

Article

Improving Rutin Biotransformation Efficiency of α -L-Rhamnosidase from *Bacteroides thetaiotaomicron* VPI-5482 via Targeted Mutagenesis Focused on General Acid Motif

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Abstract: α -L-Rhamnosidases with desirable activity and thermostability profiles could be used for the biocatalytic production of the flavonoid glucoside isoquercetin from natural rutin for functional food. Herein, to improve the catalytic activity of GH78 α -L-rhamnosidase BtRha78A from *Bacteroides thetaiotaomicron* VPI-5482, a list of residues located at the conserved general acid motif were selected for targeted mutagenesis by the sequence alignment of BtRha78A with homologous α -L-rhamnosidases. Ala-scanning mutagenesis and site-directed mutagenesis based on sequence alignment were performed, and the relative activity on rutin was evaluated. Furthermore, the reaction time curves and enzyme kinetics of better mutants were determined. The results indicate that the conversion rates of mutants V338A, V338I, S340A, and G341A were increased by 21.3%, 20.1%, 13.2%, and 1.6%, respectively, compared with the wild type when using whole-cell biotransformation. Moreover, the catalytic efficiency k_{cat}/K_M value of mutant V338A was 1.3-fold higher than that of the wild type. The best mutant, V338A, was employed for the enzymatic preparation of isoquercetin via the biotransformation of rutin at a concentration of 2 mM, and 1.80 g of isoquercetin was obtained. The identification of the best mutant V338A lays the foundation for the efficient preparation of isoquercetin via the biotransformation of rutin, which in turn provides theoretical guidance for its large-scale production.

Keywords: α -L-rhamnosidase; general acid motif; targeted mutagenesis; rutin; isoquercetin; whole-cell biotransformation



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1. Introduction

Based on amino acid sequence similarity, α -L-rhamnosidases are distributed in the glycoside hydrolase families GH78, GH106, and GH28 [1], among which the GH78 family α -L-rhamnosidases (Rha78s) have been investigated more thoroughly. To date, 28 Rha78s have been discovered and characterized according to the CAZy database. Most of the characterized bacterial Rha78s exhibit an optimal temperature higher than the growth temperature of the bacteria. Their optimal pH is weakly acidic to neutral [2–8], and most of the α -L-rhamnosidases have strong affinity for the synthetic substrate *p*-nitrophenol α -L-rhamnopyranoside (*p*NPR). The sequence lengths of different Rha78s differ greatly (523–1030 residues). The number of amino acids in general acid–base pairs is similar (254–282 residues). The general acid is Asp or Glu, and the general base is Glu.

Six crystal structures of Rha78 have been determined so far. BT1001 (BtRha78A) from *Bacteroides thetaiotaomicron* VPI-5482 contains four structural domains: domain N, domain B, domain A, and domain C [9]. Among them, domain A is the largest catalytic domain and is

mainly composed of α -helices, and the product analogue Tris is bound to this domain. The remaining three domains exist as β -folds. BsRhaB from *Bacillus* sp. GL1 has five structural domains: four β -fold structural domains and one $(\alpha/\alpha)_6$ catalytic structural domain; this enzyme was the first to aid in the reporting of the determination of the crystal structure of α -L-rhamnosidase and the identification of the $(\alpha/\alpha)_6$ -barrel structure as the catalytic structural domain [3]. DtRha from *Dictyoglomus thermophilum* possesses six structural domains [10]. KoRha from *Klebsiella oxytoca* has the smallest structure and contains only two structural domains: an $(\alpha/\alpha)_6$ catalytic domain and a β -fold domain [11]. The structure of α -L-rhamnosidase SaRha78A from *Streptomyces avermitilis* in complex with L-rhamnose contains six structural domains: an $(\alpha/\alpha)_6$ catalytic domain and a Ca^{2+} -dependent non-catalytic carbohydrate-binding domain (CBM67) [7]. A-RhA from *Aspergillus terreus* CCF 3059 contains six structural domains [12]. The structures of the six Rha78s exhibit a low sequence identity of 20–30%. The sequence alignment of GH78 α -L-rhamnosidase subfamily I yields a conserved sequence (Asp330–Asp342), which is named a general acid motif [13]. Asp335 is the catalytic general acid, and the completely conserved amino acids Asp330, Arg334, Trp339, and Asp342 are the functional residues of the enzyme.

Rutin is a disaccharide glycoside of quercetin which is derived from *Sophora japonica*, buckwheat, and other plants. Rutin displays a wide range of biological activities, exerting antioxidant [14,15], anti-inflammatory [16], hypotensive [17], hypolipidemic [18], anti-obesity [19], anti-asthmatic [20], anti-platelet [21], anti-cancer, and neuroprotective effects [22], but possesses low biological activity and bioavailability. Compared with rutin, its derhamnosyl product, isoquercetin, has higher biological activity and bioavailability [23–25]. Currently, although isoquercetin is widely distributed, it is inadequate for the food and pharmaceutical industries. Rutin can be hydrolyzed to produce isoquercetin by α -L-rhamnosidase (Figure 1). α -L-Rhamnosidase has become an important biotechnological enzyme that can hydrolyze L-rhamnose at the ends of natural products, such as rutin, hesperidin, naringin, trichostatin, ginsenoside, and epimedium [26]. Some examples of this include the hydrolysis of rutin to prepare isoquercetin [27], the hydrolysis of naringin from citrus juices to remove bitterness [28,29], and the improvement in wine aroma by the deglycosylation of terpenes [30] for applications in industry, medicinal pretreatment, and food production. Furthermore, isoquercetin can be prepared by the enzymatic hydrolysis of α -L-rhamnosidase [27,31–34].

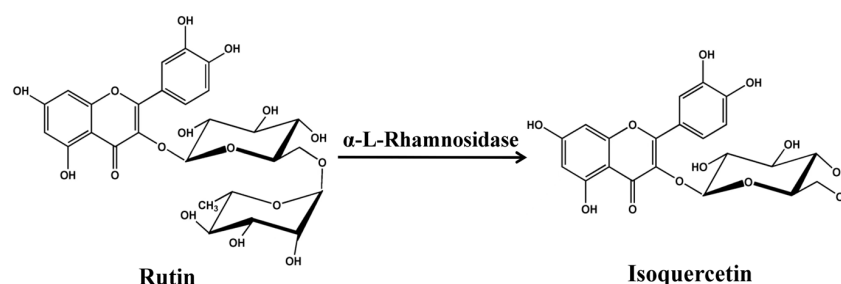


Figure 1. Chemical structure of flavonoids rutin and isoquercetin, and the biotransformation from rutin to isoquercetin by α -L-rhamnosidase.

Human gut bacteria possess powerful metabolic functions and can degrade and utilize a wide range of polysaccharides [35]. The α -L-rhamnosidase BtRha78A from *B. thetaiotaomicron* VPI-5482 is heterologously expressed in *E. coli*, and the enzymatic properties of BtRha78A have been characterized in detail [13], showing that it can hydrolyze the synthetic substrate *p*NPR efficiently. The optimal pH of BtRha78A is 6.5, and it shows the highest activity at 60 °C. BtRha78A displays good pH stability and relatively high thermal stability. BtRha78A can tolerate low concentrations of alcohol. These advantages make BtRha78A a promising alternative biocatalyst for industrial applications. The catalytic general acid Asp 335 and the catalytic general base Glu595 of BtRha78A are obtained by site-directed mutagenesis. Ala-scanning mutagenesis reveals that the conserved residues

Asp330, Arg334, Trp339, Asp342, Tyr383, Trp440, and His620 are essential for enzyme catalysis. Most of the functional residues locate on the conserved general acid motif (Asp330–Asp342) and are completely conserved in GH78 subfamily I. Despite BtRha78A possessing good enzymatic properties, it shows low hydrolytic activity on rutin [26,36]. Currently, a rational design based on sequence alignment and structural analysis is a good approach for molecular modification [37]. The catalytic activity of *Aspergillus niger* XynB has been enhanced by selecting residues at the active site [38]. The thermal stability of fungal GH11 xylanase has been improved by using site-directed mutagenesis by the guiding of sequence alignment and structural analysis [39].

In this study, we focus on the general acid motif of BtRha78A (Asp330–Asp342) by sequence alignment with the same subfamily of Rha78s. Combined with its crystal structure (PDB id 3CIH), we take the conserved and semi-conserved residues of the general acid motif, except for the functional residues, as the targeted sites. These targeted residues are mutated by the Ala-scanning mutagenesis method [40], and site-directed mutants are designed on the basis of sequence alignment. We use rutin as a substrate to analyze the catalytic activity of the mutants and characterize the enzymatic properties of the best mutant. Finally, in order to guide industrial application, the catalytic performance of mutant V338A is verified in scale-up production.

2. Results and Discussion

2.1. Structural and Sequence Analyses of General Acid Motif

The general acid motif of α -L-rhamnosidase BtRha78A from *Bacteroides thetaiotaomicron* is highly conserved and is essential for the catalytic function of this enzyme, and a series of residues are crucial for the catalytic function of the enzyme [13]. The 3D structure (Figure 2A) of BtRha78A shows that the general acid motif is located in the catalytic domain, which is a flexible loop. In addition, the general acid motif is involved in the formation of the substrate binding pocket. The general acid Asp335 and the general base Glu595 are located near the product analogue Tris (Figure 2B). As is shown in Figure 2C, the general acid motif contains six charged residues (three acidic residues and three basic residues) and two aromatic residues positioned near the substrate entry channel. And the residues Asp330, Arg334, and Asp342 form hydrogen bonds with the analogue Tris.

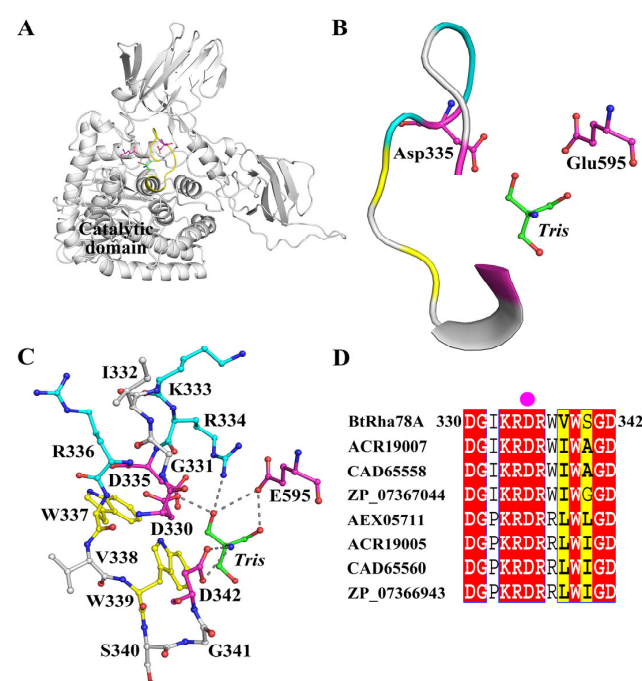


Figure 2. The location and conformation of the general acid motif in BtRha78A. (A). The crystal structure of BtRha78A, where the catalytic residues of the general acid and the general base are shown

in pink and the general acid motif is shown in yellow. (B). The flexible loop of the general acid motif of BtRha78A. (C). The residues of BtRha78A interacting with Tris. The ligand Tris 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. The images of the structures were generated by using the program PyMOL. (D). The sequence alignment of bacterial Rha78s at the general acid motif. The initial sequence alignment was generated by DNAMAN 7 software and then illustrated by the ESPript 3.0 server. Completely conserved residues are highlighted with red-shaded boxes, and moderately conserved residues are highlighted with yellow-shaded boxes. The catalytic general acid is labeled with a pink circle.

Based on amino acid sequence alignment of bacterial Rha78s at the general acid motif (Figure 2D), the residues Gly331, Lys333, Arg336, and Gly341 are also completely conserved in addition to five functional residues. Interestingly, the residues located at site 332 and site 337 are moderately conserved. One group of residues located at site 332 is Ile, and the other is Pro, while one group of residues located at site 337 is Trp and the other is Arg. The residues located at site 338 and site 340 are also moderately conserved. Previous experiments demonstrated that the eight Ala-scanning mutants at the general acid motif in addition to functional residues did not result in the loss of activity, and they could modulate the catalytic activity of α -L-rhamnosidase on the substrate *p*NPR [36], implying that they might also affect the catalytic activity of the enzyme on the natural substrate. Hence, we thought that the activity of rutin could also be increased. Based on the above analysis, we evaluated the catalytic activity of the eight Ala-scanning mutants on rutin. Furthermore, we designed the site-directed mutants I332P, W337R, V338I, V338L, S340I, and S340L based on sequence alignment and evaluated the hydrolytic activity on rutin.

2.2. Screening of the Best Mutants with Higher Catalytic Activity

The relative hydrolytic activity of the site-directed mutants of BtRha78A at the general acid motif against rutin were evaluated by a spectrophotometric method for the high-throughput screening of α -L-rhamnosidase activity combined with a thermophilic and highly active β -D-glucosidase using whole-cell biocatalysts [36]. The screening results of the Ala-scanning mutants revealed that mutants V338A, S340A, and G341A showed higher absorption values at the wavelength of 320 nm than the wild type, and the absorption values of mutants V338A and S340A at the wavelength of 400 nm were lower than that of the wild type. It was indicated that mutants V338A and S340A displayed higher hydrolytic activity on rutin than the wild type. Mutant G341A was also selected for further validation, although its absorption value at 400 nm was higher than that of the wild type (Figure 3A).

The hydrolytic activity of the site-directed mutants of BtRha78A based on sequence alignment on rutin was compared by using the UV–visible method. The results show that the absorption value of mutant V338I at 320 nm was higher than that of the wild type, and mutants I332P, W337R, V338L, S340I, and S340L had some reductions to varying degrees (Figure 3B). Mutant V338I displayed higher catalytic activity on rutin than the wild type. Therefore, mutant V338I was selected for further validation.

The conversion rates of the four mutants obtained by UV–visible screening on rutin were further determined by HPLC by using whole-cell biocatalysts. The results, as shown in Figure 4, indicate that the conversion rates of the BtRha78A wild type, V338A, V338I, S340A, and G341A were 44.8%, 66.1%, 64.9%, 58.0%, and 46.4%, respectively. Notably, the conversion rates of mutants V338A and V338I on rutin were increased by more than 20% compared with the wild type, and the conversion rate of G341A was only 1.6% higher. The four mutants showed higher catalytic activity on rutin than the wild type when using the whole-cell biocatalysts.

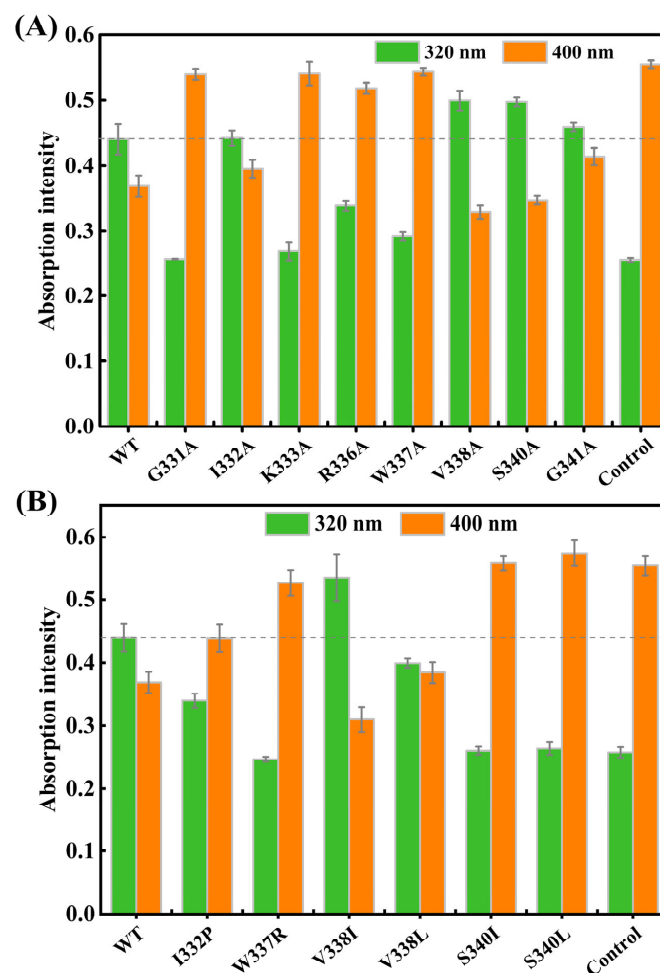


Figure 3. Relative activities of the BtRha78A mutants at general acid motif on rutin by using UV-vis method. (A). Relative activities of the Ala-scanning mutants at the general acid motif. (B). Relative activities of the site-directed mutants at the general acid motif based on sequence alignment. All reactions were performed in triplicate, and the error bars represent the standard deviations of the mean.

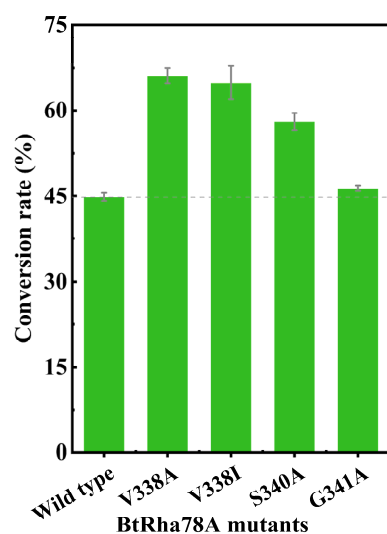


Figure 4. Bioconversion rates of the BtRha78A wild type and its best mutants on rutin by using whole-cell biocatalysts. All reactions were performed in triplicate, and the error bars represent the standard deviations of the mean.

2.3. Biotransformation of Rutin by BtRha78A Wild Type and Its Best Mutants

The reaction time curves for the whole-cell biotransformation of the BtRha78A wild type and its best mutants on rutin display that the conversion rates of mutants V338A, V338I, and S340A were higher than that of the wild type (Figure 5). The conversion rate of mutant V338I was superior to the WT, S340A, and V338A before 2.5 h. However, after 2.5 h, the conversion rate of V338A was superior to the WT, S340A, and V338I. The conversion rate of the best mutant, V338A, could reach 94.2% at 4.5 h, while the conversion rate of the WT was only 79.0% at 5.0 h.

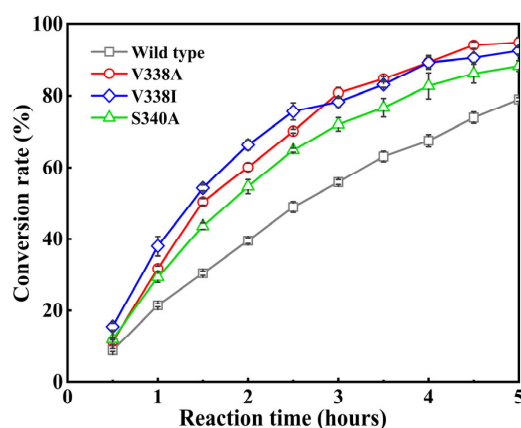


Figure 5. Effects of the reaction time on the biotransformation of rutin by the BtRha78A wild type and the mutants by using whole-cell biocatalysts. All reactions were performed in triplicate, and the error bars represent the standard deviations of the mean.

When taking the purified enzymes as the biocatalysts, the conversion rates of the BtRha78A wild type and its best mutants on rutin gradually increased with reaction time, with a rapid increase from 20 min to 180 min and a slow increasing trend from 180 min to 240 min. The conversion rates of mutant V338A were lower than those of the wild type until 40 min (Figure 6), while the conversion rates of mutant V338A became higher than those of the wild type from 40 min to 240 min. When the reaction reached the steady phase at 180 min, the conversion rates of the WT, V338A, V338I, and S340A were 83.8%, 88.0%, 81.5%, and 81.3%, respectively. The conversion rates of mutants V338I and S340A on rutin were consistently lower than that of the wild type, indicating that the hydrolytic activity of mutants V338I and S340A was reduced. However, the conversion rates of mutants V338I and S340A on rutin were higher than that of the wild type when using whole-cell biocatalysts.

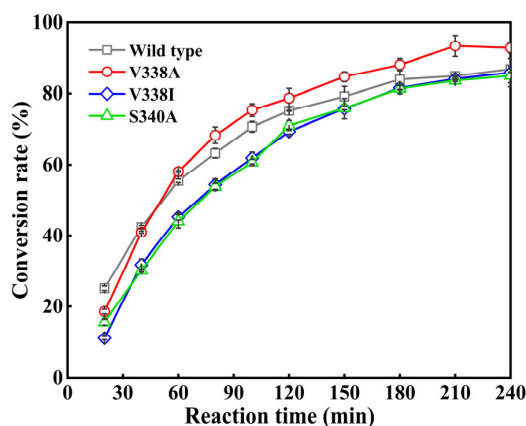


Figure 6. Effects of the reaction time on the biotransformation of rutin by the BtRha78A wild type and the mutants by using purified enzymes. All reactions were performed in triplicate, and the error bars represent the standard deviations of the mean.

2.4. Enzyme Steady-State Kinetics for BtRha78A Wild Type and Its Best Mutants on Rutin

The enzyme kinetic constants of the BtRha78A wild type and its best mutants were fitted by the Michaelis–Menten equation based on the enzyme kinetic reaction curves. The results (Table 1) show that only mutant V338A had a higher $V_{\max}$ ($0.43 \mu\text{mol min}^{-1} \text{mg}^{-1}$) than the wild type ($0.40 \mu\text{mol min}^{-1} \text{mg}^{-1}$), whereas mutants V338I and S340A had lower $V_{\max}$ than the wild type (0.39 and $0.29 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively). The k_{cat} values for the wild type, V338A, V338I, and S340A were 0.57 , 0.62 , 0.56 , and 0.42 s^{-1} , respectively. The K_{M} values of three mutants were lower, thereby indicating strong affinity for the substrate, rutin, with the lowest K_{M} value (0.48 mM) being that of mutant V338A and the highest K_{M} value (0.86 mM) that of mutant V338I. The $k_{\text{cat}}/K_{\text{M}}$ values of V338A, WT, V338I, and S340A were 1291.7 , 1000.0 , 651.2 , and $656.3 \text{ s}^{-1} \text{ M}^{-1}$, respectively. The catalytic efficiency of mutant V338S was 1.3 times higher than that of the wild type. Overall, only mutant V338A had higher $V_{\max}$, k_{cat} , and $k_{\text{cat}}/K_{\text{M}}$ than the wild type, whereas its K_{M} was lower than that of the wild type, thus revealing the reason why mutants V338I and S340A had lower conversion rates on rutin than the wild type when using purified enzymes as the biocatalysts.

Table 1. Michaelis–Menten kinetic parameters of BtRha78A wild type and site-directed mutants on hydrolysis of rutin.

Enzymes	$V_{\max}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{ M}^{-1}$)
Wild type	0.40 ± 0.01	0.57 ± 0.01	0.57 ± 0.03	1000.0
V338A	0.43 ± 0.01	0.62 ± 0.01	0.48 ± 0.03	1291.7
V338I	0.39 ± 0.02	0.56 ± 0.03	0.86 ± 0.09	651.2
S340A	0.29 ± 0.01	0.42 ± 0.01	0.64 ± 0.03	656.3

2.5. Scale-Up Reaction for Production of Isoquercetin by BtRha78A V338A

In order to make the best mutant of BtRha78A, V338A, capable of being used for the large-scale production of isoquercetin and enable its potential application in the food industry, under the condition of whole-cell biotransformation, the flavonoid glucoside isoquercetin was produced by using mutant V338A. The reaction mixture was 1 L with 2 mM substrate concentration, and the conversion rate of isoquercetin reached more than 95% at 26 h. A total of 1.80 g of isoquercetin powder was obtained.

3. Materials and Methods

3.1. Bacterial Strains, Enzymes, and Reagents

Oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). TransStart® FastPfu Fly DNA polymerase and PCR-related reagents were purchased from TransGen Biotech (Beijing, China). FastDigest *Dpn* I restriction enzyme was obtained from Thermo Fisher Scientific (Waltham, MA, USA). *E. coli* DH5 α was used as the host for mutant construction, and *E. coli* BL21 (DE3) was used for protein expression. The flavonoids (rutin and isoquercetin) were provided by Yuanye Biotech (Shanghai, China). Kanamycin, IPTG, and imidazole were obtained from Sangon Biotech (Shanghai, China). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany).

3.2. Construction, Over-Expression, and Purification of BtRha78A Site-Directed Mutants

The QuikChange mutagenesis protocol was employed to construct the site-directed mutants [41] on the recombinant plasmid pET-28a-BtRha78A, as described in a previous study [13]. The primers for the generation of the site-directed mutants are listed in Table S1. The PCR products were transformed into *E. coli* DH5 α after template digestion by FastDigest *Dpn* I and verified by DNA sequencing (Sangon Biotech, Shanghai, China).

The BtRha78A wild type and site-directed mutants were obtained by heterologous expression in *E. coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany) according to our previous report [13]. The cells were harvested and

resuspended in 50 mM NaH_2PO_4 - Na_2HPO_4 buffer (pH 8.0) and stored at $-20\text{ }^\circ\text{C}$ for further use. The purity of the purified wild type and site-directed mutants was estimated by SDS-PAGE. The protein concentrations of the purified enzymes were measured at 280 nm by NanoDrop 2000.

3.3. Activity Evaluation of BtRha78A Site-Directed Mutants on Rutin by Using Whole Cells

The enzymatic activity of different BtRha78A site-directed mutants on rutin was evaluated by using a simple spectrophotometric method based on dual-wavelength detection by combining with a highly active β -D-glucosidase with the whole-cell biocatalysts [36]. The reaction mixture of 500 μL consisted of 455 μL of 50 mM NaH_2PO_4 - Na_2HPO_4 buffer (pH 6.5), 25 μL of rutin (20 mM, in 100% methanol), 10 μL of purified β -D-glucosidase TnBgl1A-DM (final concentration, 20 $\mu\text{g mL}^{-1}$), and 10 μL of whole cells (final concentration, 8 mg mL^{-1}) of different BtRha78A site-directed mutants according to the method developed in our previous report [36]. The absorption at 320 nm and 400 nm was measured. The reaction in which whole cells were replaced with 50 mM NaH_2PO_4 - Na_2HPO_4 buffer (pH 8.0) was used as the control. The best BtRha78A mutants on rutin obtained by that spectrophotometric method were further confirmed by the high-performance liquid chromatography (HPLC) method.

3.4. Biotransformation of Rutin by BtRha78A Wild Type and Its Best Mutants

The biotransformation of rutin into isoquercetin by the BtRha78A wild type and its best mutants for different reaction times was studied. The 500 μL reaction mixture consisted of 465 μL of 50 mM NaH_2PO_4 - Na_2HPO_4 buffer (pH 6.5), 25 μL of rutin (final concentration, 1.0 mM, dissolved in 100% methanol), and 10 μL of purified enzymes (final concentration, 40 $\mu\text{g mL}^{-1}$) or whole cells (final concentration, 8 mg/mL , resuspended in pH 8.0 50 mM NaH_2PO_4 - Na_2HPO_4 buffer) of BtRha78A wild type and its best mutants. The reaction was performed by shaking for different times in 200 rpm at $37\text{ }^\circ\text{C}$. The reaction was stopped by adding 500 μL of 100% methanol, and the mixture was centrifuged at 13,000 rpm for 5 min. The rutin and isoquercetin concentrations in the reaction were determined by HPLC.

3.5. Enzyme Steady-State Kinetics for BtRha78A Wild Type and Its Best Mutants on Rutin

The enzyme kinetics of the BtRha78A wild type and its best mutants on rutin were determined with varied rutin concentrations (0.1–3.5 mM) at $37\text{ }^\circ\text{C}$ in pH 6.5 50 mM NaH_2PO_4 - Na_2HPO_4 buffer for 20 min. The kinetic parameters V_{max} and K_M were acquired by fitting enzymatic activity as a function of substrate concentrations according to the Michaelis–Menten equation by using GraphPad Prism 8. The parameter k_{cat} was calculated by the equation $k_{\text{cat}} = V_{\text{max}}/[E]$, where $[E]$ is the molar concentration of the enzyme.

3.6. Scale-Up Reaction for Production of Isoquercetin

The 1 L reaction mixture consisted of 930 mL of 50 mM NaH_2PO_4 - Na_2HPO_4 buffer (pH 6.0), 50 mL of rutin (40 mM, in 100% methanol), and 20 mL of whole cells (final concentration, 2 mg/mL , resuspended in pH 8.0 50 mM NaH_2PO_4 - Na_2HPO_4 buffer) of the best BtRha78A mutants. The reaction was performed by shaking for different times in 200 rpm at $37\text{ }^\circ\text{C}$. A total of 100 μL of each sample was taken, 900 μL of 100% methanol was added, and the mixture was centrifuged at 13,000 rpm for 5 min. The rutin and isoquercetin concentrations in the reaction were determined by HPLC. When the conversion rates were the highest and almost no residue of the substrate was observed, the reaction was stopped. The isoquercetin that precipitated from the reaction was dissolved in 150 mL of 100% methanol, and the mixture was centrifuged at 5000 rpm for 15 min. The precipitate was discarded, and the supernatant was evaporated with water and methanol. And then 30 mL of 100% methanol was used to fully dissolve the precipitate isoquercetin attached to the wall on the spin vial. After centrifuging at 8000 rpm for 15 min, the supernatant was spin-evaporated to remove methanol, and isoquercetin was attached to the wall on the

spin vial and dried in the oven at 45 °C. Finally, the powder was deducted as isoquercetin and weighed.

3.7. High-Performance Liquid Chromatography (HPLC)

HPLC analysis was determined by a Waters 1525 binary pump and a Waters 2487 dual λ absorbance detector (Waters, Framingham, MA, USA) on a reverse-phase ZORBAX SB-C18 column (4.6 $\times$ 150 mm, particle size 5 μ m; Agilent, City of Santa Clara, CA, USA) at room temperature. HPLC chromatograms were recorded at 260 nm. Rutin and isoquercetin were eluted by using 0.5% (*v/v*) acetic acid (A) and acetonitrile (B) as the mobile phase for 20 min at a flow rate of 1.0 mL/min. The linear gradient started from 18% B at 0 min to 11% B at 18 min. Flavonoid glycoside substrates and their derhamnosylated products were quantified based on their standard curves. The conversion rate was calculated by using the following equation:

$$\text{Conversion rate (\%)} = (\text{isoquercetin concentration} / \text{initial rutin concentration}) \times 100$$

4. Conclusions

In this study, four mutants, V338A, V338I, S340A, and G341A, displayed higher catalytic activity on rutin than the wild type according to the UV–visible method. On the basis of studies of the enzymatic properties of the mutants with higher catalytic efficiency, we obtained the optimum reaction time and enzyme kinetic parameters. When the reaction time was 180 min, the steady phase was reached. Thus, 180 min could be chosen as the optimal reaction time for the hydrolysis of rutin when using purified enzymes as the biocatalysts. The K_M values of three mutants and the wild type were lower, thereby indicating strong affinity for the substrate, rutin. Separately, all enzyme kinetic parameters of mutant V338A were superior to those of the wild type. The use of whole-cell biocatalysis is important for industrial applications. With the determination of the reaction time curves of the mutants, we found that the reaction basically reached the steady phase at 4.5 h. Therefore, 4.5 h could be selected as the optimal reaction time for the whole-cell biotransformation of rutin. Furthermore, the conversion rates of rutin by mutants V338I and S340A were lower compared with the wild type when using purified enzymes, whereas the conversion rates of rutin by V338I and S340A whole cells were higher compared with the wild type. Subsequently, we used the best mutant, V338A, to biotransform rutin for the scale-up preparation of isoquercetin in a 1 L reaction mixture with 2 mM substrate concentration, and we obtained 1.80 g of isoquercetin powder. The identification of the best α -L-rhamnosidase BtRha78A mutant, V338A, provides an important basis for the study of the catalytic mechanism of this enzyme and theoretical guidance for the scale-up production of isoquercetin.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal14080501/s1>, Figure S1: SDS-PAGE (15%) for the BtRha78A wild type and its best mutants, Figure S2: Enzyme kinetic curves for the hydrolysis of rutin by the BtRha78A wild type and its best mutants, Table S1: PCR primers for Ala-scanning mutagenesis and site-directed mutagenesis of BtRha78A.

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