BBA - Proteins and Proteomics 1865 (2017) 1758–1769

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

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

Structural and functional characterization of a highly secreted α-L-arabinofuranosidase (GH62) from *Aspergillus nidulans* grown on sugarcane bagasse

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ARTICLE INFO

Keywords:
α-L-Arabinofuranosidase
GH62
Aspergillus nidulans
N-glycosylation
Arabinoxylan

ABSTRACT

Carbohydrate-Active Enzymes are key enzymes for biomass-to-bioproducts conversion. α-L-Arabinofuranosidases that belong to the Glycoside Hydrolase family 62 (GH62) have important applications in biofuel production from plant biomass by hydrolyzing arabinoxylans, found in both the primary and secondary cell walls of plants. In this work, we identified a GH62 α-L-arabinofuranosidase (*An*Abf62A$_{wt}$) that was highly secreted when *Aspergillus nidulans* was cultivated on sugarcane bagasse. The gene AN7908 was cloned and transformed in *A. nidulans* for homologous production of *An*Abf62A$_{wt}$, and we confirmed that the enzyme is N-glycosylated at asparagine 83 by mass spectrometry analysis. The enzyme was also expressed in *Escherichia coli* and the studies of circular dichroism showed that the melting temperature and structural profile of *An*Abf62A$_{wt}$ and the non-glycosylated enzyme from *E. coli* (*An*Abf62A$_{deglyc}$) were highly similar. In addition, the designed glycomutant *An*Abf62A$_{N83Q}$ presented similar patterns of secretion and activity to the *An*Abf62A$_{wt}$, indicating that the N-glycan does not influence the properties of this enzyme. The crystallographic structure of *An*Abf62A$_{deglyc}$ was obtained and the 1.7 Å resolution model showed a five-bladed β-propeller fold, which is conserved in family GH62. Mutants *An*Abf62A$_{Y312F}$ and *An*Abf62A$_{Y312S}$ showed that Y312 was an important substrate-binding residue. Molecular dynamics simulations indicated that the loop containing Y312 could access different conformations separated by moderately low energy barriers. One of these conformations, comprising a local minimum, is responsible for placing Y312 in the vicinity of the arabinose glycosidic bond, and thus, may be important for catalytic efficiency.

1. Introduction

Plant biomass degradation is one of the most important fields for microbial enzyme application due to the great interest in second-generation ethanol production. The predominant method for plant biomass deconstruction consists of using Carbohydrate-Active enzymes (CAZymes) for the hydrolysis of these polysaccharides. These enzymes are categorized into different classes according to their function in the CAZy database (http://www.cazy.org). The classes include glycoside hydrolases (GHs) that hydrolyze glycosidic bonds of complex carbohydrates, glycosyltransferases (GTs) that transfer sugar molecules, polysaccharide lyases (PLs) that cleave uronic acid-containing polysaccharide chains, carbohydrate esterases (CEs) that hydrolyze ester bonds in carbohydrates and finally auxiliary activities (AAs) that promote oxidation-reduction reactions for degrading plant biomass [31].

Plant biomass, in general, is composed primarily of cellulose and hemicellulose, followed by lignin. Sugarcane bagasse is one of the most important sources of lignocellulosic residues and is composed of 25–45% cellulose, 28–32% hemicellulose and 15–25% lignin [21]. Although cellulose has been studied for cellulosic ethanol production,

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http://dx.doi.org/10.1016/j.bbapap.2017.09.001
Received 15 March 2017; Received in revised form 31 August 2017; Accepted 4 September 2017
Available online 08 September 2017

hemicellulose is a complex polysaccharide and requires a different combination of hemicellulases for its degradation [47]. In sugarcane bagasse, the main hemicellulose is arabinoxylan, more specifically composed of a xylose backbone with β-(1 → 4) linkages branched with arabinofuranosyl and 4-*O*-methyl glucopyranosyl units [57]. L-Arabinose, present in hemicelluloses, is the second most abundant pentose sugar in plants and its liberation is of interest for hemicellulose deconstruction; L-arabinose also has important medical applications as it may inhibit intestinal sucrase activity delaying sucrose digestion [27].

Arabinases or arabinofuranosidases are the enzymes capable of liberating L-arabinose from polysaccharides, arabino-oligosaccharides or from synthetic substrates, with *endo* or *exo* modes of action. An important group of these enzymes corresponds to the α-L-arabinofuranosidases (E.C. 3.2.1.55) that cleave α-(1 → 2), α-(1 → 3), or α-(1 → 5) linked L-arabinofuranosyl residues from non-reducing ends in oligo- and polysaccharides that contain arabinose. These enzymes are classified into three types according to Beldman et al. [5]. Type-A preferentially hydrolyzes ρ-nitrophenyl-α-L-arabinofuranoside (ρNP-α-L-arabinofuranoside) and arabino-oligosaccharides. Type B acts on arabinoxylan, releasing xylose and L-arabinose. The third type is called 1 → 4-β-D-arabinoxylan arabinofuranohydrolase (AXH) that is highly specific for arabinosidic linkages in different arabinoxylans, with the release of L-arabinose. This third type is not active on several synthetic substrates, including ρNP-α-L-arabinofuranoside. α-L-Arabinofuranosidases are classified into the GH3, -43, -51, -54, and GH62 families. Interestingly, GH62 is the only family composed exclusively of arabinofuranosidases, and together with GH43, belong to the clan GH-F. Among the five families, GH62 is the least biochemically and structurally studied. Recently, an interesting review of arabinofuranosidases of family GH62 has been reported showing different aspects of these enzymes [67]. To date, there are 22 GH62 enzymes characterized according to the CAZy database, of which only 6 have their crystal structure resolved [31]. The GH62's structures from *Coprinopsis cinerea*, *Podospora anserine*, *Scytalidium thermophilum*, *Streptomyces coelicolor*, *Streptomyces thermoviolaceus* and *Ustilago maydis* [24,33,55,58,62] presented a five-bladed β-propeller fold, which is similar to many GH43 enzymes. The catalytic residues are conserved and are composed of the triad aspartate, aspartate, and glutamate [24].

Aspergillus is a very important source of different CAZymes with high potential for protein secretion [6]. Several α-L-arabinofuranosidases from different GH families have been detected in secretome studies of different *Aspergillus* species. Two GH62 enzymes (AN7908 and AN2632) have been detected in the secretome of *A. nidulans* cultivated on sorghum stover [50]. Arabinofuranosidases from family 62 has also been detected in the proteome of *Aspergillus fumigatus* grown on sawdust [2]. The secretome analysis of *Aspergillus niger* grown on sugarcane showed the presence of the arabinofuranosidases GH51 and GH62 [8]. *Aspergillus* is a genus capable of performing N-glycosylation of proteins which is one of the most important post-translational modifications since it can influence secretion [69], activity and stability [54], as well as other enzyme properties. This genus may be considered of great interest for the production of homologous and heterologous proteins [40].

Recently, the biochemical characterization of α-L-arabinofuranosidase from *A. nidulans* A773 (*An*Abf62A$_{wt}$, GH62) was reported [66]. However, the N-glycosylation and the structural aspects of the enzyme were not investigated. In this work, *An*Abf62A$_{wt}$ was highly secreted by *A. nidulans* grown on NaOH-pretreated sugarcane bagasse, which was detected by a glycoproteomics approach in a previous study [46]. The gene AN7908 was cloned and transformed in *A. nidulans* and in *E. coli* for *An*Abf62A production and the enzyme was structurally and functionally characterized. The influence of N-glycosylation on the secretion of *An*Abf62A by *A. nidulans* was also investigated, as well as the influence of Y312 on enzymatic activity and kinetic parameters.

2. Material and methods

2.1. Microorganisms and cultivation

All *E. coli* strains were maintained in Luria-Bertani (LB) medium. *A. nidulans* A773 (*pyrG89*;*wA3*;*pyroA4*) was maintained in minimal medium (MM) and 1% glucose (w/v), pH 6.5. The MM composition was described by Segato et al. [51].

2.2. Vectors and gene cloning

The gene AN7908, which encodes for the protein *An*Abf62A$_{wt}$, was amplified using the genomic DNA from *A. nidulans* A773 as the template and the primers F (5′- TATATACATATGCAGTGCGGTCTCCCGT-3′) and R (5′- TATAAAGCTTTTACTTCAGGGTGAGGAGCCC-3′), and cloned into the *NotI* and *XbaI* sites of the vector pEXPYR [51] for homologous expression by *A. nidulans* A773. *E. coli* DH5α was used for propagation of plasmids. To produce the enzyme in *E. coli*, AN7908 was amplified using genomic DNA from *A. nidulans* A773 as the template, since it did not have introns. The primers used had the same sequence as those used for cloning in pEXPYR, but instead with the *NheI* and *BamHI* sites. The gene was cloned into the pET28a(+) vector. *E. coli* Arctic was used for *An*Abf62A$_{deglyc}$ production.

2.3. Site-directed mutagenesis by overlap-extension PCR

For the glycomutant *An*Abf62A$_{N83Q}$, the amplified gene AN7908 was used as a template for PCR amplification. In addition to the two primers described in the previous item, two different primers (5′-CATGTCGGACCACTGCGAGAAGAGGCCGAAATTCATG-3′ and 5′-GGCCTCTTCTCGCAGTGGTCCGACATGGCC-3′) were used, substituting the codon N83 for Q83. The amplified fragments were used as templates for overlap extension PCR [23], what resulted in a single ORF. The glycomutant N83Q was further digested with restriction enzymes (*NotI* and *XbaI*) and cloned into the vector pEXPYR.

The mutants *An*Abf62A$_{Y312S}$ and *An*Abf62A$_{Y312F}$ were amplified using the plasmid pET28a(+) containing the gene AN7908 as the template. For amplification of the vector with the mutant Y312S, the primers 5′-CTCAACCCCGGCTCCGACCTTCTGCC-3′ and 5′-GGCAGAAGGTCGGAGCCGGGGTTGAG-3′ were used. For Y312F the primers 5′- CTCAACCCCGGCTTCGACCTTCTGCC-3′ and 5′-GGCAGAAGGTCGAAGCCGGGGTTGAG-3′ were used. The vectors with the mutants were treated with the KLD enzyme mix (New England Biolabs) to degrade methylated DNA and ligate the vector.

2.4. Transformation procedures

The DNA was transformed into calcium competent *E. coli* cells by heat shock. The colonies obtained were tested by colony PCR and the genetic material was extracted. For *An*Abf62A$_{deglyc}$ production by *E. coli* Arctic, the bacterium was cultivated in LB medium with 1 mg/mL of kanamycin and protein production induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and evaluated by SDS-PAGE.

The transformation procedures in *A. nidulans* A773 were carried out as described by Segato et al. [51]. The protoplasts were obtained and the exogenous DNA was introduced into the competent cells using polyethylene glycol (PEG) solution. For enzyme production, the spores of the transformants (10^7–10^8 de spores/mL) were inoculated in MM, 2% (w/v) maltose, 100 mM HEPES, pH 6.5 for 72 h at 37 °C. Crude protein extracts were quantified by the Bradford method [9].

2.5. Protein purification and identification

*An*Abf62A$_{wt}$ produced by *A. nidulans* A773 was purified as described by Damasio et al. [14] with modifications. *An*Abf62A$_{deglyc}$ produced in *E. coli* Arctic was purified as described by Calzado et al. [10]. Protein