Archives of Biochemistry and Biophysics 648 (2018) 1–11

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Structural and functional insights into RHA-P, a bacterial GH106 α-L-rhamnosidase from *Novosphingobium* sp. PP1Y

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ARTICLE INFO

Keywords:
Rhamnosidase
Flavonoid
Homology modeling
Site-directed mutagenesis
Novosphingobium sp. PP1Y

ABSTRACT

α-L-Rhamnosidases (α-RHAs, **EC 3.2.1.40**) are glycosyl hydrolases (GHs) hydrolyzing terminal α-L-rhamnose residues from different substrates such as heteropolysaccharides, glycosylated proteins and natural flavonoids. Although the possibility to hydrolyze rhamnose from natural flavonoids has boosted the use of these enzymes in several biotechnological applications over the past decades, to date only few bacterial rhamnosidases have been fully characterized and only one crystal structure of a rhamnosidase of the GH106 family has been described. In our previous work, an α-L-rhamnosidase belonging to this family, named RHA-P, was isolated from the marine microorganism *Novosphingobium* sp. PP1Y. The initial biochemical characterization highlighted the biotechnological potential of RHA-P for bioconversion applications. In this work, further functional and structural characterization of the enzyme is provided. The recombinant protein was obtained fused to a C-terminal His-tag and, starting from the periplasmic fractions of induced recombinant cells of *E. coli* strain BL21(DE3), was purified through a single step purification protocol. Homology modeling of RHA-P in combination with a site directed mutagenesis analysis confirmed the function of residues D503, E506, E644, likely located at the catalytic site of RHA-P. In addition, a kinetic characterization of the enzyme on natural flavonoids such as naringin, rutin, hesperidin and quercitrin was performed. RHA-P showed activity on all flavonoids tested, with a catalytic efficiency comparable or even higher than other bacterial α-RHAs described in literature. The results confirm that RHA-P is able to hydrolyze both α-1,2 and α-1,6 glycosidic linkages, and suggest that the enzyme may locate different polyphenolic aromatic moities in the active site.

1. Introduction

α-L-Rhamnosidases (EC 3.2.1.40) are glycosyl hydrolases (GHs) that cleave terminal α-L-rhamnose from a large number of natural products [1]. The action of α-L-rhamnosidases (α-RHAs) has been reported, among others, on different complex substrates such as heteropolysaccharides or glycosylated proteins containing rhamnose units (gellan gum, rhamnogalacturonan and arabinogalactan-proteins) [2–5]. Among α-RHAs substrates, natural flavonoids are gaining much interest among the food and nutraceutic industry. Natural flavonoids are polyphenolic compounds generally characterized by a three-ring structure, which consists of two phenyl rings (A and B) and a heterocyclic ring (C). These molecules are naturally produced in plants in glycosylated forms, showing the presence of either a rutinoside (6-α-L-rhamnosyl-β-D-glucoside) or a neohesperidoside (2-α-L-rhamnosyl-β-D-glucoside) disaccharidic unit bound in different positions. These molecules are very interesting due to their potential antioxidant, antitumor and anti-inflammatory properties [6,7]. In humans, endogenous β-glycosidases and α-L-arabinosidases in the small intestine are responsible for removing the glucose (or possibly arabinose or xylose) moiety from

Abbreviations: α-RHA, α-L-rhamnosidase; ABS, absorbance; BSA, bovin serum albumin; *E. coli*, *Escherichia coli*; GHs, glycosyl hydrolases; GTs, glycosyl transferases; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria Bertani; *N.* sp. PP1Y, *Novosphingobium* sp. PP1Y; OD, optical density; ORF, open reading frame; *p*NPR, *para*-nitrophenyl-α-L-rhamnopyranoside; RT, room temperature; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography

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https://doi.org/10.1016/j.abb.2018.04.013
Received 13 February 2018; Received in revised form 12 April 2018; Accepted 16 April 2018
Available online 18 April 2018
0003-9861/

flavonoids thus facilitating their absorption. These enzymes, however, are not able to cleave terminal rhamnose units, thereby limiting the bioavailability of rhamnosylated flavonoids that are converted to more bioactive forms by the colon microflora [8,9]. Therefore, enzymatic rhamnose removal from potentially bioactive flavonoids may be the key for improving their intestinal absorption and thus their beneficial properties for human health [10,11].

Due to their ability to hydrolyze rhamnose from natural flavonoids, α-RHAs are used in several biotechnological applications. Some examples include the hydrolysis of naringin to improve beverage quality by debittering grapefruit and citrus juices [12,13], and the removal of hesperidin crystals from orange-derived preparations [14]. Other applications of α-RHAs are gaining popularity in the oenological industry, where these enzymes are used to hydrolyze terpenyl glycosides to enhance aroma in wine, grape juices and derived beverages [15]. Moreover, an α-RHA has been implemented for the synthesis of rhamnose-containing chemicals by reverse hydrolysis, suggesting a yet unexplored potential of this enzymatic class in the chemical and pharmaceutical industry [16]. Despite their potential as industrial biocatalysts, to date only a limited number of bacterial rhamnosidases has been fully characterized [17–21]. The commercial preparations of α–L-rhamnosidases, naringinases and hesperidinases available and currently used in oenology, are all isolated from fungal sources such as *Aspergillus niger* and *Penicillium decumbens* [22–24]. The different operational parameters observed among the bacterial and fungal sources, with bacterial RHAs being more efficient at higher pH and temperature, suggest this class of α-RHAs to be an alternative source of biocatalysts to use for carbohydrates biotransformation at high pH values.

Few attempts have been made so far to engineer bacterial α-RHAs to unravel the molecular details underlying their catalytic mechanism, to modify their substrate specificity or to optimize their catalytic efficiency towards different substrates. A major obstacle is the limited number of α-RHAs crystal structures that are currently available among the different families of GH enzymes, which include the GH28, GH78, and GH106 families according to the CAZy database [25–27]. To the best of our knowledge only five crystal structures of bacterial α-L-rhamnosidases are currently available, four of which belong to the GH78 family, which show an $(\alpha/\alpha)_6$ 3D-structure, and include the putative α-L-rhamnosidase BT1001 from *Bacteroides thetaiotaomicron* VPI-5482 [28], the α-L-rhamnosidase B (BsRhaB) from *Bacillus* sp. GL1 [29], the α-L-rhamnosidase (SaRha78A) from *Streptomyces avermitilis* [30], and the α-L-rhamnosidase (KoRha) from *Klebsiella oxytoca* [31].

Among the members of the GH106 family, which groups 319 different sequences, a single 3D-structure has been reported, the BT0986 from *Bacteroides thetaiotaomicron* that shows a $(\beta/\alpha)_8$ barrel and catalyzes the hydrolysis of an α-L-rhamnopyranoside bound to the C2 position of an arabinofuranoside (L-Rha*p*-α-1,2-L-Ara*f*). In this same family of glycosidases only two enzymes have been characterized so far, and the reaction mechanism and the catalytic residues have been inferred from the 3D-structure of BT0986 [27].

In our previous work, a novel α-L-rhamnosidase was isolated from the marine microorganism *Novosphingobium* sp. PP1Y, a Gram-negative bacterium isolated from a polluted marine environment in the Pozzuoli harbor (Naples, Italy) [32–34]. The α-RHA from *N.* sp. PP1Y was isolated from the native bacterium, expressed in *E. coli* and partially characterized [35]. This enzyme, named RHA-P, was characterized as an *inverting* monomeric glycosidase of *ca.* 120 kDa belonging to the GH106 family. A preliminary biochemical characterization using the synthetic substrate *p*NPR (*para*-nitrophenyl-α-L-rhamnopyranoside) showed that RHA-P has moderate tolerance to organic solvents and optimal activity between pH 6.0–7.5 at 35–45 °C. Moreover, TLC analysis showed that RHA-P is able to hydrolyze rhamnose from natural flavonoids such as naringin, rutin and neohesperidin dihydrochalcone [35].

Here we extend our previous analysis on the functional and structural features of RHA-P, subcloning the enzyme with a C-terminal His-tag, improving the yield and purity of the recombinant protein, and making way for a detailed kinetic characterization of RHA-P activity on natural flavonoids. In addition, an homology model of RHA-P based on BT0986 3D-structure in combination with the characterization of the substrate specificity of both the wild type and mutant enzymes, revealed a new specificity for GH106 enzymes expanding the possible applicative field of this glycosidase family.

2. Materials and methods

2.1. General

Bacterial cultures, plasmid purifications and transformations were performed according to Sambrook et al. [36]. Bacterial growth was followed by measuring the optical density expressed as OD/mL at 600 nm (from now on defined as OD_{600}). When cells were centrifuged and the supernatant discarded, we indirectly referred to the wet weight of the collected cells as "total OD_{600}", a value obtained by multiplying the volume of cells suspension by the optical density.

The pET22b(+) expression vector and BL21(DE3) *E. coli* strain were from Amersham Biosciences, whereas the Top10 *E. coli* strain was purchased from Life Technologies.

The recombinant DNA polymerase used for PCR amplification was TAQ Polymerase from Microtech Research Products, and dNTPs were purchased from Promega.

The Wizard PCR Preps DNA purification system for elution of DNA fragments from agarose gels was from Promega. The QIAprep Spin Miniprep Kit for plasmid DNA purification was from QIAGEN. T4 DNA Ligase was from Promega, enzymes and other reagents for DNA manipulation were from New England Biolabs. Oligonucleotides were synthesized by MWG-Biotech (http://www.mwg-biotech.com).

Ni Sepharose 6 Fast Flow was obtained from GE Healthcare. Solvents used in enzymatic assays were obtained from Applichem (DMSO). Both chicken egg white lysozyme, and *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) were purchased from Sigma Aldrich, IPTG (isopropyl β-D-1-thiogalactopyranoside) was obtained from Applichem.

TLC silica gel plates were obtained from E. Merck (Darmstadt, Germany). Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich.

2.2. Construction of the pET22b(+)/rha-his expression vector

The *orf* PP1Y_RS05470, coding for the wild-type RHA-P protein, was previously cloned in pET22b(+) vector; the corresponding recombinant plasmid was named pET22b(+)/*rha-p* [35]. To obtain the pET22b(+)/*rha-his* plasmid, which expressed recombinant RHA-P with a C-terminal His-tag (from now on defined as RHA-Phis), our cloning strategy involved a single point mutation (TGA to CGA) to convert the stop codon to an arginine, and the extension of *rha-p* coding sequence on the pET22b(+) plasmid to include a linker sequence and the coding region of a 6-His-tag domain at 3′of the gene. The plasmid pET22b(+)/*rha-dw*, bearing only the C-terminal 1625 bp-long coding fragment of *rha-p* and already available in our laboratory [35], was chosen as template for the PCR procedure that allowed the insertion of the 6-His-tag at the 3′ of the gene. The use of pET22b(+)/*rha-dw* as template was useful to avoid the amplification of the entire *rha-p* gene, which is 3441 bp long. The mutagenesis experiment was performed using specific designed complimentary primers RHAmutUP (5′ACCACGGGCGGGCA TCGAGCCG**TCG**ACAAGC3′) and RHAmutDW (5′GCTTGT**CGA**CGGCTC GATGCCCGCCCGTGGT3′) containing the desired mutated codon (highlighted in bold). Quickchange II Site Directed Mutagenesis kit (Agilent technologies) was used for this experiment, following the manufacturer protocol. The mutation was verified by DNA sequencing. A fragment containing the mutated codon was identified between the single restriction sites *Aat*II - *Not*I. The cassette was excised from the pET22b(+)/*rha-dw* plasmid, purified from an agarose gel and