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The metagenome-derived enzyme RhaB opens a new subclass of bacterial B type α-L-rhamnosidases

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ABSTRACT

A combined sequence- and function-based analysis of a metagenomic library DNA derived from elephant feces led to the identification of a novel bacterial α-L-rhamnosidase belonging to glycoside hydrolase family 78 (GH78). The gene was designated *rhaB* (4095 bp) and encoded for a putative protein of 1364 amino acids. The *C*-terminal part of the enzyme revealed an amino acid (AA) sequence identity of 58% to a predicted bacterial α-L-rhamnosidase from *Bacteroides nordii*. Interestingly, the *N*-terminal region of the deduced enzyme RhaB contained a GDSL-like lipase motif and an acetyl-xylan esterase (DAP2) motif. While heterologous expression of the complete *rhaB* failed, subcloning of the gene identified the most active open reading frame (ORF) to be of 3081 bp, which we designated *rhaB1*. The enzyme RhaB1 was overexpressed in *Escherichia coli* BL21 (DE3) and was purified to an amount of 75 mg/L of culture medium. In accordance to the intestinal origin, RhaB1 showed a preference for mesophilic conditions with an optimum activity at a temperature T_{Opt} of 40 °C and a pH_{Opt} of 6.5, respectively. The recombinant protein had a K_m value of 0.79 mM and a specific activity v_{max} of 18.4 U for *p*NP-α-L-rhamnose, a calculated K_m of 6.36 mM and v_{max} of 2.9×10^{-3} U for naringin, and a K_m of 6.75 mM and specific activity v_{max} of 8.63×10^{-2} U for rutin, respectively. Phylogenetic analysis and amino acid domain architecture comparison revealed that RhaB1 belongs to a new subclass of bacterial B type α-L-rhamnosidases of GH 78. To our knowledge RhaB1 is the first biochemically-characterized enzyme of this subclass.

1. Introduction

The α-L-rhamnosidases (EC 3.2.1.40) are inverting glycoside hydrolases that catalyze the cleavage of terminal α-L-rhamnose residues (Yadav et al., 2010). Most known bacterial α-L-rhamnosidases possess the glycoside hydrolase (GH) motif of GH family 78 (GH78) (Henrissat and Davies, 1997). Only few examples are known that are members of GH family 106 (Miyata et al., 2005). The eukaryotic α-L-rhamnosidases RgxB from *Aspergillus niger* CBS51388 and RhaR from *A. niger* DLFCC-90 are grouped within the GH-families GH28 and GH13, respectively (Liu et al., 2012; Martens-Uzunová et al., 2006).

The majority of characterized bacterial α-L-rhamnosidases to date originate from Gram-positive bacteria, i.e. *Bacillus*, *Lactobacillus*, *Staphylococcus*, *Clostridium*, *Streptomyces* and *Thermomicrobium* species (Avila et al., 2009; Beekwilder et al., 2009; Birgisson et al., 2004; Hashimoto et al., 1999; Ichinose et al., 2013; Michlmayr et al., 2011; Puri and Kaur, 2010; Zverlov et al., 2000). RhaB, one of the two published enzymes of *Bacillus* sp. GL1 was successfully crystallized and its structure determined by X-ray crystallography (Cui et al., 2007).

Some of the so far characterized α-L-rhamnosidases originate from intestinal bacteria including *Bacteroides* spp. isolated of the human gut (Bokkenheuser et al., 1987; Jang and Kim, 1996; Park et al., 2008). Until now the intestinal tract of several animals has been shown to be a rich source for polysaccharide degrading bacteria and their enzymes (Feng et al., 2009; Gloux et al., 2011; Ilmberger et al., 2012; Warnecke et al., 2007). These bacteria and their so called carbohydrate active enzymes (CAZYmes, (Cantarel et al., 2009)) play an important role for their hosts in digestion but also affecting even more than alimentation (Hehemann et al., 2012; Maslowski and Mackay, 2011; Nicholson et al., 2012). A predominant bacterial phylum of gut organisms are the *Bacteroidetes* (Thomas et al., 2011). A putative α-L-rhamnosidase from *Bacteroides thetaiotaomicron* VPI-5482 was already crystallized (*PDB: 3CIH)*.

From metagenomic approaches to date most genes of putative GH78 enzymes were annotated though rarely tested for

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α-L-rhamnosidase activity. For instance, two GH78 genes were identified in one clone from the metagenome of a human fecal sample (Tasse et al., 2010). In a more recent study on the termite abdominal microbiome also sequences possessing GH78 motifs were published but not characterized (Bastien et al., 2013). Further on the functional level, two α-L-rhamnosidases were identified by activity assays in a comprehensive functional study on glycoside hydrolases (GHs) from cow rumen (Ferrer et al., 2012).

The α-L-rhamnosidases are of interest for chemical industry, especially pharmaceutical and food industries (Manzanares et al., 2007). The commercial enzyme preparations naringinase and hesperidinase, both of fungal origin, comprise α-L-rhamnosidase next to β-D-glucosidase activity (Monti et al., 2004; Young et al., 1989). Naringinases and hesperidinases are applied for debittering and clarifying of fruit juices (Ribeiro, 2011; Yadav et al., 2010). The aroma optimization of wine is a further potential field of application of α-L-rhamnosidases (Manzanares et al., 2003; Michlmayr et al., 2011). The derhamnosylating function of α-L-rhamnosidases can enhance the pharmacological properties of drugs like antibiotics as chloropolysporin, steroids as ruscin or gypenosides (Di Lazzaro et al., 2001; Feng et al., 2005; Takatsu et al., 1987; Yu et al., 2004). As a byproduct of derhamnosylations L-rhamnose is produced from natural substances. L-rhamnose itself is a demanded precursor as a chiral compound for chemical synthesis but also for glycodiversification of natural compounds (Manzanares et al., 2007; Puri, 2012; Thibodeaux et al., 2007). A rich source of L-rhamnose containing biomolecules are flavonoids. The use of citrus fruit peel waste from juice making as a source of naringin and hence prunin and L-rhamnose was already demonstrated by Kaur and colleagues (Kaur et al., 2010). Flavonoids are well known for their beneficial effects on human health (Ververidis et al., 2007) whereof the mono-glycosylated forms like isoquercitrin and prunin show a higher bioavailability then the respective disaccharides (Hollman et al., 1999). Because of the bioactivity flavonoids meet an increasing demand in the cosmetic, the pharma- and nutraceutical industries (Leonard et al., 2008; Schütz et al., 2006; Wang et al., 2006).

For the discovery of novel α-L-rhamnosidases the metagenome of the intestinal microbiome of an Asian elephant was chosen. In a combined sequence and function based screening approach a metagenome library of fresh feces was analyzed where the putative α-L-rhamnosidase gene *rhaB* was discovered. The gene was subsequently cloned for functional analyses and the active enzyme RhaB1 was characterized biochemically.

2. Material and methods

2.1. Bacterial strains, plasmids and chemical reagents

Bacterial strains and plasmids used in the present work are listed in Table S1 and primers are listed in Table S2. If not otherwise stated *Escherichia coli* was grown at 37 °C in LB media (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with appropriate antibiotics. All used reagents and chemicals used were of laboratory grade. Protease Inhibitor cocktail for purification of histidine-tagged proteins as a DMSO solution and *para*-nitrophenyl-α-L-rhamnopyranoside (*p*NP-rham) and were purchased from Sigma Aldrich. The flavonoids quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin), naringenin-7-*O*-α-L-rhamnopyranosyl-(1,2)-β-D-glucopyranoside (naringin), naringenin-7-*O*-β-D-glucopyranoside (prunin), quercetin, and quercetin-3-*O*-α-L-rhamnopyranosyl-(1,6)-β-D-glucopyranoside (rutin) were kindly provided by Merck KGaA from the suppliers Roth, ABCR, Sigma Aldrich and AppliChem. Stock solutions of each flavonoid were prepared in DMSO.

2.2. Isolation of DNA and fosmid library construction

The dung sample for construction of the elephant feces library was derived from a healthy six year old female Asian elephant (*Elephas maximus*) named Kandy living in the Hagenbeck Zoo (Hamburg, Germany). Construction of the metagenomic library and storage was performed as described before (Rabausch et al., 2013). The elephant feces library encompassed a total of 20,000 clones. Average insert size of the fosmids was 35 kb.

2.3. Molecular cloning strategies

Amplification of the present open reading frames (ORFs) was performed with purified fosmid pUR16A2 DNA as a template for PCR. The reactions were performed in 35 cycles using *Pfu*-polymerase enzyme. To amplify the different ORFs *rhaB*, *rhaB1*, *rhaB2*, and *rhaB3* the primers rhaB-*Sal*I-for, rhaB1-*Sal*I-for, rhaB2-*Sal*I-for, and rhaB3-*Bam*HI-for, respectively, were used each in combination with rha-*Hin*dIII-rev (see Table S2). The purified PCR fragments were ligated into *Sma*I-prepared *p*UC19 vector and *E. coli* DH5α was transformed by heat shock. Insert carrying clones were identified by blue white screening on LB agar plates containing 10 μM 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and 400 μM isopropyl-β-D-thiogalactopyranoside (IPTG) after overnight growth. Correct clones were identified by plasmid purification, followed by RFLP analysis. The DNA sequences were verified by di-deoxy DNA sequencing. The resulting plasmids were designated *p*UC-B, *p*UC-B1, *p*UC-B2, and *p*UC-B3, respectively (Table S1).

Directed ligation of the respective ORF into expression vector *p*ET21a (Merck, Darmstadt, Germany) was achieved applying the primer born endonuclease restriction sites, *Sal*I or *Bam*HI at the 5′end and the *Hin*dIII-site at the 3′end. Heat shock transformed *E. coli* DH5α *p*ET21-clones were analyzed for the correct insert by direct colony PCR using the respective-forward primers rhaB-*Sal*I-for, rhaB1-*Sal*I-for, rhaB2-*Sal*I-for, or rhaB3-*Bam*HI-for and T7 terminator primer. Additionally, the inserts of *p*ET21*rhaB*, *p*ET21*rhaB1*, *p*ET21*rhaB2*, and *p*ET21*rhaB3* were sequenced using T7 promotor and T7 terminator primers to certify the constructs.

2.4. Overproduction and purification of enzymes

For overproduction of the hexa-histidin (His_6-) tagged proteins RhaB, RhaB1, RhaB2, and RhaB3 *E. coli* BL21 (DE3) was transformed with the respective *p*ET21*rha* construct. All four strains harboring, respectively, *p*ET21*rhaB*, *p*ET21*rhaB1*, *p*ET21*rhaB2*, or *p*ET21*rhaB3* were treated equally for enzyme production. An overnight preculture was harvested by centrifugation. The expression cultures were inoculated with 1% of resuspended cells and grown at 22 °C until 0.7 OD_{600}. The cultures were transferred to 17 °C and overexpression was induced by 400 μM of IPTG. After 16 h, the cultures were harvested by centrifugation at 7500 × *g* and 4 °C. The cells were resuspended in 50 mM phosphate buffer saline (PBS) with 0.3 M NaCl at pH 7.4 and disrupted by ultrasonication with a S2 sonotrode in a UP200S (Hielscher, Teltow, Germany) at a cycle of 0.5 and an amplitude of 75%. To avoid degradation protease inhibitor cocktail (Sigma Aldrich, Basel, Switzerland) was added.

Crude cell extracts were centrifuged at 15,000 × *g* and 4 °C. The supernatants were loaded on 1 mL HisTrap FF Crude columns in an ÄKTAprime plus apparatus (GE Healthcare, Freiburg, Germany). The enzymes were purified according to the manufacturer protocol for gradient elution of His-tagged proteins. The fractions were analyzed on a denaturing 10% SDS-PAGE. Fractions of purified protein were pooled and dialyzed twice against 1000 vol. of 50 mM MES,