Characterization of a glycoside hydrolase family 78 α -L-rhamnosidase from *Bacteroides thetaiotaomicron* VPI-5482 and identification of functional residues

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Abstract

A putative glycoside hydrolase family 78 α -L-rhamnosidase BtRha78A from *Bacteroides thetaiotaomicron* VPI-5482 was heterologously over-expressed in *Escherichia coli*. Enzymatic properties of recombinant BtRha78A were characterized in detail. Recombinant BtRha78A might efficiently hydrolyze *p*-nitrophenyl α -L-rhamnopyranoside. BtRha78A displayed the highest activity at 60 °C in pH 6.5. BtRha78A exhibited a good pH stability and relatively high thermostability. BtRha78A could be tolerant of a low concentration of alcohols. These attractive advantages made it a promising alternative biocatalyst for industrial applications. The catalytic general acid Asp335 and general base Glu595 of BtRha78A were confirmed by site-directed mutagenesis. Alanine scanning mutagenesis based on sequence alignment and structural analysis revealed that the conserved residues Asp330, Arg334, Trp339, Asp342, Tyr383, Trp440, and His620 were crucial for enzyme catalysis. Most functional residues located at the conserved general acid motif (Asp330–Asp342) and were completely conserved in the subfamily I Rha78s.

Keywords α -L-Rhamnosidase · Glycoside hydrolase family 78 · General acid · General base · General acid motif

Introduction

α -L-Rhamnosidase [E. C. 3.2.1.40] specially cleaves the terminal α -L-rhamnose from a number of natural products including flavonoid glycosides rutin, troxerutin, naringin, hesperidin, diosmin and quercitrin, ginsenosides Re and Rg2, terpenyl glycosides and many other natural glycosides containing terminal α -L-rhamnose (Yadav et al. 2010). α -L-Rhamnosidase has turned out to be a biotechnologically important enzyme due to its biocatalytic applications in a variety of processes such as debittering of citrus fruit juices (Alvarenga et al. 2013), biotransformation of rutin into isoquercetin (Weignerová et al. 2012; You et al. 2010; Lee

et al. 2013; Zhang et al. 2015; Wang et al. 2012), enzymatic preparation of prunin from naringin, improving bioavailability (González-Barrio et al. 2004), biological activities (de Araújo et al. 2013; Lee et al. 2012; da Silva et al. 2013) of flavonoids, enzymatic biosynthesis of α -L-rhamnosides by reverse hydrolysis (Lu et al. 2015; De Winter et al. 2013), and bioconversion of gypenoside-5 into ginsenoside Rd (Yu et al. 2004).

α -L-Rhamnosidases are classified into three glycoside hydrolase families: GH28, GH78, and GH106 based on sequence similarity according to the CAZy database (Lombard et al. 2014). Up to now, sixteen genes encoding bacterial GH78 α -L-rhamnosidases (Rha78s) have been cloned and characterized, and most originate from lactic acid bacteria (LAB), such as *Bifidobacterium breve* (Zhang et al. 2015), *B. dentium* (Bang et al. 2015), *Lactobacillus acidophilus* (Beekwilder et al. 2009), *L. plantarum* NCC245 (Avila et al. 2009), *L. plantarum* WCFS1 (Beekwilder et al. 2009), and *Pediococcus acidilactici* (Michlmayr et al. 2011). To date, only four crystal structures of Rha78s have been determined and contain various domains. The largest structure of Rha78s is SaRha78a from *Streptomyces avermitilis* MA-4680, which contains six domains, including a catalytic

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domain and a carbohydrate binding domain CBM67 (Fujimoto et al. 2013). RhaB from *Bacillus* sp. GL1 is composed of five distinct domains (Cui et al. 2007), and BT1001 from *Bacteroides thetaiotaomicron* VPI-5482 contains four domains (Kumaran et al. to be published). However, KoRha from *Klebsiella oxytoca* consists of two domains only, without a CBM67 (O'Neill et al. 2015). The catalytic domain of Rha78s is a typical $(\alpha/\alpha)_6$ -barrel structure, and Rha78s hydrolyze the glycosidic bonds through general acid base-assisted inverting mechanism (single displacement). The general acid and base of Rha78s have been ascertained by site-directed mutagenesis, Glu and Glu (Fujimoto et al. 2013; Cui et al. 2007), or by analyzing the enzyme-product complex, Asp and Glu (O'Neill et al. 2015).

Bacteroides thetaiotaomicron VPI-5482, a prominent member of human gut microbiota, is able to utilize a wide variety of indigestible dietary plant polysaccharides including amylose, amylopectin, dextran, polygalacturonate and larch arabinogalactan, and mucin polysaccharides such as hyaluronate, heparin, chondroitin sulfate and ovomucoid (Salysers et al. 1977). *B. thetaiotaomicron* can also degrade the pectin with different degrees of esterification (Dongowski et al. 2000) and efficiently metabolizes fructan exopolysaccharide synthesized by probiotic *Lactobacilli* (Lammerts van Bueren et al. 2015). *B. thetaiotaomicron* utilizes complex glycans using a series of similarly patterned, cell envelope-associated multiprotein systems, the starch utilization system (Sus) (Martens et al. 2009). In *Bacteroidetes*, the genes encoding the carbohydrate-active enzymes (CAZymes) that target a specific carbohydrate or related groups of carbohydrates are often found in large gene clusters termed polysaccharide utilization loci (PUL) (Bjursell et al. 2006). The ability of *B. thetaiotaomicron* foraging on both host and dietary glycans resides in its genome which encodes a wide variety of GH families, GHs (Xu and Gordon 2003), and PULs (Martens et al. 2008, 2011; Cuskin et al. 2015). The molecular mechanisms and PULs of *B. thetaiotaomicron* degrading the starch (Koropatkin et al. 2008), pectin (Ndeh et al. 2017), yeast mannan (Cuskin et al. 2015), and N-glycan (Zhu et al. 2010) have been elucidated.

Herein, we focused on the putative Rha78 BT1001 from *B. thetaiotaomicron* VPI-5482. The crystal structure of BT1001 has been determined by New York SGX Research Center for Structural Genomics (NYSGXRC) in 2008 (Bonanno et al. 2005). BT1001 might cleave the α 1-3' glycosidic linkage of Rha β -Apif in the depolymerization of pectin rhamnogalacturonan-II using HPAEC-PAD and ESI-MS for identifying the generated monosaccharides and oligosaccharides, respectively (Ndeh et al. 2017). However, the catalytic function, enzymatic properties, and catalytic key residues of BT1001 remained unclear. In this paper, BT1001, designated as BtRha78A, was heterologously over-expressed in *E. coli* BL21(DE3). Enzymatic properties were characterized

in detail. Moreover, the residues crucial for enzyme catalysis were confirmed via site-directed mutagenesis based on sequence alignment and structural analysis of the complex with Tris, an analog of the product rhamnose.

Materials and methods

Bacterial strains, enzymes and reagents

Bacteroides thetaiotaomicron VPI-5482 was obtained from China General Microbiological Culture Collection Center (No. 1.5132). *TransStart*[®] *FastPfu* Fly DNA polymerase and PCR-related reagents were purchased from TransGen Biotech (Beijing, China). Restriction enzymes QuickCut[™] *Nde* I and QuickCut[™] *Xho* I were obtained from TaKaRa (Otsu, Japan). Restriction enzyme *Dpn* I was obtained from Fermentas (Burlington, Canada). Oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). *E. coli* Trans1-Blue (TransGen Biotech, Beijing, China) was used as the host for DNA manipulation, and *E. coli* BL21(DE3) was used for protein expression. The substrate *p*-nitrophenyl α -L-rhamnopyranoside (*p*NPR) used for enzyme activity assays and kinetics was purchased from Sigma-Aldrich (St. Louis, USA). Other reagents were of analytical grade or higher.

Sequence analysis

B. thetaiotaomicron VPI-5482 genome information was extracted from GenBank (accession: AE015928). CAZymes, GH families, and GHs from the genome of *B. thetaiotaomicron* VPI-5482 were obtained from CAZy database. The PULs containing the Rha78s genes were analyzed by the database PULDB (Terrapon et al. 2015). Multiple sequence alignment was performed by the software DNAMAN 7 and illustrated with the server ESPrpt 3.0 (Robert and Gouet 2014). The phylogenetic tree was constructed using the software MEGA 7.0 (Kumar et al. 2016) on the basis of the neighbor-joining method.

Gene cloning of BtRha78A and construction of the site-directed mutants

The *BtRha78A* gene (BT1001, GenBank accession: AAO76108, UniProtKB accession: Q8A916) was amplified from genomic DNA of *B. thetaiotaomicron* VPI-5482 using the primers *BtRha78A-Nde* I-F and *BtRha78A-Xho* I-R (as shown in Table S1). PCR product of target gene was digested with *Nde* I and *Xho* I, and ligated into pET-28a expression vector. The ligation product was transformed into chemically competent cell *E. coli* Trans1-Blue. The sequence of *BtRha78A* gene was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

The site-directed mutants were constructed using the QuikChange mutagenesis protocol (Wang and Malcolm 1999) on the recombinant plasmid pET-28a-BtRha78A. The primers used to generate the site-directed mutants are listed in Table S1. Mutagenic PCR was performed under the following conditions: 95 °C for 5 min; 20 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 4 min; and a final extension at 72 °C for 30 min. The template was then eliminated by digestion with 5 U of *Dpn* I at 37 °C for 4 h. The remaining PCR product was purified using Axygen PCR clean-up kit (Corning, New York, USA). Each purified PCR product was transformed into chemically competent cell *E. coli* Trans1-Blue. Successful introduction of desired mutation was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

Over-expression and purification of recombinant BtRha78A

The BL21(DE3) cells carrying the recombinant plasmid pET28a-BtRha78A with wild-type or mutant genes were grown in 2 × YT medium containing 50 µg mL⁻¹ kanamycin at 37 °C. Protein expression was induced for overnight at 16 °C by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) until optical density at 600 nm (OD₆₀₀) reached 0.8–1.0. Then, the cells were harvested and resuspended in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 8.0) containing 10 mM imidazole. After sonication, the cell lysate was cleared by centrifugation at 9000×*g* for 30 min at 4 °C. The recombinant enzyme was purified by Ni–NTA affinity chromatography (Qia-gen, Hilden, Germany). The purified enzyme was further dialyzed twice in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 8.0). Protein concentration of the purified enzyme was measured at 280 nm by NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Enzyme activity assays

The reaction mixture of 500 µL contained 20 µL enzyme solution (1.2 µM), 10 µL the substrate *p*NPR (10 mM), and 470 µL of 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5). After the incubation for 5 min at 50 °C, the reaction was stopped by adding an equal volume of 1 M Na₂CO₃. The released *p*-nitrophenol (*p*NP) was determined at 405 nm. An extinction coefficient 16,000 M⁻¹ cm⁻¹ for *p*NP at 405 nm was used. One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 µmol *p*NP from the substrate per min at 50 °C in pH 6.5. All assays were performed in triplicate.

Characterization of recombinant BtRha78A

Effect of pH and temperature on enzyme activity

The effects of pH on enzymatic activity of BtRha78A were determined by measuring catalytic activities at 50 °C in 50 mM of varied buffers within pH range of 5.0–9.0. The pH buffers included citric acid–sodium citrate buffer (pH 5.0–6.0), NaH₂PO₄–Na₂HPO₄ buffer (pH 6.0–8.0), Tris–HCl buffer (pH 8.0–8.6), and glycine–NaOH buffer (pH 8.6–9.0).

The effects of temperature on enzymatic activity of BtRha78A were determined by measuring catalytic activities at the temperatures from 30 to 70 °C in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5).

Effect of pH and temperature on enzyme stability

The pH stability of BtRha78A was evaluated by incubating the enzyme (1.2 µM) for 60 min at 40 °C in 50 mM of a series of buffers pH ranging from 5.0 to 9.0, respectively. The buffers included citric acid–sodium citrate buffer (pH 5.0–5.5), NaH₂PO₄–Na₂HPO₄ buffer (pH 6.0–8.0), and glycine–NaOH buffer (pH 8.6–9.0). The residual activities were measured in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5) at 50 °C.

Thermal inactivation experiments of BtRha78A were performed by incubating the enzyme (11.6 µM) for specified time intervals at 45 or 50 °C. The enzyme samples were then cooled on ice and residual activities were measured in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5) at 50 °C. The first-order rate constant k_{inact} of thermal inactivation was obtained by plotting logarithmic percentages of residual activity against time of heat treatment. The half-life $t_{1/2}$ of thermal inactivation was calculated using the equation $t_{1/2} = \ln 2 / k_{\text{inact}}$.

Thermal inactivation experiments of BtRha78A were also performed by incubating the enzyme (11.6 µM) for 30 min at different temperatures (40, 42, 44, 46, 48, 50, 52, 54, and 56 °C). The enzyme samples were then cooled on ice and residual activities were measured in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5) at 50 °C. The half-inactivation temperature T_{50} of BtRha78A was estimated.

Enzyme steady-state kinetics

Enzyme kinetics of BtRha78A for *p*NPR were determined in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5) at 50 °C for 30 s. The initial rate of hydrolysis reaction was measured with varied concentrations of *p*NPR (0.05–2.5 mM). Kinetic parameters V_{max} and K_M were acquired by fitting enzymatic activities as a function of substrate concentrations to the Michaelis–Menten equation using non-linear regression of the software GraphPad Prism 6. The parameter k_{cat} was

obtained using the equation $k_{\text{cat}} = V_{\text{max}}/[E]$, where $[E]$ was molar concentration of the enzymes.

Effect of organic solvents on enzyme activity

The effects of organic solvents on enzymatic activity of BtRha78A were determined by measuring catalytic activities at 50 °C in 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5) with different concentrations (1 and 5%, v/v) of organic solvent methanol, ethanol, isopropanol, ethylene glycol, DMSO, DMF, acetonitrile, and acetone, respectively.

Results and discussion

Genome mining and gene Loci

Bacteroides thetaiotaomicron played a key role in maintaining human health by harnessing abundant GH families to exploit dietary polysaccharides and host glycans as nutrients (Xu and Gordon 2003). Genome mining revealed that the genome of *B. thetaiotaomicron* VPI-5482 (Xu et al. 2003) contains 4780 protein-coding genes (CDS), 428 CAZymes (9.0%), 91 PULs, 47 GH families, 271 GHs (5.7%), 9 α -L-rhamnosidases (GH78 and GH106), and 6 Rha78s. The genes BT1001, BT1013, BT1019, BT2523, BT2524, and BT4076 are annotated to the putative Rha78s. The gene BT1001 is located at the PUL89 (Ndeh et al. 2017) and the RG-II PUL1 (Martens et al. 2011) in *B. thetaiotaomicron* VPI-5482.

Sequence analysis

The putative Rha78 BT1001, designated as BtRha78A, is composed of 2190 bp and correspondingly encodes 729 amino acid residues with a theoretical molecular mass of 83.9 kDa. Recombinant bacterial Rha78s varied greatly in the number of amino acid residues from 523 to 1030 (Table S2). BtRha78A shared a low sequence identity (< 35%) with reported bacterial Rha78s. Phylogenetic tree (Fig. 1) indicated that BtRha78A clustered together with seven bacterial Rha78s and these Rha78s had the same proposed general acid, Asp (Fig. 2), but the proposed general acid of the other bacterial Rha78s was Glu (Table S2). It was concluded that the bacterial Rha78s might be divided into two subfamilies based on the general acid residue. The subfamily I Rha78s taking the residue Asp as the general acid contained fewer amino acid residues than the subfamily II Rha78s using the residue Glu as the general acid (Table S2). Furthermore, several charged residues Asp330, Lys333, Arg334, Arg336, and Asp342 along the proposed general acid Asp335 were completely conserved in the subfamily I Rha78s, and thus formed a conserved amino acid

Fig. 1 Phylogenetic tree of 17 recombinant bacterial Rha78s. The phylogenetic tree is constructed using the software MEGA 7.0 based on the neighbor-joining method. ACR19007, RhaB1 from *Lactobacillus plantarum* NCC245; CAD65558, Ram1 from *L. plantarum* WCFS1; ZP_07367044, Ram from *Pediococcus acidilactici*; AEX05711, KoRha from *Klebsiella oxytoca*; ZP_07366943, Ram2 from *P. acidilactici*; ACR19005, RhaB2 from *L. plantarum* NCC245; CAD65560, Ram2 from *L. plantarum* WCFS1; BAB62315, RhaB from *Bacillus* sp. GL1; AAR96047, RhmB from *Thermomicrobia bacterium* PRI-1686; AGH13557, RhaB1 from elephant feces metagenome; AHJ22585, BbRha from *Bifidobacterium breve*; AAV43293, RamA from *L. acidophilus*; CAB53341, RamA from *Clostridium stercorarium*; BAC68538, SaRha78A from *Streptomyces avermitilis*; AAR96046, RhmA from *Thermomicrobia bacterium* PRI-1686; AGS77942, BdRham from *Bifidobacterium dentium*. BtRha78A is marked with an orange circle

Fig. 2 Sequence alignment of BtRha78A with homologous bacterial Rha78s (subfamily I Rha78s). The Rha78s indicated by the GenBank accession ACR19007, CAD65558, ZP_07367044, AEX05711, ACR19005, CAD65560, and ZP_07366943 are the same as the Fig. 1. The initial alignment is generated using the software DNAMAN 7, manually adjusted, and then illustrated with the server ESPrnt 3.0. Strictly conserved residues are highlighted with red shaded boxes, and moderately conserved residues are boxed. The proposed general acid and base are marked with blue circle. The residues forming hydrogen bonds with Tris are marked with green triangle. Two aromatic residues Trp interacting with the rhamnose by hydrophobic stacking are marked with pink triangle

motif (Asp330–Asp342), described as the general acid motif (Fig. 2).

Expression and purification of recombinant BtRha78A

The gene *BtRha78A* was ligated to pET28a vector to construct the recombinant plasmid pET28a-BtRha78A, which

Fig. 3 SDS-PAGE (15%) analysis for purified BtRha78A. M, protein marker; Lane 1, the purified BtRha78A. Molecular mass of recombinant BtRha78A containing an N-terminal (His)₆ tag is 86.1 kDa

was multiplied in *E. coli* Trans1-Blue and was used for protein expression in *E. coli* BL21(DE3). The site-directed mutants of BtRha78A were successfully constructed through site-directed mutagenesis. There were no obvious differences in expression level among BtRha78A wild-type and site-directed mutants. BtRha78A wild-type and site-directed mutants were produced as the fusion protein with an N-terminal (His)₆ tag. After purification with Ni-NTA affinity chromatography, the purified BtRha78A was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). Apparent molecular mass of approximately 86 kDa was consistent with the theoretical value.

Enzymatic properties of recombinant BtRha78A

Enzymatic activity assay showed that BtRha78A might efficiently hydrolyze the synthetic substrate *p*NPR. However,

Fujimoto had given the observation that BtRha78A was catalytically inactive (Fujimoto et al. 2013).

Effect of pH and temperature on enzyme activity

pH and temperature are two important physical factors that influence catalytic activity of an enzyme. The effects of pH and temperature on BtRha78A were studied using *p*NPR as substrate. BtRha78A had the highest activity in pH 6.5 with a decline in enzymatic activity observed in acidic (< 5.5) and alkaline pH (> 8.6) (Fig. 4a). Most recombinant bacterial Rha78s displayed the highest enzymatic activity in neutral or slightly acidic buffer (Table 1) and most recombinant bacterial Rha78s originated from lactic acid bacteria (LAB). Moreover, BtRha78A showed high activity (> 60%) at alkaline pH 7.5–8.6. This was necessary that alkaline environment contributed to dissolution of flavonoid glycosides, the natural substrates of α -L-rhamnosidases. Enzymatic activity of BtRha78A was inhibited approximately threefold by Tris-HCl buffer in comparison with the corresponding NaH₂PO₄-Na₂HPO₄ buffer or glycine-NaOH buffer. This might be due to the similar molecular structure of Tris with the product rhamnose. Molecular structure of Tris contained three hydroxyls and one amino which might form the hydrogen bonds with active site as suggested by the complex structure of BtRha78A with Tris. The optimum temperature of BtRha78A was 60 °C, and more than 50% of catalytic activity was maintained in the wide temperature range from 35 to 65 °C (Fig. 4b). The most recombinant bacterial Rha78s displayed the highest enzymatic activity at high temperature (> 50 °C) (Table 1). However, all recombinant bacterial Rha78s originated from the mesophiles, except RhmA and RhmB from *Thermomicrobia* bacterium PRI-1686. Optimum temperature of BtRha78A only was lower than that of Ram2 from *P. acidilactici*, and RhmA and RhmB from *Thermomicrobia* bacterium PRI-1686. High temperature was helpful for dissolving the flavonoid glycosides in the biotransformation reaction.

Effect of pH and temperature on enzyme stability

BtRha78A exhibited pH stability over a wide range from 5.5 to 9.0 (Fig. 4c). In this pH range, more than 95% of the activity was retained after the incubation of 1 h at 40 °C. Interestingly, the activity of BtRha78A was enhanced significantly after the incubation in alkaline environment. BtRha78A retained 142 and 133% of initial activity after the incubation of 1 h at 40 °C in 50 mM glycine-NaOH buffer (pH 8.6 and pH 9.0), respectively. Thermal inactivation curves (Fig. 4d) showed that the enzyme had relatively high thermostability. The half-life of BtRha78A was approximately 100 min at 50 °C, and BtRha78A kept 60% of initial activity after the

Fig. 4 Enzymatic properties of recombinant BtRha78A. **a** Effects of pH on enzymatic activities of BtRha78A at 50 °C. 50 mM citric acid-sodium citrate buffer (pH 5.0–6.0, square), NaH₂PO₄–Na₂HPO₄ buffer (pH 6.0–8.0, circle), Tris–HCl buffer (pH 8.0–8.6, diamond), and glycine–NaOH buffer (pH 8.6–9.0, triangle) were used. Enzymatic activities are normalized as percentages of maximum activity in pH 6.5. **b** Effects of temperature on enzymatic activities of BtRha78A in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5). Enzymatic activities are normalized as percentages of maximum activity at 60 °C. **c** pH stability profiles of BtRha78A. Enzymatic activity of

BtRha78A without incubation is taken as the 100%. **d** Thermal inactivation profiles of BtRha78A varying the time. Enzymatic activity of BtRha78A without incubation is taken as the 100%. The data for residual activities at 45 and 50 °C are shown in square and circle, respectively. **e**. Thermal inactivation profiles of BtRha78A varying the temperature. Enzymatic activity of BtRha78A without incubation is taken as the 100%. The half-inactivation temperature T_{50} is marked with the dashed line. **f** Enzyme kinetics curve of BtRha78A. All reactions were performed in triplicate, and error bars represent the standard deviations of mean

incubation of 4 h at 45 °C. Thermal inactivation experiment performed by incubating the enzyme for 30 min at different temperature displayed that the half-inactivation temperature T_{50} of BtRha78A was about 51 °C (Fig. 4e). Most Rha78s were thermostable. This characteristic of α -L-rhamnosidases was required for biocatalytic application of the enzyme complex naringinase containing α -L-rhamnosidase and β -D-glucosidase to cleave terminal α -L-rhamnose from natural products specially.

Enzyme steady-state kinetics

Enzyme kinetic parameters were determined on the hydrolysis of *p*NPR at 50 °C and in pH 6.5 (Fig. 4f). Enzyme kinetics indicated that BtRha78A had a $V_{\max}$ of 48.7 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, the turnover number k_{cat} of 69.8 s^{-1} , the Michaelis constant K_M of 0.53 mM, and the catalytic efficiency k_{cat}/K_M was $1.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). The k_{cat} value of BtRha78A was high and only lower than Ram (311 s^{-1}) from *P. acidilactici* and RhaB (140 s^{-1}) from *Bacillus* sp. GL1 (Table 1). The K_M value of BtRha78A was low, and the most recombinant bacterial Rha78 s had a low K_M value ($< 1.0 \text{ mM}$, Table 1), which suggested that the Rha78 s showed a high affinity with *p*NPR.

Effect of organic solvents on enzyme activity

Organic solvents were frequently used for dissolving the substrates in the biocatalysis process. We observed the effects of organic solvents on enzymatic activity of BtRha78A. BtRha78A exhibited a great tolerance on low concentration of alcohols and retained 78–85% of catalytic activity at the alcohols of 1% (v/v), and maintained 67% of catalytic activity at ethylene glycol of 5% (v/v) (Fig. 5). This was very significative for biotransformation of flavonoid glycosides because these natural substrates were well soluble in the alcohols such as methanol. The catalytic activity of BtRha78A was markedly inhibited by *N, N*-dimethylformamide (DMF) and acetonitrile of 5% (v/v); only 10% of the activity was residual.

Identification of functional residues

We explored the functional residues of BtRha78A crucial for enzyme catalysis by site-directed mutagenesis based on sequence alignment and structural analysis. Multiple sequence alignment indicated that the Rha78s were completely conserved in the proposed general base (Glu), but the proposed general acid was Asp or Glu. Interestingly,

Table 1 Enzymatic properties of recombinant bacterial Rha78s

Enzyme	Organism	Genbank accession	pH _{opt}	T _{opt} (°C)	k _{cat} (s ⁻¹)	K _M (mM)	References
BtRha78A	<i>B. thetaiotaomicron</i> VPI-5482	AAO76108	6.5	60	69.8	0.53	This study
RhaB	<i>Bacillus</i> sp. GL1	BAB62315	7.0	40	140	0.28	Hashimoto et al. (2003), Cui et al. (2007)
BbRha	<i>Bifidobacterium breve</i>	AHJ22585	6.5	55	2.5	2.2	Zhang et al. (2015)
BdRham	<i>Bifidobacterium dentium</i>	AGS77942	6.0	35	NA	1.06	Bang et al. (2015)
RamA	<i>Clostridium stercorarium</i>	CAB53341	7.5	60	NA	NA	Zverlov et al. (2000)
KoRha	<i>Klebsiella oxytoca</i>	AEX05711	5.0	NA	0.98	0.21	O'Neill et al. (2015)
RamA	<i>Lactobacillus acidophilus</i>	AAV43293	6.0	37–45	NA	0.7	Beekwilder et al. (2009)
RhaB1	<i>L. plantarum</i> NCC245	ACR19007	7.0	50	NA	0.57	Avila et al. (2009)
RhaB2	<i>L. plantarum</i> NCC245	ACR19005	5.0	60	NA	0.51	Avila et al. (2009)
Ram1	<i>L. plantarum</i> WCFS1	CAD65558	7.0	37–45	64	6.3	Beekwilder et al. (2009)
Ram2	<i>L. plantarum</i> WCFS1	CAD65560	NA	NA	NA	NA	Beekwilder et al. (2009)
Ram	<i>Pediococcus acidilactici</i>	ZP_07367044	5.5	50	311	16.2	Michlmayr et al. (2011)
Ram2	<i>P. acidilactici</i>	ZP_07366943	4.5	70	3.7	22.3	Michlmayr et al. (2011)
SaRha78A	<i>Streptomyces avermitilis</i>	BAC68538	6.0	50	26.4	0.03	Ichinose et al. (2013), Fujimoto et al. (2013)
RhmA	Thermomicrobia bacterium PRI-1686	AAR96046	7.9	70	NA	0.46	Birgisson et al. (2004)
RhmB	Thermomicrobia bacterium PRI-1686	AAR96047	5.0–6.9	70	NA	0.66	Birgisson et al. (2004)
RhaB1	Elephant feces metagenome	AGH13557	6.5	40	36.38	0.79	Rabausch et al. (2014)

NA: not available

pH_{opt}: optimum pH; T_{opt}: optimum temperature; k_{cat} and K_M: enzyme kinetic parameters for hydrolysis of the substrate *p*NPR

Table 2 Michaelis-Menten kinetic parameters of recombinant BtRha78A on the hydrolysis of *p*NPR

$V_{\max}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)
48.7 ± 0.9	69.8 ± 1.2	0.53 ± 0.03	1.3×10^5

All reactions were performed in 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5) at 50 °C for 30 s

the number of amino acid residues between putative general acid and base was close (254–282) among all reported Rha78s (Table S2). The proposed general acid of BtRha78A was Asp335, located at the general acid motif at the loop linking α_3 -helix and α_4 -helix. The proposed general base of BtRha78A was Glu595, located at the loop linking α_{13} -helix and α_{14} -helix (Fig. 6a). The distance between the closest atoms of catalytic side-chains of Asp335 and Glu595 was 6.0 Å (Fig. 6a), which was the shortest distance of a typical inverting glycoside hydrolase (6–11 Å) (McCarter and Withers 1994). However, the distance of the other three Rha78s with 3D structure was shorter, 5.5 Å for KoRha, 5.6 Å for RhaB, and 5.7 Å for SaRha78A, suggesting that the Rha78s might employ a new inverting mechanism for enzyme catalysis (Cui et al. 2007). To verify whether Asp335 and Glu595 were the general acid and base, the site-directed mutants D335A, D335N, D335E, E595A, and E595Q were constructed. These mutations produced a loss of 560- to 19,170-fold in enzymatic activity on the hydrolysis of *p*NPR (Table 3), confirming the catalytic function of Asp335 and Glu595. The general base of the four Rha78s with 3D structures all was Glu, but the general acid was

Fig. 5 Effects of organic solvent on enzymatic activity of BtRha78A. Enzymatic activity of BtRha78A in the absence of organic solvent is taken as the control. The data for enzymatic activities at 1 and 5% (v/v) are shown in green and orange, respectively. All reactions were performed in triplicate, and error bars represent the standard deviations of mean

different, Asp or Glu. The mutant D335E of BtRha78A had a decrease of 2770-fold in enzymatic activity (Table 3), and the mutation of the general acid Glu636 in SaRha78A into Asp brought a 377-fold reduction of catalytic activity (Fujimoto et al. 2013). This revealed that general acid Asp and Glu could not be exchanged in the Rha78s. The residue Asp was preceded by an unusual nonproline cis-peptide bond,

Fig. 6 Comparisons of the residues involved in the product or analog binding between BtRha78A (PDB id: 3CIH) and KoRha (PDB id: 4XHC). **a** The catalytic domain of BtRha78A. The general acid motif is labeled in yellow. Tris is shown in green ball and sticks, the general acid Asp335 and general base Glu595 in pink ball and sticks, and the distance between side-chain of general acid and that of general base is displayed in gray dashed line. **b** The residues of BtRha78A

interacting with Tris. **c** The residues of KoRha interacting with rhamnose. The ligand Tris or rhamnose is shown in green ball and sticks, the acidic residues in pink ball and sticks, the aromatic residues in yellow ball and sticks, and the basic residues in cyan ball and sticks. Hydrogen bonds are shown in gray dashed lines and the distance is labeled. These images of the structures are generated using the program PyMOL

Table 3 Enzymatic activity of recombinant BtRha78A wild type and the site-directed mutants

Enzymes	Function or interaction	Specific activity ^a (U g ⁻¹)	Reduction fold
Wild type	–	$2.3 \times 10^4 \pm 2.2 \times 10^2$	–
D335A	General acid	41.0 ± 1.1	560
D335 N	General acid	19.6 ± 0.2	1170
D335E	General acid	8.3 ± 0.4	2770
E595A	General base	3.3 ± 0.4	6970
E595Q	General base	1.2 ± 0.2	19,170
D330A	Hydrogen bond	9.8 ± 1.3	2350
R334A	Hydrogen bond	83.4 ± 2.8	280
W339A	π – π interaction	18.7 ± 0.1	1230
D342A	Hydrogen bond	3.0 ± 0.3	7670
Y383A	Hydrogen bond	31.0 ± 0.6	740
W440A	Hydrogen bond	43.3 ± 0.9	530
	π – π interaction		
H620A	Hydrogen bond	584.3 ± 7.9	40

^aThe enzymatic activity was determined in 50 mM NaH₂PO₄–Na₂HPO₄ buffer (pH 6.5) containing 1.0 mM *p*NPR at 50 °C

which assisted in projecting the carboxyl group into active center in BtRha78A and KoRha (O'Neill et al. 2015).

The complex structure of BtRha78A with the product analog Tris would be helpful for understanding enzyme hydrolysis for the rhamnosides. Structural analysis for the complex of BtRha78A with Tris revealed that most residues interacting with Tris were charged and aromatic (Fig. 6b). It was common in the Rha78s. The side-chains of the residues Asp330, Arg334, Asp342, Tyr383, Trp440, and His620 made nine hydrogen bonds with Tris, two aromatic residues Trp339 and Trp440 might sandwich the rhamnose by hydrophobic stacking (π – π interaction), and the residues Ile380, Phe437, Tyr610, and Trp622 formed hydrophobic interaction with Tris (Fig. 6b). The residues of BtRha78A interacting with Tris superimposed well with those of KoRha interacting with the product rhamnose (Fig. 6b, c). Furthermore, these residues were completely conserved in the subfamily I Rha78s, except Ile380 (Fig. 2). Alanine scanning mutagenesis had been a widespread and rapid technique for probing the contribution of side chain of an individual residue to the functionality of an enzyme (Morrison and Weiss 2001). The substitution of the residues Asp330, Arg334, Trp339, Asp342, Tyr383, Trp440, and His620 with alanine resulted in a significant decrease in catalytic performance by 40- to 7670-fold (Table 3), suggesting that these residues played important roles in enzyme catalysis. The key residues Asp330, Arg334, Asp335, Trp339, and Asp342 located at the general acid motif, suggesting that the conserved general acid motif might be important for enzyme catalysis in the subfamily I Rha78s. The conserved residues Asp330

and Asp342 might assist in enzyme catalysis by the acidic side-chains to offset the short distance between general acid and base for the inverting catalytic mechanism in the Rha78s.

Conclusions

In this study, we characterized a putative GH78 α -L-rhamnosidase BtRha78A from *B. thetaiotaomicron* VPI-5482. Recombinant BtRha78A might efficiently hydrolyze *p*NPR. Recombinant BtRha78A had significant advantages, such as high activity at alkaline pH, high activity at high temperature, high thermostability, good pH stability, and alcohol tolerance. Furthermore, the key residues of BtRha78A for enzyme catalysis were confirmed by site-directed mutagenesis based on sequence alignment and structural analysis. The general acid and base of BtRha78A were Asp335 and Glu595, respectively. The bacterial Rha78s might be divided into two subfamilies based on the general acid residue. The conserved residues Asp330, Arg334, Trp339, Asp342, Tyr383, Trp440, and His620 were important for enzyme catalysis. Our findings might discover a promising alternative biocatalyst for biotransformation and provide an opportunity for better understanding of the catalytic mechanism of GH78 α -L-rhamnosidase.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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