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SCIENCE DIRECT
Enzyme and Microbial Technology 38 (2006) 649–656
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Characterization of the *Aureobasidium pullulans* α-glucuronidase expressed in *Saccharomyces cerevisiae*

B.J.M. de Wet, W.H. van Zyl, B.A. Prior *

Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

Received 11 April 2005; received in revised form 6 July 2005; accepted 20 July 2005

Abstract

The α-glucuronidase gene (*aguA*) of *Aureobasidium pullulans* NRRL Y-2311-1 was amplified by PCR and sequenced. Based on its deduced amino acid sequence, AguA was found to be a member of family 67 of the glycoside hydrolases. It shares greater than 60% identity and between 34% and 42% identity with fungal and with bacterial α-glucuronidases, respectively. The open reading frame lacks introns and encodes a polypeptide of 836 amino acids that contains a putative signal peptide of 15 amino acids resulting in a mature protein with a calculated molecular mass of 91.0 kDa. A construct of the *aguA* gene encoding an additional C-terminal hexahistidine tag was cloned on an episomal plasmid under control of the *ADH2* promoter and terminator and expressed in *Saccharomyces cerevisiae* Y294. The heterologous α-glucuronidase was purified to homogeneity by Ni-chelation affinity chromatography, and displayed an electrophoretic mobility of 157 kDa on SDS–PAGE. Maximal activity was measured at 65 °C and at pH 5 and pH 6. The enzyme had K_m values in the millimolar range for the series of substrates from aldobiouronic acid to aldopentaouronic acid, but was unable to hydrolyze an internally substituted aldopentaouronic acid.

Keywords: Hemicellulose; Enzyme; α-Glucuronidase; Yeast-like fungus

1. Introduction

Xylan is one of the main non-cellulose polysaccharides found in plant cell walls and contributes to between 10% and 35% of the dry weight of the cell wall depending on the plant species and the tissue type. Xylan is a substituted heteropolysaccharide that consists of a backbone of 1,4-linked β-D-xylopyranose units substituted with 1,2-linked α-4-*O*-methyl glucuronic acid, 1,3-linked α-L-arabinofuranose and acetic acid esters at positions 2 and 3. Arabinofuranose moieties may contain ester-linked cinnamic acids at the fifth position [1]. The enzymatic degradation of xylan is a crucial process in the carbon cycle in nature and is of industrial relevance. As a result of the structural complexity of the xylan polymer, the cooperative action of several enzymes is required for the complete hydrolysis of xylan. The internal glycosidic bonds of the main chain are hydrolyzed by *endo*-β-1,4-xylanase (E.C. 3.2.1.8) releasing smaller xylo-oligosaccharides. β-Xylosidase (E.C. 3.2.1.37) subsequently removes single unsubstituted xylose moieties from the non-reducing ends of xylo-oligosaccharides, while the xylanolytic accessory enzymes, α-arabinofuranosidase (E.C. 3.2.1.55) and α-glucuronidase (E.C. 3.2.1.139) remove the side-chains from substituted xylo-oligosaccharides [2].

The side-chain cleaving accessory enzymes, such as α-glucuronidase, have received little attention compared to the main-chain cleaving *endo*-β-1,4-xylanases. A number of fungal and bacterial α-glucuronidases have been purified and characterized, although few of the genes encoding these enzymes have been sequenced [3]. Currently, all of the sequenced α-glucuronidases are classified in family 67 of the glycoside hydrolases, and α-glucuronidase is the only enzyme activity found in this family (http://afmb.cnrs-mrs.fr/CAZY/). Microbial α-glucuronidases cluster into two groups within the family, defined by either bacterial or fungal origin. Bacterial α-glucuronidases are dimeric proteins with calculated monomeric molecular masses of ∼70 kDa, while

* Corresponding author. Tel.: +27 21 808 5856; fax: +27 21 808 5846.
E-mail address: bap@sun.ac.za (B.A. Prior).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.07.018

fungal α-glucuronidases are monomeric proteins with calculated molecular masses of ∼90 kDa. In spite of the differences in molecular mass and quaternary structure, it is likely that the family 67 enzymes share a highly conserved active site architecture as the catalytically important amino acids identified in the crystal structure of the *Cellvibrio japonicus* α-glucuronidase were found to be conserved in all of the family 67 enzymes [4]. Predictably, family 67 α-glucuronidases are also conserved in terms of substrate specificity. They release 4-*O*-methyl glucuronic acid only when it is linked to the terminal non-reducing end xylopyranosyl unit of small xylo-oligosaccharides and cannot hydrolyze *p*-nitrophenyl α-D-glucuronide [3]. Microbial α-glucuronidases with the ability to release 4-*O*-methyl glucuronic acid from polymeric glucuronoxylan have been described, although it is not known if they are family 67 enzymes [5,6]. Specific industrial applications for microbial α-glucuronidases have not emerged to date. Even so, microbial α-glucuronidases play an important synergistic role in combination with other lignocellulolytic enzymes in processes involving the breakdown of complex plant cell wall polymers [7]. α-Glucuronidase has been shown to have a positive effect on the release of reducing sugars from xylan by *endo*-β-1,4-xylanase and β-xylosidase [8,9], and 4-*O*-methyl-glucuronic acid has been shown to be cross-linked to lignin, increasing the recalcitrance of lignocellulosic material to enzymatic degradation [10].

Aureobasidium pullulans is an euascomycetous fungus [11] and colour-variant strains of this organism have been isolated for the high-level production of xylanolytic enzymes [12,13]. The main *endo*-β-1,4-xylanase has been purified [14], and the *xynA* gene cloned [15] and expressed in *Saccharomyces cerevisiae* [16]. The presence of several xylanolytic accessory enzyme activities has been noted in the culture fluid of *A. pullulans* NRRL Y-2311-1 cultivated on lignocellulosic carbon sources [17]. α-Glucuronidase activity was detected in the culture supernatants of *A. pullulans* NRRL Y-2311-1, but the enzyme proved very difficult to purify [18]. Consequently, recombinant expression of the *A. pullulans aguA* gene in *S. cervisiae* was selected as an alternative method of obtaining the enzyme for study. The present report describes the cloning of the *A. pullulans aguA* gene, its expression in *S. cerevisiae* and the characterization of the heterologous enzyme.

2. Materials and methods

2.1. Microbial strains, plasmids and culture conditions

A. pullulans NRRL Y-2311-1 was obtained from the ARS culture collection, located at the National Center for Agricultural Utilization Research (Peoria, IL, USA) and cultivated in YPD medium (containing per litre, 10 g yeast extract, 20 g peptone, 20 g glucose and for solid medium, 20 g agar) at 30 °C and defined medium (containing per litre, 10 g birchwood xylan, 6.7 g yeast nitrogen base, 2 g L-asparagine and 5 g monobasic potassium phosphate). *Escherichia coli* XL1-Blue (Stratagene) was used as a host for recombinant plasmids and pGEM-T Easy (Promega) as a plasmid vector for the cloning of purified PCR products. *E. coli* XL1-Blue cells harbouring insert-containing plasmid were selected on Luria–Bertani medium containing 100 mg l^{-1} ampicillin (Sigma), 40 mg l^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma) and 100 μmol l^{-1} isopropyl-β-D-thiogalactopyranoside (Sigma) [19]. *S. cerevisiae* Y294 (ATCC 201160; *α leu2-3,112 ura3-52 his3 trp1-289*) was routinely cultured in YPD medium or synthetic complete (SC) medium (containing per litre, 6.7 g yeast nitrogen base and 20 g glucose, and for solid medium 20 g agar, medium adjusted to pH 6). The yeast URA3-based episomal expression vector pDLG1 [20] was used for heterologous production of α-glucuronidase in *S. cerevisiae*. Autoselective *fur1* strains of *S. cerevisiae* expressing the *aguA* gene were generated by transforming the yeast with a *fur1::LEU2* DNA fragment obtained from plasmid pDF [20].

2.2. Amplification of the A. pullulans aguA gene

Genomic DNA was extracted from *A. pullulans* by glass bead disruption and phenol/chloroform extraction [21]. Amino acid sequences of α-glucuronidases from *Aspergillus niger* (CAC38119), *Aspergillus tubingensis* (CAA75605), *Talaromyces emersonii* (AAL33576) and *Trichoderma reesei* (CAA92949), were aligned using CLUSTAL-W [22]. Areas of invariant sequence were used to design degenerate primers. An internal segment of the *aguA* gene was amplified from *A. pullulans* genomic DNA using the degenerate primers AGUdegF2 and AGUdegR2 (Table 1). *Taq* DNA polymerase (Promega) was used for all PCR reactions unless otherwise stated, and all PCR products were gel purified using a Wizard SV Gel and PCR Clean-Up System (Promega), cloned as described above and sequenced. Circular DNA molecules for inverse PCR were generated by partial digestion of *A. pullulans* genomic DNA followed by ligation at low DNA concentration [23,24]. Partial digests of *A. pullulans* genomic DNA were made by incubating 10 μg DNA with between 0.05 U mg^{-1} and 1 U mg^{-1} *Sau*3AI (Roche). Digested DNA between 2000 bp and 5000 bp was gel purified by the freezing and phenol extraction [25]. Intramolecular ligation was achieved by performing reactions at DNA concentration of 2.4 μg ml^{-1} using 5 U of T4 DNA ligase (Roche). Regions flanking the known internal segment of the *aguA* gene were amplified from the circular DNA template using the primers AGUinvL1 and AGUinvR1 (Table 1). RNA was extracted from *A. pullulans* by glass bead disruption and phenol/chloroform extraction after cultivation in defined medium containing 1% birchwood xylan [26]. mRNA was reverse transcribed using a First strand cDNA Synthesis kit (Roche) with an oligo-p$(dT)_{15}$ primer. The 3′-end of the *aguA* gene was amplified from cDNA using AGU-F4 and oligo$(dT)_{15}$ as