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RHA-P: Isolation, expression and characterization of a bacterial α-L-rhamnosidase from *Novosphingobium* sp. PP1Y

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ABSTRACT

α-L-Rhamnosidases (α-RHAs) are a group of glycosyl hydrolases of biotechnological potential in industrial processes, which catalyze the hydrolysis of α-L-rhamnose terminal residues from several natural compounds. A novel α–RHA activity was identified in the crude extract of *Novosphingobium* sp. PP1Y, a marine bacterium able to grow on a wide range of aromatic polycyclic compounds. In this work, this α-RHA activity was isolated from the native microorganism and the corresponding *orf* was identified in the completely sequenced and annotated genome of strain PP1Y. The coding gene was expressed in *Escherichia coli*, strain BL21(DE3), and the recombinant protein, rRHA-P, was purified and characterized as an inverting monomeric glycosidase of ca. 120 kDa belonging to the GH106 family. A biochemical characterization of this enzyme using *p*NPR as substrate was performed, which showed that rRHA-P had a moderate tolerance to organic solvents, a significant thermal stability up to 45 °C and a catalytic efficiency, at pH 6.9, significantly higher than other bacterial α-RHAs described in literature. Moreover, rRHA-P was able to hydrolyze natural glycosylated flavonoids (naringin, rutin, neohesperidin dihydrochalcone) containing α-L-rhamnose bound to β-D-glucose with either α-1,2 or α-1,6 glycosidic linkages. Data presented in this manuscript strongly support the potential use of RHA-P as a biocatalyst for diverse biotechnological applications.

1. Introduction

α-L-*R*hamnosidases (α-RHAs) are a group of glycosyl hydrolases (GHs) that catalyze the hydrolysis of terminal residues of α-L-rhamnose from a large number of natural compounds [1]. L-Rhamnose is widely distributed in plants as component of flavonoid glycosides, terpenyl glycosides, pigments, signaling molecules, and in cell walls as a component of complex heteropolysaccharides, such as rhamnogalacturonan and arabinogalactan-proteins [2–7]. In bacteria, L-rhamnose appears to be included in membrane rhamnolipids [8,9] and polysaccharides [10]. According to the similarities among their amino acidic sequences, α-RHAs are grouped in the CAZy (carbohydrate-active enzymes) database (www.cazy.org) into four different families: GH28, GH78, GH106, and NC (non-classified).

In the last decade, α-RHAs have attracted a great deal of attention due to their potential application as biocatalysts in a variety of industrial processes and in particular in the food industry [1]. Several dietary products are rich in glycosylated flavonoids that show the presence of either a rutinoside (6-α-L-rhamnosyl-β-D-glucose)

Abbreviations: α-RHAs, α-L-rhamnosidases; GHs, glycosyl hydrolases; GTs, glycosyl transferases; PPMM, potassium phosphate minimal medium; *p*NPR, *p*-nitrophenyl-α-L-rhamnopyranoside; MOPS, 3-(*N*-morpholino) propanesulfonic acid; *p*NP, *p*-nitrophenolate; BSA, bovine serum albumin; LB, Luria Bertani medium; LB-N, Luria Bertani medium containing a final concentration of 0.5 M NaCl; LB-BS, Luria Bertani medium supplemented with 1 mM of both betaine and sorbitol; LB-NBS, Luria Bertani medium containing a final concentration of 0.5 M NaCl and 1 mM of both betaine and sorbitol; IPTG, Isopropyl β-D-1-thiogalactopyranoside.

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or a neohesperidoside (2-α-L-rhamnosyl-β-D-glucose) disaccharidic unit. In particular, naringin, hesperidin and rutin, flavanone glycosides found in grapefruit juices, lemons, sweet oranges and vegetables, have gained increasing recognition for their potential antioxidant, antitumor and anti-inflammatory properties [11–14].

The ability to hydrolyze glycosylated flavonoids has been used to mitigate the bitterness of citrus juices, which is primarily caused by naringin. Rhamnose removal from naringin allows softening the bitter taste of citrus juice [15,16]. Moreover, the corresponding de-rhamnosylated compound, prunin, is endowed with antimicrobial properties [14], and shows an improved intestinal assimilation when compared to naringin. Other applications of α-RHAs are gaining popularity in the oenological industry, where these enzymes are used to hydrolyze terpenyl glycosides and enhance aroma in wine, grape juices and derived beverages [17–19].

Application of α-RHAs to improve flavonoids bioavailability has also been recently described [20]. In humans, flavonoids absorption occurs primarily in the small intestine where the attached glucose (or possibly arabinose or xylose) is removed by endogenous β-glucosidases [21–23]. Terminal rhamnose is not a suitable substrate for human β-glucosidases. Therefore, unabsorbed rhamnosylated flavonoids arrive unmodified in the colon, where they are hydrolyzed by α-rhamnosidase activities expressed by the local microflora [24]. To improve intestinal absorption of rhamnosylated flavonoids, and thus their bioavailability in humans, a removal of the terminal rhamnose group catalyzed by α-RHAs would be indeed beneficial [25–27].

The absence of human α-RHAs has been the key to the development of a novel targeted drug delivery strategy, indicated as LEAPT (Lectin-directed enzyme activated prodrug therapy) [28,29], a bipartite system based on the internalization of an engineered α-RHA bearing a glycosidic moiety that is recognized by specific lectins present on the surface of different eukaryotic cell lines. In the LEAPT system, the intake of a rhamnosylated prodrug, which cannot be processed by mammalian enzymes, allows a site-selective action of the drug in cells where the engineered α-RHA has been prelocalized.

To date, microbial α-RHAs have been mainly purified from fungal strains such as *Penicillium* and *Aspergillus* [30–32]; only one example of α-RHA isolated from a viral source has been described [33], and it is noteworthy that only a limited number of bacterial rhamnosidases has been fully characterized [34–39]. One of the main differences between fungal and bacterial α-RHAs is their different optimal pH values, with the fungal enzymes showing more acidic pH optima when compared to the bacterial counterparts, for which neutral and alkaline values have generally been described. This characteristic suggests diverse applications for fungal and bacterial enzymes, making bacterial α-RHAs suitable in biotechnological processes requiring good activity in more basic solutions such as, for example, the production of L-rhamnose from the hydrolysis of naringin or hesperidin, flavonoids whose solubility strongly increases at higher pH values [40]. In addition, bacterial rhamnosidases could be ideal candidates for dietary supplements having activity across the entire gastrointestinal (GI) tract, and more specifically in the small intestine where flavonoids absorption should mostly occur to enhance the beneficial effect of these molecules on human health.

In order to elucidate the real biotechnological potential of bacterial α-RHAs, further investigation is undoubtedly needed. Few details on the catalytic mechanism of bacterial α-RHAs are available, and most importantly, to the best of our knowledge, no attempt to improve the catalytic efficiency or modify substrate specificity of these enzymes by mutagenesis has been performed yet. This is in part consequential to the fact that only a very limited number of crystal structures of α-RHAs are currently available, such as the α-L-rhamnosidase B (BsRhaB) from *Bacillus* sp. GL1 [41], and the α-L-rhamnosidase from *Streptomyces avermitilis* [42]. Therefore, it is evident that bacterial α-RHAs represent a yet unexplored reservoir of potential biocatalysts for which more functional and structural data are required.

A member of the order of the Sphingomonadales, recently isolated and microbiologically characterized, *Novosphingobium* sp. PP1Y [43,44], appears to be a valuable source for the isolation of α-RHA activities. Sphingomonadales are a group of Gram-negative α-proteobacteria whose genomes show the presence of a great abundance of both glycosyl hydrolases (GHs) and glycosyltransferases (GTs). These activities are probably involved in the biosynthesis of complex extracellular polysaccharides and microbial biofilms [45]. The interest for *Novosphingobium* sp. PP1Y carbohydrate-active enzymes has grown as the sequencing and annotation of the whole genome, recently completed, allowed the identification of a great number of genes encoding for both GHs (53 *orfs*) and GTs (57 *orfs*) [46].

Recently, a α-RHA activity in *Novosphingobium* sp. PP1Y crude extract was described, which showed an alkaline pH optimum and a moderate tolerance to organic solvents [47]. *Novosphingobium* sp. PP1Y crude extract expressing this enzymatic activity was used for the bioconversion of naringin, rutin and hesperidin. Based on these preliminary results, a more detailed biochemical characterization of the α-RHA activity was essential.

In this work, the isolation, recombinant expression and partial characterization of a α-RHA from *Novosphingobium* sp. PP1Y is reported. This enzyme, named RHA-P, belongs to the GH106 family [48], a subgroup for which, according to our knowledge, no crystal structure is available yet. As evident from our analyses, RHA-P is a promising candidate for several biotechnological applications.

2. Materials and methods

2.1. Generals

General molecular biology techniques were performed according to Sambrook et al. [49]. Bacterial growth was followed by measuring the optical density at 600 nm (OD_{600}). pET22b(+) expression vector and *E. coli* strain BL21(DE3) were from Amersham Biosciences; *E. coli* strain Top10 was purchased from Life Technologies. *N.* sp. PP1Y was isolated from polluted seawater in the harbor of Pozzuoli (Naples, Italy) as previously described [43].

The thermostable recombinant DNA polymerase used for PCR amplification was TAQ Polymerase from Microtech Research Products. dNTPs, T4 DNA ligase, and the Wizard PCR Preps DNA purification system for elution of DNA fragments from agarose gels were purchased from Promega. The QIAprep Spin Miniprep Kit for plasmid DNA purification was from QIAGEN. Enzymes and other reagents for DNA manipulation were from New England Biolabs. Oligonucleotides synthesis and DNA sequencing were performed by MWG-Biotech. N-terminus of rRHA-P was sequenced by Proteome Factory AG. The presence and location of a potential signal peptide cleavage site on the amino acidic sequence of RHA-P was analyzed using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP).

Q-Sepharose Fast Flow and *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) were from Sigma Aldrich; Sephacryl S200 High Resolution was purchased from Amersham Biosciences. IPTG (isopropyl β-D-1-thiogalactopyranoside) was obtained from Applichem.

Solvents used in enzymatic assays were either from Applichem (DMSO) or from Romil (acetone). TLC silica gel plates were from E. Merck (Darmstadt, Germany).

2.2. Growth of Novosphingobium sp. PP1Y cells

N. sp. PP1Y cells were grown in Potassium Phosphate Minimal Medium (PPMM) at 30 °C for 28 h under orbital shaking at 220 rpm. A pre-inoculum in LB was prepared by transferring 50 μL from a glycerol stock stored at −80 °C to a 50 mL Falcon tube containing 12.5 mL of sterile LB. The pre-inoculum was allowed to grow at 30 °C O/N under orbital shaking and then used to inoculate 1 L of PPMM at an initial cell concentration of 0.01- 0.02 OD_{600}. 0.3 mM naringine was added to the medium and used as an inducer of the α-RHA activity, as previously described [47].

2.3. Cloning of orfPP1Y_RS05470 and construction of the pET22b(+)/rha-p expression vector

Genomic DNA was extracted from a 50 mL saturated culture of *N.* sp. PP1Y as described elsewhere [44]. *Orf*PP1Y_RS05470 coding for the α-RHA activity was amplified in two contiguous fragments, owing to the considerable length of the *orf* (3441 bp). The first fragment, named *rha-up* (1816 bp), was amplified using an internal downstream primer, RHA-Intdw (5′-AGGCGGCCATGGGAATGT-3′), which included an internal *Nco*I site already present in *orf*PP1Y_RS05470, and an upstream primer, RHA-up (5′-GGGAATTCCATATGCCGCGCCTTTCGCT-3′), designed to add a *Nde*I restriction site at 5′ of *orf*PP1Y_RS05470. The second half of the gene, named *rha-dw* (1625 bp), was amplified using the upstream primer RHA-Intup (5′-ACATTCCCATGGCCGCCT-3′), complementary to RHA-Intdw, and the downstream primer RHA-dw (5′-AAAACCGAGCTCTCAATGCCCGCCCGTG-3′) that was intended to incorporate a *Sac*I restriction site downstream of the amplified *orf*.

The amplified fragments, *rha-up* and *rha-dw*, were purified from agarose gel, digested, respectively, with *Nde*I/*Nco*I and *Nco*I/*Sac*I, and individually cloned in pET22b(+) vector previously cut with the same enzymes.

Ligated vectors were used to transform *E. coli*, strain Top10, competent cells. The resulting recombinant plasmids, named pET22b(+)/*rha-up* and pET22b(+)/*rha-dw*, were verified by DNA sequencing. Next, the construction of complete *rha-p* gene in pET22b(+) was performed. First, both pET22b(+)/*rha-dw* and pET22b(+)/*rha-up* were digested with *Nco*I/*Sac*I restriction endonucleases to obtain, respectively, fragment *rha-dw* and linearized pET22b(+)/*rha-up*.

Digestion products were purified from agarose gel electrophoresis, eluted and ligated. Ligation products were used to transform *E. coli* Top10 competent cells and the resulting recombinant plasmid, named pET22b(+)/*rha-p* was verified by DNA sequencing.

2.4. α-L-Rhamnosidase recombinant expression

Protein expression was carried out in *E. coli* BL21(DE3) strain transformed with pET22b(+)/*rha-p* plasmid.

All the media described in this paragraph contained 100 μg/mL of ampicillin.

2.4.1. Analytical expression

E. coli BL21(DE3) competent cells transformed with plasmid pET22b (+)/*rha-p* were inoculated in a sterile 50 mL Falcon tube containing 12.5 mL of either LB [50] or LB containing a final concentration of 0.5 M NaCl (LB-N). Cells were grown under constant shaking at 37 °C up to 0.6–0.7 OD_{600}. This preinoculum was diluted 1:100 in 12.5 mL of either one of the four following media: LB, LB-N, LB supplemented with 1 mM of both betaine and sorbitol (LB-BS), or LB containing a final concentration of 0.5 M NaCl and 1 mM of both betaine and sorbitol (LB-NBS). Cells were grown under constant shaking at 37 °C up to 0.7–0.8 OD_{600}. RHA-P recombinant expression was induced with 0.1 mM IPTG at either 23 °C or 37 °C; growth was continued in constant shaking for 3 h. Cells were collected by centrifugation (5,524 × *g* for 15 min at 4 °C) and suspended in 25 mM MOPS pH 6.9 at a final concentration of 14 OD_{600}. Cells were disrupted by sonication (12 times for a 1-min cycle, on ice) and an aliquot of each lysate was centrifuged at 22,100 × *g* for 10 min at 4 °C. Both soluble and insoluble fractions were analyzed by SDS-PAGE. The soluble fraction was assayed for the presence of α-RHA enzymatic activity.

2.4.2. Large scale expression

Fresh transformed cells were inoculated into 10 mL of LB-N and incubated in constant shaking at 37 °C O/N. The preinoculum was diluted 1:100 in four 2 L Erlenmeyer flasks containing each 500 mL of LB-NBS and incubated in constant shaking at 37 °C up to 0.7–0.8 OD_{600}.

Expression of the recombinant protein, named rRHA-P, was induced with 0.1 mM IPTG and growth was continued for 3 h at 23 °C. Cells were collected by centrifugation (5,524 × *g* for 15 min at 4 °C) and stored at −80 °C until needed.

2.5. Native and recombinant α-L-rhamnosidase purification

Both native and recombinant RHA-P were purified following three chromatographic steps. Cell paste was suspended in 25 mM MOPS pH 6.9, 5% glycerol (buffer A), at a final concentration of 100 OD_{600} and cells were disrupted by sonication (10 times for a 1-min cycle, on ice). Cell debris were removed by centrifugation at 22,100 × *g* for 60 min at 4 °C and the supernatant was collected and filtered through a 0.45 μm PVDF Millipore membrane.

Afterwards, cell extract was loaded onto a Q Sepharose FF column (30 mL) equilibrated in buffer A. The column was washed with 50 mL of buffer A, after which bound proteins were eluted by using a 300 mL linear gradient of buffer A from 0 to 0.4 M NaCl at a flow rate of 15 mL/h. The chromatogram was obtained by analyzing fractions absorbance at λ = 280 nm and the presence of the recombinant α-RHA activity was detected using the *p*NPR assay. Relevant fractions were analyzed by SDS-PAGE, pooled and concentrated at a final volume of ∼ 0.5 mL using a 30 kDa Amicon ultra membrane, Millipore. The sample was then loaded onto a Sephacryl HR S200 equilibrated with buffer A containing 0.2 M NaCl (buffer B).

Proteins were eluted from the gel filtration column with 250 mL of buffer B at a flow rate of 12 mL/h. Fractions were collected, analyzed and screened for the presence of α-RHA activity as previously described. At this stage, NaCl was removed from pooled fractions by repeated cycles of ultrafiltration and dilution with buffer A. The sample was then loaded on a Q Sepharose FF column (30 mL) equilibrated in buffer A. The column was washed with 50 mL of buffer A, after which bound proteins were eluted with 300 mL of a linear gradient of buffer A from 0 to 0.25 M NaCl at a flow rate of 13 mL/h. Fractions were collected, analyzed by SDS-PAGE and screened for the presence of α-RHA activity. Relevant fractions were pooled, concentrated, purged with nitrogen, and stored at −80 °C until use.

2.6. Analytical gel filtration

Analytical gel-filtration experiments were carried out as follows: 100 μL of a 40 pM protein sample was loaded on a Superdex 200 HR 10/300 column previously equilibrated in buffer B, installed on an AKTA™FPLC™ (GE Healthcare Life Science). Samples were eluted isocratically at RT at a flow rate of 0.5 mL/min. Protein elution was monitored at λ = 280 nm. A molecular weight calibration was performed in the same buffer with the following proteins of known molecular weight: β-amylase (200 kDa), glyceraldehyde-3-phosphate dehydrogenase (143 kDa), carbonic anhydrase (29 kDa).

2.7. Enzyme activity assays

α-RHA activity was determined using *p*NPR as substrate (*p*NPR assay). Otherwise stated, all activity assays were performed at RT. The reaction mixture contained, in a final volume of 0.5 mL of 50 mM MOPS pH 6.9, *p*NPR at a final concentration of 600 μM and variable amounts of the sample tested. The reaction was blocked after 10 and 20 min by adding 0.5 M Na_2CO_3; the product, *p*-nitrophenolate (*p*NP), was detected spectrophotometrically at λ = 405 nm. The extinction coefficient used was $\varepsilon_{405} = 18.2\ mM^{-1}\ cm^{-1}$. One unit of enzyme activity was defined as the amount of the enzyme that releases one micromole of *p*NP per min.

2.7.1. Kinetic parameters determination

Kinetic parameters were obtained at pH 6.9 using a *p*NPR concentration in the range 0.025–1 mM. All kinetic parameters were determined by a non-linear regression curve using GraphPad Prism (GraphPad Software; www.graphpad.com).

2.7.2. pH optimum

pH optimum for α-RHA activity was determined in the range 4.7–8.8. Enzyme assays were performed as described above, using the following buffers: 50 mM potassium acetate (pH 4.7–5.7), 50 mM MOPS/NaOH (pH 5.7–7.7) and 50 mM Tris/HCl (pH 7.7–8.8).

2.7.3. Temperature optimum and stability

Optimum temperature was evaluated by performing the standard *p*NPR assay and incubating the reaction mixture at different temperatures, in the range 25–55 °C.

The thermal stability of the enzyme was determined by incubating the enzyme for one and 3 h at 30, 40, 50, 60 °C and measuring, after each incubation, the residual specific activity.

2.7.4. Organic solvents tolerance

The tolerance of the enzymatic activity to the presence of organic solvents in the reaction mixture, such as DMSO, acetone or ethanol, was evaluated by performing the standard *p*NPR assay in 50 mM MOPS pH 6.9 to which either 10% or 50% of solvent was added.

2.8. Substrate specificity

Reactions were carried out in 0.6 mL of 50 mM Na-phosphate buffer pH 7.0 under magnetic stirring at 40 °C in the presence of 20 mM of either aryl glycoside and 0.25 U of rRHA-P. Reactions were monitored over time (0–24 h) by TLC analysis (system solvent: EtOAc:MeOH:H_2O 70:20:10). Compounds on TLC plates were visualized under UV light or charring with α-naphthol reagent.

Hydrolysis reactions of maltose, pullulan, starch, amylopectin, sucrose, raffinose, lactose, xylan from birchwood, xylan from oat spelt, hyaluronic acid, α – cellulose, cellobiose, chitosan, β-Glucan from barley, laminarin, curdlan, fucoidan from *Fucus versiculosus*, rhamnogalacturonan, rutinose were performed using 2.5 mg of each substrate, which was suspended in 0.5 mL of 50 mM Na-phosphate buffer pH 7.0. The reaction was carried out at 40 °C under magnetic stirring, in the presence of 0.25 U of rRHA-P. Hydrolysis products were monitored by TLC analysis (system solvent HCOOH:HAc:H_2O:2-propanol: EtOAc:1:10:15:5:25).

Flavonoidic compounds such as naringin, rutin, neohesperidin dihydrochalcone, were screened as possible substrates. In this case, a 6 mM solution of each compound in a final volume of 1 mL of 50 mM Na-phosphate buffer pH 7 was incubated at 40 °C in the presence of 0.25 U of rRHA-P. Reactions were checked over time by TLC analysis (t = 0, 15′, 30′, 60′, 90′, 120′, 150′, 180′, 24 h) with the following solvent system: EtOAc:MeOH:H_2O 70:20:10. An additional hydrolysis reaction of naringin was performed in conditions similar to those reported above in the presence of 10% DMSO, and the reaction was monitored over time.

In all experiments, TLC standard solutions of pure reagents and products were used for comparison.

2.9. Mass spectrometry analysis

Identification of native and recombinant RHA-P by mass spectrometry was performed by enzymatic digestion, either in solution or *in situ* after separation by SDS-PAGE.

When digesting in solution, the protein sample was lyophilized and then dissolved in denaturing buffer (300 mM Tris/HCl pH 8.8, 6 M urea, 10 mM EDTA). Cysteines were reduced with 10 mM dithiothreitol in denaturing buffer at 37 °C for 2 h, and then carbamidomethylated with 55 mM iodoacetamide dissolved in the same buffer at RT for 30 min, in the dark. Protein sample was desalted by size exclusion chromatography on a Sephadex G–25 M column (GE Healthcare, Uppsala, Sweden). Fractions containing RHA-P were lyophilized and then dissolved in 10 mM NH_4HCO_3 buffer (pH 8.0). Enzyme digestion was performed using proteases with different specificity such as trypsin, chymotrypsin and endoproteinase GluC (V8-DE) (Sigma), using a protease/RHA-P ratio of 1:50 (w/w) at 37 °C for 16 h.

For the enzymatic digestion *in situ*, Coomassie blue stained bands excised from polyacrylamide gels were destained by several washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile. Cysteines were reduced for 45 min in 100 μL of 0.1 M NH_4HCO_3, 10 mM dithiothreitol, pH 7.5. Carbamidomethylation of thiols was achieved after 30 min in the dark by adding 100 μL of 55 mM iodoacetamide dissolved in the same buffer. Enzymatic digestion was carried out on different bands of RHA-P by using 100 ng of proteases with different specificity (same as above) in 10 mM NH_4HCO_3 buffer, pH 7.5. Samples were incubated for 2 h at 4 °C. Afterwards, the enzyme solution was removed and a fresh protease solution was added; samples were incubated for 18 h at 37 °C. Peptides were extracted by washing the gel spots/bands with acetonitrile and 0.1% formic acid at RT, and were filtered using 0.22 um PVDF filters (Millipore) following the recommended procedures.

Peptide mixtures were analyzed either by matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS) or capillary liquid chromatography with tandem mass spectrometry detection (LC–MS/MS).

MALDI-MS experiments were performed on a 4800 Plus MALDI TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). The peptide mixture (1 μL) was diluted (1:1, v/v) in acetonitrile/50 mM citrate buffer (70:30 v/v) containing 10 mg/mL of α-cyano-4-hydroxycinnamic acid. Mass calibration was performed using external peptide standards purchased from Applied Biosystems.

Spectra were acquired using a mass (*m*/*z*) range of 300–4000 amu and raw data were analyzed using Data Explorer Software provided by the manufacturer. Experimental mass values were compared with calculated masses derived from an *in silico* digestion of RHA-P obtained with different proteases, using MS-Digest, a proteomics tool from Protein Prospector software (prospector.ucsf.edu/).

Peptide mixtures were analyzed by nanoLC–MS/MS on a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, Ca). After sample loading, the peptide mixture was first concentrated and then washed at 4 nL/min in a 40 nL enrichment column (Agilent Technologies chip) with 2% acetonitrile + 0.1% formic acid.

The sample was then fractionated on a C18 reverse-phase capillary column at a flow rate of 400 nL/min by using a two-solvents

system consisting of 2% acetonitrile + 0.1% formic acid (solvent A) and 95% acetonitrile + 0.1% formic acid (solvent B). Separation was carried out with a 50 min linear gradient from 5 to 60% of solvent B.

Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 2,000 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan.

MS/MS spectra were acquired automatically when the MS signal detected upraised the threshold of 50,000 counts. Double and triple charged ions were isolated and fragmented. The acquired MS/MS spectra were transformed in Mascot Generic format (.mgf) and used to query either a NCBInr 02-2015 (21,322,359,704 amino acid sequences; 10,835,265,410 residues) or a *N.* sp. PP1Y (4635 aminoacid sequences; 1,493,994 residues) sequence database.

A licensed version (2.4.0.) of MASCOT software (www.matrixscience.com) was used with the following search parameters: trypsin, chymotrypsin or GluC endoproteinase as enzyme; 3 or 2 as allowed number of missed cleavages; carbamidomethylation of cysteines as fixed modification; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from +2 to +3. Oxidation of methionine and formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides were considered as variable modifications. Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions score >14 indicate identity or extensive homology ($p < 0.05$). Protein scores derive from ion scores as a non-probabilistic basis for ranking protein hits. Individual ion score threshold provided by MASCOT software, necessary to evaluate the quality of matches in MS/MS data and to calculate protein score, was 52 in NCBInr database and 14 in PP1Y Database. In Table 1 only peptides with ion score ≥ 10 are reported.

2.10. NMR Analysis

All the experiments were performed at 298 K on a Bruker 600 MHz DRX spectrometer equipped with a cryo probe. *p*NPR was dissolved in D_2O to a final concentration of 3 mM in a 5-mm NMR tube. The ligand proton resonances were assigned by a combination of 1D and 2D NMR experiments including COSY, TOCSY and HSQC. ^{1}H NMR spectra were acquired with 32 k data points. Double quantum-filtered phase sensitive COSY and TOCSY experiments were performed by using data sets of 2096×256 (t1 x t2) points. Total Correlation Spectroscopy (TOCSY) spectrum was recorded with a spin lock time of 100 ms. Heteronuclear single quantum coherence (HSQC) experiment was measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, by using data sets of 2048×256 points. Experiments were carried out in the phase-sensitive mode according to the method of States and coworkers [51]. In all homonuclear spectra the data matrix was zero-filled in the F1 dimension to give a matrix of 4096×2048 points and resolution was enhanced in both dimensions by a cosine-bell function before Fourier transformation.

After the acquisition of the initial spectra of the ligand alone, rRHA-P was added to a final concentration of ~1 μM (~60 μg) in the NMR tube from a ~5.9 μM solution in 25 mM MOPS containing 5% glycerol. The NMR sample was immediately placed in the spectrometer probe to record experiments on the mixture.

Data acquisition and processing were performed with TOPSPIN 2.1 software.

2.10. Analytical methods

Protein concentration was measured using the Bio-Rad Protein System [52] using bovine serum albumin (BSA) as standard. Polyacrylamide gel electrophoresis was carried out using standard techniques [53]. SDS–PAGE 15% Tris–glycine gels were run under denaturing conditions and proteins were stained with Coomassie brilliant blue G-250. "Wide range" (200–6.5 kDa) molecular weight standard was from Sigma (ColorBurst™ Electrophoresis Marker).

3. Results

3.1. Isolation and identification of a α-RHA from Novosphingobium sp. PP1Y

In a previous work [47], a α-RHA activity was detected in the crude extract of *N.* sp. PP1Y grown in minimal medium and in the presence of 0.3 mM naringin. In order to identify and characterize this enzymatic activity, *N.* sp. PP1Y cells were grown under the same experimental conditions described by the authors [47]. Cells were collected by centrifugation after 24 h of growth in PPMM (optical density of ~1 OD_{600}) at 5,524 × *g* for 15 min at 4 °C. α-RHA purification was carried out following the procedures described in this work in Materials and Methods. In all three chromatographic steps used for the purification of *N.* sp. PP1Y crude extract, a unique peak of α-RHA activity was always detected, but no major protein band was ever evident from the SDS-PAGE analysis of the corresponding active fractions (data not shown).

Peak fractions obtained from the first two chromatographic steps were digested with trypsin and analyzed by LC–MS/MS analysis. Raw data were used to search NCBI database (Bacteria as taxonomy restriction) with the MS/MS ion search program of Mascot software. Among the identified proteins, a member of the glycoside hydrolase family (*Novosphingobium* sp. PP1Y:WP_013837086.1) was identified with 10 (20% of sequence coverage) and 32 peptides (46% of sequence coverage) in the first and in the second chromatographic step, respectively (data not shown). The putative MW, deduced from the amino acid sequence, was of 124,225 Da.

Peak fractions obtained from the last chromatographic step (Q-sepharose) showed the presence of a relatively limited number of protein bands (Fig.S1, Supplementary material). Five bands migrating as expected for proteins of MW higher than 100 kDa were selected and excised from the polyacrylamide gel stained with Colloidal Coomassie Blue. Bands were digested *in situ* with trypsin, and the resulting peptides mixture was analyzed by LC–MS/MS. Raw data were used to search NCBI database (Bacteria as taxonomy restriction) with MASCOT software.

One of the protein bands analyzed, indicated in Fig. S1 by a red arrow, was identified (52 peptides, 71% sequence coverage, Table 1) as the protein encoded by the *orf*PP1Y_RS05470; this latter is located in one of the extrachromosomal elements of *N.* sp. PP1Y, the Megaplasmid referred to as Mpl [44]. This protein, from now on indicated as RHA-P, had been previously annotated as a member of the GHs family and is composed by 1,146 aa for a calculated molecular weight of about 124,225 Da.

3.2. Cloning, recombinant expression and purification of recombinant RHA-P

The recombinant plasmid pET22b(+)/*rha-p* (Materials and Methods), in which *orf* PP1Y_RS05470 was cloned, was used to transform *E. coli* BL21(DE3) strain. First attempts of expression of the recombinant protein at 37 °C showed the presence of a protein band with the expected molecular weight of recombinant RHA-P (rRHA-P) almost exclusively in the insoluble fraction of the induced culture (Fig. S2). As evident from Fig. S2 of the Supplementary material, when *E. coli* was instead induced at 23 °C [54], the presence of a protein band with the expected MW for rRHA-P in the soluble fraction (lane 5) appeared to be markedly increased when compared to the soluble fraction obtained after an induction performed

Table 1
Identification of native RHA-P by *in situ* digestion followed by LC–MS/MS analysis.

Protein [Species] NCBInr Accession number	Mass	Protein score	Sequence coverage (%)	n° of peptides	Ion score[a] and peptide sequence
Glycoside hydrolase family protein [*Novosphingobium* sp. PP1Y] gi|334145605|ref| WP_013837086.1|	124,225	2318	71	52	34 R.TGDAIK.A 31 R.VGGFYGK.G 38 R.GVVQNEK.R 28 R.LDNELVR.G 21 R.GPEIEPVK.A 11 R.VLPITGRK.A 19KVGSKNEPR.G 37 R.AVAEAAFDK.V 42 R.GVVQNEKR.Q 48KLSSPNAMALR.A 13KLSSPNAMALR.A+Oxidation (M) 37 R.DITDQVPVLR.L 13 R.ITVVGDITMDR.L 41 R.ITVVGDITMDR.L+Oxidation (M) 32 R.LGRPEDYYVK.L 17KAPIVSVTLYYK.V 24KVEKWFGQIPR.G 55KVGLHILGGLASSR.L 52 R.VKAQAEAELAQK.L 61 R.LDATAIAEAQER.L 35 R.TFVVSAPVQADR.T 28 R.MGWLLPAVTQDK.L 15 R.MGWLLPAVTQDK.L+Oxidation (M) 27 R.KAPIVSVTLYYK.V 52 R.DLAAMAALTPAQVK.A 70 R.DLAAMAALTPAQVK.A+Oxidation (M) 74 R.AVVSTLSGEIGGLER.V 32 R.LKPMLEQAFGAWK.A 31 R.LKPMLEQAFGAWK.A+Oxidation (M) 74KGATLAEGQVYSDDPAK.Y 28 R.RAVVSTLSGEIGGLER.V 50KFVLANGLTTIVHTDR.K 18KSAAPVKPLGAAVPAAQGR.I 23 R.NWTAPGINDPDTPALK.V 37 R.MGWLLPAVTQDKLDK.Q 33 R.MGWLLPAVTQDKLDK.Q+Oxidation (M) 47 R.AVAEAAFDKVLADYLR.D 65 R.ALPNQFETNAAVQAAIR.K 25KGWSYGVYSSVTQPTGPR.T 29KWMSRPVYALNVVPGPR.T 26KWMSRPVYALNVVPGPR.T+Oxidation (M) 20 R.TFVVSAPVQADRTGDAIK.A 18KGATLAEGQVYSDDPAKYK.R 36 R.IVLIDRPNSPQSVIMAGR.V 43 R.IVLIDRPNSPQSVIMAGR.V+Oxidation (M) 58KADTEALGLANDVLGGGFLSR.L 47 R.GPEIEPVKAGPVTLPAPLSR.D 55 R.HATIGSMADLDAATLTDVRK.W 56 R.HATIGSMADLDAATLTDVRK.W+Oxidation (M) 21 R.EEKGWSYGVYSSVTQPTGPR.T 46KAIIADVGAFPGQKPITQEELQR.V 70 R.TNYVETVPTGALDLALFMESDR.M 50 R.AIEASANEFLRPGGMTYVVVGDR.K 59 R.AIEASANEFLRPGGMTYVVVGDR.K+Oxidation (M) 65 R.AIEASANEFLRPGGMTYVVVGDRK.I 54 R.QGDNQPYGLFDYAQADGLLPVGHPYR.H+ Gln->pyro-Glu (N-term Q) 71 R.QGDNQPYGLFDYAQADGLLPVGHPYR.H 23 R.LNKDLREEKGWSYGVYSSVTQPTGPR.T 38KWFTEHYGPNNVVLVLSGDIDAATARPK.V 35KIVEPQLEGLGLPIDVQQAPQAAADDQSVE.- 55 R.KIVEPQLEGLGLPIDVQQAPQAAADDQSVE.- 40 R.ALGSTLFGQHPYAQPTDGLGNAASLAALTPAALR.A

[a] In the table only peptides with ion score ≥10 are reported.

at 37 °C (lane 3). Neither the use of different IPTG concentrations nor the variation of the induction time seemed to improve rRHA-P solubility.

To increase the yield of active rRHA-P in the soluble fraction of cell cultures induced at 23 °C, a novel set of analytical expression experiments using either LB or LB-N (Materials and Methods) supplemented with 1 mM of both betaine and sorbitol (two osmolytes), were performed. Data in literature suggest that the addition of these molecules to the growth medium of induced recombinant cells of *E. coli* might increase protein solubility. This effect should be improved by the co-presence in the medium of a high salt concentration (LB-N medium), which might ***i)*** increase the uptake rate in the cytoplasm of betaine and sorbitol from the extracellular

Table 2
Purification Table of rRHA-P.

	Total Units[b]	Specific Activity (Units/mg)[b]	P.F.[a]	Yield (%)
Cell lysate	13,728.8	49.8		
I Q-Sepharose	5,939.6	54.7	1.1	43.3
Gel filtration	1,370.6	66.1	1.3	9.9
II Q-Sepharose	920.9	346.9	6.9	6.7

[a] Purification Factor.
[b] Standard deviations, expressed as percentage errors, were always within 20%.

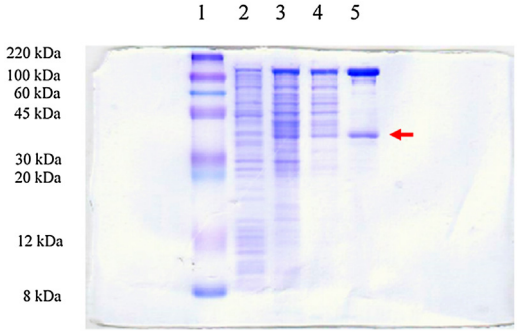

Fig. 1. SDS-PAGE analysis of rRHA-P purification steps. Lane 1: MW standard. Lane 2: cell lysate of *E. coli* strain BL21(DE3). Lane 3: peak fraction after the first Q-sepharose. Lane 4: peak fraction after S200-gel filtration chromatography. Lane 5: purified rRHA-P; the red arrow in this line indicates the contaminant protein found at the end of purification. For each sample an amount of ca. 4 μg of total proteins was loaded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environment [55], and/or ***ii)*** induce the expression of additional heat-shock proteins assisting in protein folding [56].

For these experiments, IPTG concentration and induction time were respectively 0.1 mM and 3 h. Activity assays showed that the best expression of active rRHA-P was obtained inducing the cultures at 23 °C and using LB-NBS (LB-N to which 1 mM of both betaine and sorbitol were added) as growth medium. In this case, specific activity of cell extract was 46 times higher than what obtained with the soluble fraction of recombinant cells grown in LB and induced at 37 °C.

Large-scale recombinant expression was performed by using the optimized conditions suggested by the analytical experiments.

rRHA-P purification was carried out following three different chromatographic steps. The purification table (Table 2) showed a final purification factor (PF) of 6.9. SDS-PAGE analysis of peak fractions representative of each purification step (Fig. 1) showed in the final purified sample of rRHA-P the additional presence of a major contaminant protein with an approximate MW of ~35 kDa.

The 35 kDa protein band was excised, digested *in situ* with trypsin and analyzed by LC–MS/MS. The protein, identified by searching the *E. coli* sequence database (Materials and Methods), resulted to be glyceraldehyde 3-phosphate dehydrogenase, as identified by 17 peptides matches for a sequence coverage of 71%.

Purified rRHA-P amino acidic sequence was verified up to a 94% coverage, by MS Mapping analysis carried out by MALDI-TOF and LC–MS/MS. However, it is worth noting that the protein N-terminus, detected by MS analysis, lacks the first 23 amino acids from the expected cloned sequence (Fig. 2 and Fig. S3).

To confirm the lack of the putative N-terminal peptide, purified rRHA-P was subjected to both a MALDI-TOF and LC–MS/MS analysis after an *in situ* digestion with either trypsin or chymotrypsin, and to the N-terminus sequencing. Mass spectrometry analysis allowed the identification of peptides ^{23}ESRDDAAEVAPSTRPEPSLEQAF^{45} (MH+ 2502.17 ^{ppm}) and ^{23}ESRDDAAEVAPSTR^{36} (MH$^+$ 1503.70) when rRHA-P was digested with chymotrypsin or trypsin, respectively. This evidence was also confirmed by N-terminus sequencing results, which showed that the first 5 amino acids of the recombinant protein are **ESRDD**. Interestingly, also in native RHA-P the same N-terminal peptide, obtained by fragmentation spectrum of the triple charged ion at 834.73 *m*/*z* from the *in situ* digestion of the protein with chymotrypsin (Fig. S4), had been detected in the first place. The scarce amount of native protein, however, did not allow us to confirm the effective lack of the N-terminus peptide by direct amino acidic sequencing.

Table 3
TLC results of RHA-P-catalyzed reaction using different *p*NP-α- and *p*NP-β- derivatives as substrates.

	Substrates[a]	Hydrolysis		Transglycosylation	
		3 h	20 h	3 h	20 h
1	*p*NP-α-D-Glc*p*	–	–	–	–
2	*p*NP –β-D-Glc*p*	–	–	–	–
3	*p*NP –α-D-Gal*p*	–	–	–	–
4	*p*NP –β-D-Gal*p*	–	–	–	–
5	*p*NP –α-D-Man*p*	–	–	–	–
6	*p*NP –β-D-Man*p*	–	–	–	–
7	*p*NP –β-D-Xyl*p*	–	–	–	–
8	*p*NP –α-L-Fuc*p*	–	–	–	–
9	*p*NP –β-D-Fuc*p*	–	–	–	–
10	*p*NP –α-L-Ara*f*	–	–	–	–
11	*p*NP –β-L-Ara*f*	–	–	–	–
12	*p*NP –α-L-*R*ha*p*	+	+	–	–
13	*p*NP β-GlcA*p*	–	–	–	–
14	*p*NP β-D-NAGlc*p*	–	–	–	–
15	*p*NP β-D-NAGal*p*	–	–	–	–

[a] Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; NAGlc, *N*-acetylglucosamine; NAGal: *N*-acetylgalactosamine, GlcA: glucuronic acid, Rha, Rhamnose; Xyl, xylose.

3.3. Biochemical characterization of rRHA-P

3.3.1. Analytical gel filtration

The oligomeric state of rRHA-P was verified by analytical gel filtration using a Superdex 200 analytical column; the protein eluted as a single peak with an apparent molecular weight of 101,500 ± 5,000 Da, which indicates that rRHA-P is a monomer.

3.3.2. Substrate specificity

To investigate rRHA-P substrate specificity in hydrolysis and self-condensation processes, several *p*NP-α- and *p*NP-β- derivatives were used as substrates in enzymatic assays performed as described in Materials and Methods. Reactions were followed over time by TLC analysis and data are reported in Table 3. As evident from Table 3, the purified enzyme showed activity only on *p*NPR, confirming that rRHA-P is indeed a α-L-rhamnosidase. Moreover, no activity on *p*NP-β-D-Glc was detected, excluding that rRHA-P might act as a naringinase, a class of glycosyl hydrolase having both α-L-rhamnosidase and β-D-glucosidase activities [40]. Furthermore, oligo- and polysaccharides such as rutinose, maltose, sucrose, lactose, pullulan, starch, amylopectin, raffinose, xylan from birchwood, xylan from oat spelt, hyaluronic acid, α-cellulose, cellobiose, chitosan, β-Glucan from barley, laminarin, curdlan, fucoidan from *Fucus versiculosus*, were also tested as substrates for rRHA-P. TLC analysis of all these reactions, performed as described in Materials and Methods, showed the lack of activity of this enzyme on any of these compounds.

3.3.3. Mechanism of action

To understand whether rRHA-P behaves as an inverting or a retaining glycosidase [1], ^{1}H NMR spectroscopy was used to monitor the stereochemical course of *p*NPR cleavage. As shown in Fig. 3, after the addition of the enzyme, a mixture of α and β

```
1    PRLSLRIVLCLATALSTLPVHAESRDDAAEVAPSTRPEPSLEQAFKDPPSSARPRVWWHWMNGNITKDGIRKDLEWMKRV 80
81   GIGGLQNFDANLQTPQIVDHRLVYMTPEWKDAFRFAAHEADRLDLELAIAASPGWSETGGPWVKPQDGLKKLVWSETTLA 160
161  GGQRFVGRLASPPGTTGPFQTLHPPVTIEEIISGVPAETGGVSYAGEVGVLAFPVPDIASLPVPRALDGAGNVLAGKALV 240
241  DADIAGGVTLARVDGKAPLLRLDYQRPVTVRSATVFVPNVRIPFAGAAFAGTLESSQDGKTWTPIKALELSNVPTTISFA 320
321  PVEAAHFRLVLNPGQPDAALGSPAPGVAGNDLFGAIASKRAGQPIMVGQFELHSDALVDRYETKAGFVMSRDYYALVGPH 400
401  DNVTGVDPDSVIDLTDKLKADGTLDWAAPKLPAGQHWRVLRLGYSLLGTTNHPAPPEATGLEVDKFDGEAVREYLEHYIG 480
481  MYKDAAGPDMVGKRGVRALLTDSIEVGEANWTPRMLEQFQRLRGYDARPWLPALTGTLVGTREQSDRFLYDYRRTLADLL 560
561  ASEHYGTVADVAHENDLKVYGEGLEDHRPMLGDDMAMRSHADIPMAALWTFNRDEGPRQTLIADMKGAASVAHLYGQNLV 640
641  AAESMTASMAPWAFAPKDLKRFIDLEFVTGVNRPVIHTSVHVPVDDKKPGLSLAIFGQYFNRQESWAEMARPWVDYIARS 720
721  SLLLQTGRNVADVAYFYGEEAPLTGLYGDEPVADAPVRYAYDYINFNALTELLANDGEDLVAPSGARYKTIYLGGSSSHM 800
801  TLAALRKLAALVVGGATVVGKAPIATPSNTSAQEGDLTEWSSLVARLWPGSGDARVGKGRVIASQDIESALQAMDVAPDF 880
881  TFTGADAGVKIPFVHRRDGKGEIYYLVNQQEAAQSIEAHFRVTGKQPELWHPETGKSEPISYRISGGETVVPLHLDGDEA 960
961  VFVVFRKAAARDRVTLARQGERAVATLDGAWQVAFQADRGAPASIELARLEPLDKSADPGVKYFSGIATYSRNFRVTGKY 1040
1041 GEGRSLWLDLGRVGDLAQVSVNGVDVGTAWHAPYRLDIGKAVRKGQNTLEIRVANTWVNRLIGDQQEGAQKITWTAMPTY 1120
1121 RADAPLRPSGLIGPVRLIEETTGGH 1145
```

Fig. 2. rRHA-P sequence coverage by MS analysis. Highlighted in grey are the peptides, identified by MS analysis (MALDI-TOF and/or LC–MSMS), of the enzymatic digestions of rRHA-P carried out either *in situ* or in solution with proteases of different specificity: trypsin, chymotrypsin and endoproteinase GluC (V8-DE). A global sequence coverage of 94% was obtained. Crossed is the N-terminus sequence expected for rRHA-P (reference sequence WP_013837086.1) and not retrieved, either by N-terminus or by MS analysis.

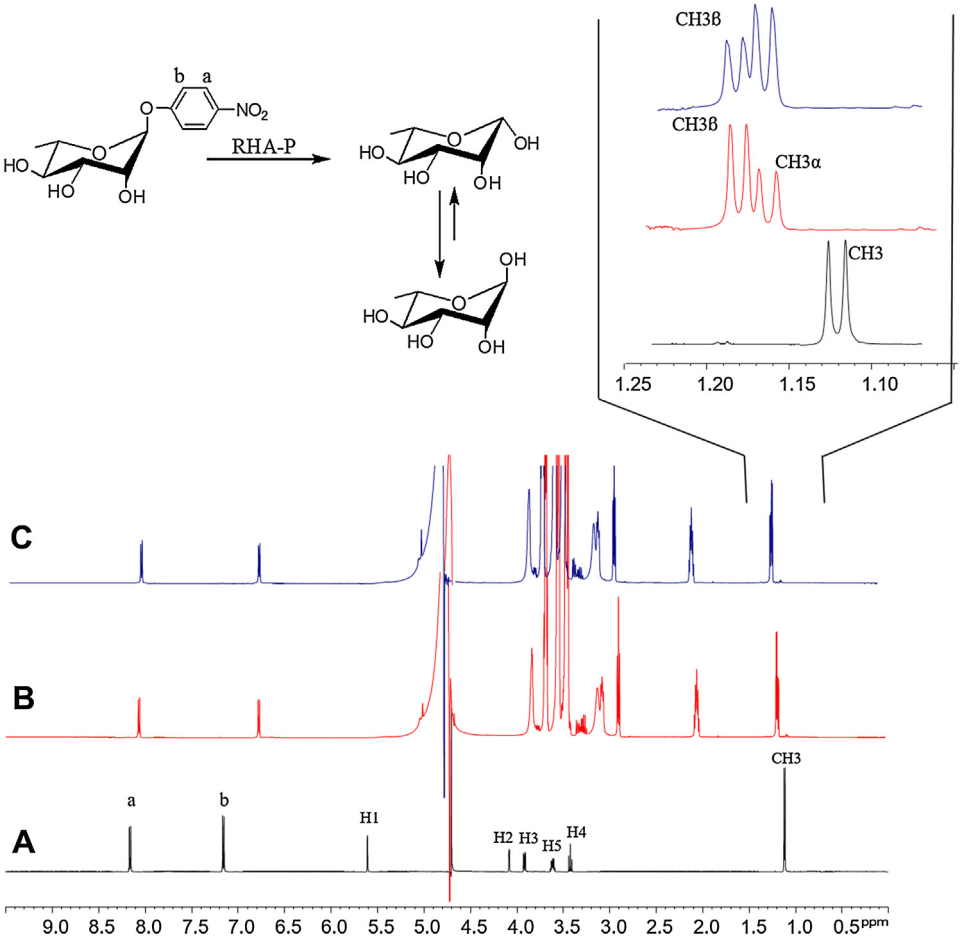

Fig. 3. ^{1}H NMR analysis of pNPR after rRHA-P catalyzed hydrolysis. A. Spectrum of the ligand alone in solution (3 mM). B. Spectrum following the addition of the enzyme to the NMR tube (~60 μg), which showed the appearance of CH_3 signals relative to α- and β-anomers. C. Spectrum obtained after a 30 min reaction; a mixture of α- and β-anomers with a different relative ratio was observed due to emiacetal equilibrium.

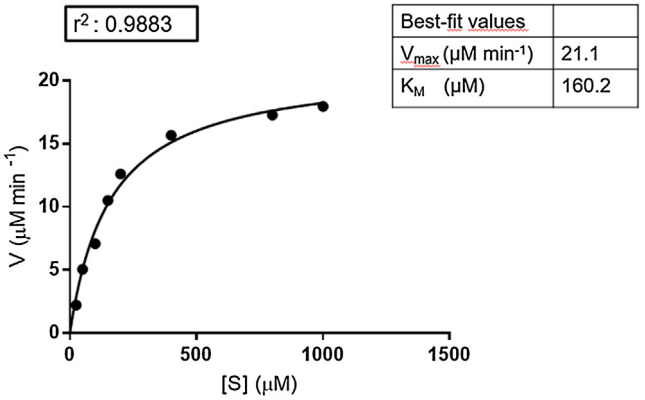

Fig. 4. rRHA-P kinetic behavior. Reaction rate expressed in μM/min is plotted as a function of *p*NPR concentration.

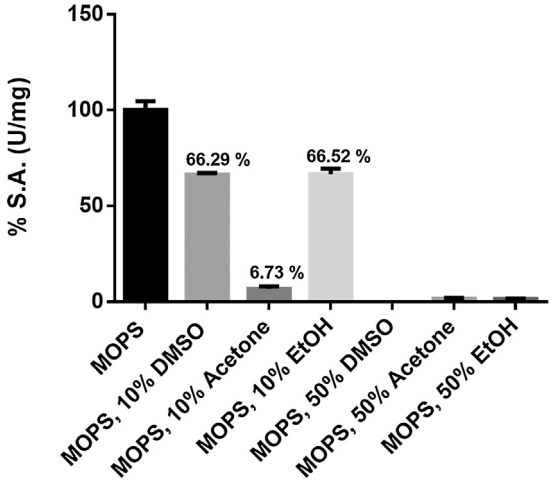

Fig. 6. rRHA-P tolerance to organic solvents. Data are reported as percentage of residual specific activity (S.A.) compared to the control (25 mM MOPS, pH 6.9). MOPS buffer is used at a final concentration of 25 mM and at pH 6.9.

rhamnose anomers was observed in solution, as evident from the presence of two different methyl group resonances. TOCSY spectrum allowed the assignment of both α and β spin systems up to the methyl group resonances located at position 6 of each rhamnose residue. Therefore, the less shielded methyl signal was assigned to β-rhamnose. The analysis of the intensity of the different methyl proton signals showed that the thermodynamically unfavorable β-rhamnose prevailed soon after the addition of rRHA-P (Fig. 3B). Afterwards, as a consequence of the equilibrium established by a reducing monosaccharide in water, the relative ratio of the two peaks changed up to the point in which the α anomeric product was predominant (Fig. 3C). Hence, the data suggested that rRHA-P acts as an inverting enzyme as it cleaves the α-glycoside ligand yielding a β-anomer, which in water solution equilibrates according to the anomeric effect.

3.3.4. Enzymatic characterization

All enzymatic assays described in this subsection are detailed in Materials and Methods section and were performed using *p*NPR as substrate. Kinetic constants on *p*NPR were determined using a substrate concentration ranging from 0.025 to 1 mM. The reaction rate (μM/min), plotted as a function of the substrate concentration (μM), showed a typical Michaelis-Menten trend and is reported in Fig. 4. K_M constant was of 160.2 (±17.3) μM, a value significantly lower than most other α-RHAs described in literature [32,57], indicating a higher affinity of rRHA-P for *p*NPR. A V_{max} of 21.1 (±8.0) μM/min, a k_{cat} of 734.4 (±212.9) sec^{-1} and a K_s of 4.6 (±1.7) sec^{-1} μM^{-1} were obtained.

rRHA-P revealed an optimal activity in the range 6.0-7.5 with an optimum at pH 6.9 (Fig. 5A), retaining 47% and 36% of activity at pH 7.9 and 8.8, respectively. In addition, rRHA-P activity was assayed in the temperature range 25 °C–55 °C. Data are presented in Fig. 5B and show an optimal activity temperature at ca. 40.9 °C, with a 44% retaining of its initial value up to 50 °C. To evaluate the thermal stability of rRHA-P in the same range used to determine the optimal temperature, an incubation of the purified protein for either one or 3 h was performed at different temperatures; afterwards, the residual rhamnosidase activity of the enzyme was calculated (Fig. S5). The enzymatic activity appeared to be stable between 25 and 40 °C, independently from the duration of the incubation. At 40 °C, corresponding to the temperature optimum, the activity is stable up to 24 h (data not shown).

In order to establish the tolerance of the enzymatic activity of rRHA-P to the presence of organic solvents, *p*NPR assay in 50 mM MOPS pH 6.9 containing 10% or 50% of either DMSO, acetone or ethanol was performed (Fig. 6). While activity dramatically decreased in all concentration of acetone and in 50% of ethanol and DMSO, a 66% of activity was still recovered in the presence of 10% of either DMSO or ethanol.

3.3.5. Activity on flavonoids

The ability of rRHA-P to hydrolyze natural flavonoids was evaluated. Enzymatic assays using naringin, rutin and neohesperidin

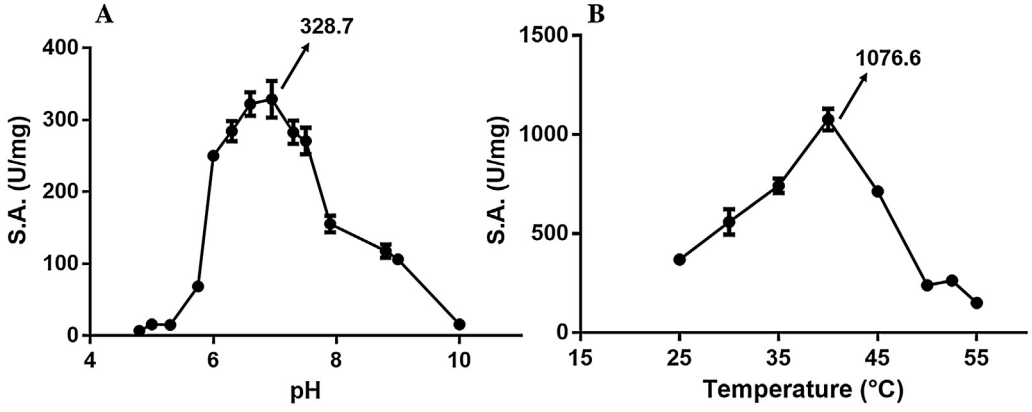

Fig. 5. rRHA-P activity assays. pH (A) and temperature (B) optimum curves. In both graphs specific activity (S.A.) is plotted as a function of pH and temperature, respectively. The maximum value of S.A. obtained is reported in both graphs and indicated by a black arrow.

dihydrochalcone were performed and followed by TLC analysis, as described in Materials and Methods. To this purpose, a 6 mM solution of each compound was incubated with purified rRHA-P in 50 mM Na-phosphate buffer pH 7.0 at 40 °C, and the reaction was monitored by TLC analyses over time. After 3 h of incubation, TLC analysis of the reaction mixtures showed a 40–60% hydrolysis of neohesperidin dihydrochalcone and rutin in the corresponding derhamnosylated neohesperidin dihydrochalcone and quercetin-3-β-glucopyranoside (isoquercitrin). Besides, a total conversion of naringin was observed as the corresponding products, rhamnose and prunin, were the only compounds observed on the TLC plate. Reaction on naringin was also carried out in the presence of 10% DMSO. In this case, in our experimental conditions, the complete conversion of naringin in prunin and rhamnose occurred after just 1h.

4. Discussion

α-RHAs are a group of glycosyl hydrolases (GHs) that have attracted a great deal of attention due to their potential application as biocatalysts in a variety of industrial processes. These enzymes are of particular interest for the biotransformation of several natural compounds used in pharmaceutical and food industry. Enzymatic derhamnosylation catalyzed by α-RHAs can be used, for example, in functional foods and beverages containing molecules with enhanced health-related properties. Some examples encompass the biotransformation of natural steroids, antibiotics, flavonoids, and terpenyl glycosides responsible for wine aromas [2,40].

Bacterial α-RHAs are still poorly characterized, but their biochemical properties might be of key importance for biotransformation processes involving reactions conditions that are unfavorable for fungal rhamnosidases. In this work, we have successfully expressed and partially characterized a novel recombinant α-RHA from *Novosphingobium* sp. PP1Y, named RHA-P. The α-RHA activity has been isolated from the native source and the corresponding protein sequence was verified by MS analysis. The sequence was used for a BLAST search against all protein databases, and resulted to be homologous to GHs present in the genomes of several Sphingomonads; among these, a 48% sequence identity of RHA-P with the α-RHA (rhaM) from *Sphingomonas paucimobilis* FP2001, was retrieved [58]. Noteworthy, an analysis of the amino acidic sequence of RHA-P highlighted the presence of a 23 amino acids long signal peptide [59] located at the N-terminus of the protein.

The new α-RHA identified is encoded by *orf*PP1Y_RS05470, a 3,441 bp gene located in Megaplasmid Mpl of *N.* sp. PP1Y [44], which was amplified and cloned into pET22b (+) plasmid to allow for the recombinant expression in *E. coli* BL21(DE3) strain. First attempts of expression of the recombinant protein, rRHA-P, resulted in the foremost presence of the protein in the insoluble portion of the induced cultures. A lower induction temperature, shifted from 37 °C to 23 °C, along with the use of a high-salt LB formulation containing both betaine and sorbitol, which presumably act as "chemical chaperones", efficiently concurred in improving the yield of soluble, active rRHA-P [54–56].

rRHA-P was purified following a three-step purification protocol. The relatively poor yield obtained at the end of the purification was not unexpected at this stage, as we chose after each step to avoid collecting rRHA-P active fractions that showed the presence of too many protein contaminants.

Purified rRHA-P sequence was verified by MS mapping. It is interesting to underline that the protein N-terminus expected from the cloned sequence was never detected, either by MS analysis (both native and recombinant RHA-P) or by N-terminus sequencing (only recombinant RHA-P). The lack of this peptide, which had been expected by a preliminary analysis of the amino acidic sequence, confirmed the presence of a signal peptide presumably cleaved through a post-translational proteolytic processing. A similar evidence has been described for the α-RHA isolated from *S. paucimobilis* FP2001 and recombinantly expressed in *E. coli* [58]. Takeshi M. and coworkers reported in this case the presence of a putative signal peptide cleaved between positions A24 and N25 [58]. Similarly, in rRHA-P the cleavage site is located between A23 and E24. The amino acidic sequence of the N-terminus of both α-RHA from strain FP2001 and strain PP1Y revealed several peculiarities owned by bacterial signal peptides, such as the presence of a charged region (2–5 residues) followed by a hydrophobic stretch of ~12 aa, along with the presence of small and apolar residues located 1 and 3 aa upstream of the cleavage site [60]. The functional role of this cleavage, as well as the possible sorting of these processed proteins in the periplasmic space of the native bacteria, needs to be further investigated.

A preliminary structural characterization showed that rRHA-P is a monomeric protein with an approximate molecular weight of 101,500 ± 5,000 Da. Mechanism of action, kinetic parameters and optimum reaction conditions were determined using a synthetic substrate, *p*NPR.

As for the reaction mechanism, GHs are generally grouped in two main classes, inverting and retaining. A typical inverting glycosidase requires the presence of a catalytic acid residue and a catalytic basic residue, whereas a typical retaining glycosidase contains a general acid/base residue and a nucleophile. These different hydrolysis mechanisms lead, respectively, to an inversion or to a retention of the anomeric oxygen configuration in the product compared to the substrate [61]. NMR experiments showed that rRHA-P acts as an inverting GH; further investigation will give additional insights in identifying residues in the active site of this enzyme, which are directly involved in catalysis.

rRHA-P catalytic efficiency, defined by a K_s value of 4.6 s^{-1} μM^{-1}, underlined that the activity of this enzyme resulted to be comparable or even higher than that of other bacterial α-RHAs described in literature. When clearly reported, such as in lactic acid bacteria *Lactobacillus plantarum,* and *Pediococcus acidilactici,* K_s values on *p*NPR range in fact from 0.01 s^{-1} μM^{-1} to 0.019 s^{-1} μM^{-1} [38,39]. In addition, K_s of rRHA-P resulted to be approximately 3 times higher than the K_s of 1.74 s^{-1} μM^{-1} reported for the α-RHA isolated from *S. paucimobilis* FP2001 [57]. The comparison among the K_M values present in literature, ranging from 1.18 mM for *S. paucimobilis* FP 2001 to 16.2 mM for the α-RHA isolated from *P. acidilactici*, showed a higher apparent affinity of rRHA-P for *p*NPR (K_M of 160 μM), which might be in part responsible for the great differences found in K_S values.

To investigate the effective potential of using rRHA-P in biotechnological processes involving the biotransformation of flavonoids, optimal reaction conditions in terms of pH, temperature and presence of organic solvents were evaluated. In particular, rRHA-P, in line with other bacterial α-RHAs [34–38,55], has an optimal activity at pH 6.9, a value that highlights a marked difference with fungal α-RHAs acting at pH values ranging from 4 to 5 [17,31,32]. rRHA-P shows an optimal activity at 40.9 °C and a significant overall thermal stability, retaining ~66% of the activity up to 45 °C, a temperature range mainly used in most industrial processes. In this respect, also a great majority of other fungal and bacterial enzymes exhibit similar values of temperature stability and optimal activity [17,32,34,37,38,55], whereas only a limited number of α-RHAs is more thermophilic and has an optimum of temperature at ca. 60 °C [31,36,37,39]. rRHA-P has a moderate tolerance to organic solvents, usually employed for the biotransformation of flavonoids that are poorly soluble in water, retaining the 66% of activity on *p*NPR in solutions containing 10% of either DMSO or ethanol. Naringin con-

version is even faster in 10% DMSO than in the absence of this solvent, probably owing to the fact that this flavonoid is more soluble in the presence of DMSO. Noteworthy, flavonoids conversion catalyzed by other α-RHA has been described in the presence of 2–5% DMSO [37–39]; higher concentration of DMSO, as the one used for our bioconversion, might further increase the solubility of many flavonoids of commercial interest. These data altogether suggest a higher tolerance of rRHA-P to the presence of solvents compared to α-RHAs described in literature for which a residual 20 − 30% activity in 12% ethanol and a 24% activity in 25% DMSO has been reported [17,39]. It is worth mentioning that rRHA-P residual activity in ethanol-containing buffers might be of additional importance as many biologically relevant rhamnosylated flavonoids are found in wine and possibly citrus derived alcoholic beverages.

As previously underlined, the kinetic data obtained so far for this enzyme undoubtedly encourage its use for the bioconversion of glycosylated natural flavonoids that are more soluble at high temperature (ca. 50 °C) [62], basic pH values, and in the presence of organic solvents. In this context, experiments to verify the hydrolytic properties of rRHA-P on few natural flavonoids containing either α-1,2 or α-1,6 glycosidic linkages between α-L-rhamnose and β-D-glucose were performed. TLC analysis on naringin (α-1,2), rutin (α-1,6) and neohesperidin (α-1,6) showed a total conversion of naringin in the corresponding prunin and rhamnose, while a partial hydrolysis of rutin and neohesperidin was observed, thus confirming the ability of this enzyme to hydrolyze both α-1,2 and α-1,6 linkages. Likewise, the majority of other bacterial and fungal α-RHAs exhibit similar substrate specificity, even though these enzymes often show a slight preference for the hydrolysis of α-1,6 linkages [31,32,37–39].

In conclusion, the results described in this work boost the interest toward a further biochemical characterization of RHA-P activity using for example a wide array of glycosylated flavonoids, to investigate the effective potential of this novel enzyme for various biomass and food-processing applications aimed at recovering high-value added compounds from complex vegetable matrixes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.10.002.

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