**AGGTT	60.2 °C	Antisense
JE-20LR	ATGTGGAGCGGCATTCCT**R**TA TTCGC	62.9 °C	Sense
JE-21LR	CTAATC**Y**ACAATTCC**Y**GGTG GCAGCAC	61.4 °C	Antisense
JE-30LR	ATGTGGAGCGGCATTCCTGT ATTC	59.4 °C	Sense
JE-31LR	CTAATCTACAATTCCCGGTGG CAGCA	60.7 °C	Antisense
JE-32LR	AGATACA**GAATTC**ATGTGGAGC GGCATTCCTG	62.9 °C	Sense
JE-34LR	AGA**TCTAGA**TAATCTACAATTC CCGGTGGCAGC	61.9 °C	Antisense

Degenerations are marked in bold while restriction sites for *Eco*RI and *Xba*I are marked in bold and underlined.