A spectrophotometric method for high-throughput screening of α -L-rhamnosidase activity on rutin coupled with a β -D-glucosidase assay

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Abstract

α -L-Rhamnosidase may biotransform rutin into isoquercetin with better bioavailability and bioactivity. To date, the high-throughput screening for the activity of α -L-rhamnosidases on rutin could not be achieved. Herein, based on the spectral differences between rutin and its aglycone quercetin in alkaline pH 10.0, we have developed a novel and simple spectrophotometric method for high-throughput screening of α -L-rhamnosidase activity on rutin by combining with a highly active β -D-glucosidase. Quercetin showed the maximum absorbance at 320 nm in alkaline pH 10.0, and could be considered as the characteristic peak of quercetin because rutin had low absorption at 320 nm. Meanwhile, rutin exhibited the maximum absorption at 400 nm and quercetin showed low absorption at 400 nm in pH 10.0. With this novel spectrophotometric method, the relative abilities of nine different α -L-rhamnosidases on rutin had been evaluated by monitoring the absorption values of the reaction mixture in alkaline pH 10.0 at 320 nm and 400 nm, and the trend in the activity on rutin was consistent with that obtained by HPLC. Moreover, the library from site-directed saturation mutagenesis at the residue Val338 in the α -L-rhamnosidase BtRha78A from *Bacteroides thetaiotaomicron* was constructed for high-throughput screening by this novel spectrophotometric method, and the mutant V338S with improved activity on rutin was obtained. The conversion rate of the mutant V338S on rutin increased by 21.7% and 16.8% than wild type when using whole cells and purified enzymes, respectively. Our findings demonstrated that this novel spectrophotometric method coupled with the β -D-glucosidase assay might be applied for high-throughput screening of different α -L-rhamnosidases and a great number of mutants from semi-rational design and directed evolution for α -L-rhamnosidase.

Keywords α -L-Rhamnosidase · High-throughput screening · Spectrophotometric · Isoquercetin · Rutin

Introduction

The natural flavonoid rutin (Fig. 1a) consists in the flavonol quercetin 3-O-linked to the disaccharide rutinose (6-O- α -L-rhamnopyranosyl- β -D-glucopyranoside), and isoquercetin (Fig. 1a) is the 3-O- β -D-glucopyranoside of the aglycone quercetin (Fig. 1a). They exhibit a wide variety of biological

activities (Chua 2013; Valentová et al. 2014) including anti-oxidant (Yu et al. 2015), anti-inflammatory (Mascaraque et al. 2014), (Lee et al. 2013a), anti-hypertensive (Larson et al. 2012), hypolipidemic (Santos et al. 1999), anti-cancer (Wu et al. 2018; Jeong et al. 2009) activities, and as the inhibitors of α -glucosidase (Li et al. 2009b) and α -amylase (Li et al. 2009a). Quercetin glycosides rutin and isoquercetin show higher water solubility than quercetin due to the hydrophilicity of the sugar moieties. Isoquercetin displays better bioavailability (Makino et al. 2009; Cermak et al. 2003; Reinboth et al. 2010) than quercetin and rutin, and higher bioactivity (Li et al. 2009b, 2016) than rutin. Although isoquercetin is widely distributed, it is difficult to obtain sufficient pure compound for food and pharmaceutical industry, since isoquercetin is rare in nature. Isoquercitrin can now be prepared on a large scale by enzymatic hydrolysis of rutin with the α -L-rhamnosidases (Weignerova et al. 2012; You

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Fig. 1 Molecular structures, de-glycosylated pathway, and UV–visible spectra of rutin, isoquercetin, and quercetin. **a** Molecular structures and de-glycosylated pathway of rutin, isoquercetin, and quercetin. The α -L-rhamnosyl group is colored with blue, and the β -D-glucosyl group is red. **b** UV–visible spectra of rutin, isoquercetin, and quercetin at the concentration of 25 μ M in 50 mM sodium carbonate (pH 10.0)

et al. 2010; Lee et al. 2013b; Zhang et al. 2015; Wang et al. 2012).

α -L-Rhamnosidase [E. C. 3.2.1.40] hydrolyzes terminal α -L-rhamnose specifically from many natural products including flavonoid glycosides rutin, troxerutin, naringin, hesperidin, diosmin and quercitrin, ginsenosides Re and Rg2, terpenyl glycosides and other natural glycosides containing terminal α -L-rhamnose (Yadav et al. 2010). α -L-Rhamnosidase has turned out to be a biotechnologically important enzyme owing to the biocatalytic applications in improving the bioavailability (Gonzalez-Barrio et al. 2004) and biological activities (de Araujo et al. 2013; Lee et al. 2012; da Silva et al. 2013) of the flavonoids, enzymatic preparation of prunin from naringin, biosynthesis of α -L-rhamnosides by reverse hydrolysis (Lu et al. 2015; De Winter et al. 2013), biotransformation of gypenoside-5 into ginsenoside Rd (Yu et al. 2004), and debittering of citrus fruit juices (Alvarenga et al. 2013).

A convenient method has been widely used for measuring the activity of α -L-rhamnosidase by using the synthetic substrate *p*-nitrophenyl- α -L-rhamnopyranoside (*p*NPR) and monitoring the liberation of *p*-nitrophenol spectrophotometrically at 405 nm. However, the substrate *p*NPR is expensive, and α -L-rhamnosidases possess the substrate selectivity, especially molecular structure of natural substrates is larger and the linkages between glycosyl groups are varied. The measurement of α -L-rhamnosidase activity on natural substrate rutin relies on quantification of the product isoquercetin by high performance liquid chromatography (HPLC). Therefore, high-throughput screening for α -L-rhamnosidases could not be achieved. Recently, a spectrophotometric method has been reported for quantifying the activity of β -rutosidase for biotransformation rutin

into quercetin based on spectral difference between rutin and quercetin in pH 10.0 (Weiz et al. 2017). The convenience of this method is in the quantification of β -rutosidase activity on rutin by UV–visible spectrometry. However, this method was only compatible with the β -rutosidase, naringinase and hesperidinase, not with the α -L-rhamnosidase, and had not been used for high-throughput screening of a deal of enzymes or mutants.

If β -D-glucosidase activity is present along with α -L-rhamnosidase activity, the generated isoquercetin from rutin will be fully converted into quercetin as shown in Fig. 1a. In this study, with simplification and modification of previous UV–visible method, according to spectral properties of rutin and the aglycone quercetin in alkaline pH 10.0, an optimized spectrophotometric method has been established for high-throughput screening of α -L-rhamnosidase activity on rutin by combining with a highly active β -D-glucosidase. This novel spectrophotometric method has been used for evaluating the relative activities of nine different GH78 family α -L-rhamnosidases (Rha78s) on rutin and high-throughput screening of the mutants library for site-directed saturation mutagenesis at the residue Val338 in the α -L-rhamnosidase BtRha78A.

Materials and methods

Bacterial strains, enzymes and reagents

The flavonoids rutin, isoquercetin and quercetin were purchased from Yuanye Biotech (Shanghai, China). Chromatographic grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). 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). *E. coli* BL21(DE3) was used as the host for protein expression. Kanamycin, IPTG and imidazole were purchased from Sangon Biotech (Shanghai, China). Other chemicals for buffer and medium preparation were of analytical grade or higher.

UV-visible spectra of flavonoids

The flavonoids rutin, isoquercetin, and quercetin (0.5 mM) were solubilized in 100% methanol. The spectrophotometric assays were performed by adding 50 μ L flavonoids (0.5 mM) into 950 μ L 100% methanol, 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5), and 50 mM sodium carbonate (pH 10.0), respectively. After incubating for 30 min at room temperature, the spectra from 250 to 500 nm at 25 °C were obtained by a spectrophotometer UV-5800PC (Metash, Shanghai, China).

Gene cloning of the β -D-glucosidase TnBgl1A-DM

The double mutant N221S/P342L (designated as TnBgl1A-DM) of the β -D-glucosidase TnBgl1A (GenBank accession, AAB95492) from *Thermotoga neapolitana* was used for combining with the α -L-rhamnosidase activity. The gene of TnBgl1A-DM with codon optimization was synthesized by Sangon Biotech (Shanghai, China), and was cloned into the vector pET-28a by prolonged overlap extension polymerase chain reaction (POE-PCR) (You et al. 2012) using the primers as shown in Table S1. The linear pET-28a was amplified with the primers pET-28a-F and pET-28a-R. The PCR system consisted of dNTP, 0.2 mM for each; primers, 0.5 μ M for each; plasmid template, 1 ng/ μ L; and *TransStart[®] FastPfu* Fly DNA polymerase, 0.05 U/ μ L. The PCR program was 95 °C denaturation, 5 min; 30 cycles of 95 °C denaturation,

30 s; 63 °C annealing, 30 s; 72 °C extension, 3 min; and 10 min extension at 72 °C. Similarly, the TnBgl1A-DM gene was amplified by the primers TnBgl1A-DM-F and TnBgl1A-DM-R. Two linearized DNA fragments were purified with a SanPrep PCR product purification kit (Sangon Biotech, Shanghai, China) after digesting the template by FastDigest *Dpn* I restriction enzyme (Thermo Fisher Scientific, Waltham, USA). In POE-PCR, each tube contained 0.2 mM dNTP, 1 ng/ μ L purified TnBgl1A-DM gene, 4 ng/ μ L purified linear pET-28a, and 0.05 U/ μ L *TransStart[®] FastPfu* Fly DNA polymerase without the addition of primers. The POE-PCR was conducted as follows: 95 °C denaturation, 5 min; 30 cycles of 95 °C denaturation, 30 s; 55 °C annealing, 30 s; 72 °C extension, 4.5 min; and 10 min extension at 72 °C. 5 μ L POE-PCR products were directly transformed into 100 μ L chemically competent cell *E. coli* DH5 α .

Over-expression and purification of recombinant Rha78s and TnBgl1A-DM

Nine α -L-rhamnosidases Rha78s and the β -D-glucosidase TnBgl1A-DM (Table 1) were over-expressed in *E. coli* BL21(DE3) as described in that study (Li et al. 2018a). The cells were harvested and resuspended in 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 8.0) and readied for enzyme purification or activity screening using whole cells. The TnBgl1A-DM, and BtRha78A wild type and its mutant were purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany).

Activity screening assay for α -L-rhamnosidase on rutin by UV-visible spectroscopy

The enzyme reaction mixture of 500 μ L contained 455 μ L 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5), 25 μ L rutin (20 mM, in 100% methanol), 10 μ L purified TnBgl1A-DM (1 mg/mL), and 10 μ L various concentrations of purified

Table 1 Sequence information for the α -L-rhamnosidases and β -D-glucosidase used in this study

Enzymes	Organism	GenBank accession	Gene length (bp)	Number of residues	References
BtRha78A	<i>Bacteroides thetaiotaomicron</i> VPI-5482	AAO76108	2190	729	Li et al. (2018a)
BtRha78D	<i>Bacteroides thetaiotaomicron</i> VPI-5482	AAO77630	3459	1152	Li et al. (2017)
BtRha78E	<i>Bacteroides thetaiotaomicron</i> VPI-5482	AAO77631	2646	881	Li et al. (2017)
HFM-RhaA	Human fecal metagenome	QBM20340	2499	832	Unpublished
HFM-RhaB	Human fecal metagenome	QBM20341	1725	574	Unpublished
HFM-RhaC	Human fecal metagenome	QBM20342	2676	891	Unpublished
HFM-Rha78	human fecal metagenome	AYD38341	2166	721	Shi et al. (2018)
PhRha78E	<i>Pedobacter heparinus</i>	ACU04972	2205	734	Li et al. (2018b)
PhRha78G	<i>Pedobacter heparinus</i>	ACU06078	2715	904	Li et al. (2018b)
TnBgl1A	<i>Thermotoga neapolitana</i>	AAB95492	1335	444	Turner et al. (2006)

wild-type BtRha78A (0–80 µg/mL). The reaction for the α -L-rhamnosidase activity was performed by incubating for different time intervals (0–120 min) at 45 °C, subsequently, for the β -D-glucosidase activity by incubating for 20 min at 80 °C. The reaction was stopped by adding the 100% methanol of 500 µL, and the mixture was centrifuged at 13,000 rpm for 5 min to remove the denatured enzymes. And then 50 µL supernatant was added into 950 µL 50 mM sodium carbonate (pH 10.0). After incubating for 30 min at room temperature, the absorption values at 320 nm and 400 nm were measured, respectively. For the effects of reaction times on enzymatic reaction process, the enzyme concentration was determined as 80 µg/mL, and the reaction time was fixed to 120 min for the effects of enzyme concentrations on enzymatic reaction process. The reaction replacing purified enzymes with 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 8.0) was used as the control.

Activity screening of different α -L-rhamnosidases on rutin using whole cells

For activity screening of different α -L-rhamnosidase Rha78s, the reaction mixture of 500 µL contained 455 µL 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5), 25 µL rutin (20 mM, in 100% methanol), 10 µL purified TnBgl1A-DM (1 mg/mL), and 10 µL whole cells (400 mg/mL) of different Rha78s. The reaction for the α -L-rhamnosidase activity was performed by shaking for 2 h in 200 rpm at 37 °C, and then the β -D-glucosidase activity by incubating for 20 min at 80 °C. The reaction was stopped by adding the 100% methanol of 500 µL, and the mixture was centrifuged at 13,000 rpm for 5 min to remove denatured enzymes and cell debris. The supernatant was analyzed by this spectrophotometric method and HPLC method, respectively. The reaction replacing whole cells with 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 8.0) was used as the control.

Construction and high-throughput screening of the α -L-rhamnosidase mutant library

The site-directed saturation mutagenesis of BtRha78A at the residue Val338 were performed using the QuikChange mutagenesis protocol (Wang and Malcolm 1999) on the recombinant plasmid pET-28a-BtRha78A as described before (Li et al. 2018a). The primers used for site-directed saturation mutagenesis are listed in Table S1. After digesting the template by FastDigest *Dpn* I restriction enzyme (Thermo Fisher Scientific, Waltham, USA), PCR products were transformed into chemically competent cell *E. coli* BL21(DE3). The mutant clones were picked with sterile toothpicks into 96-well plates. The cells were cultured overnight at 37 °C by shaking the plates at 150 rpm. The

plates were duplicated by transferring 10 µL culture from each well into new plates. The original plates were stored, and the duplicated plates were shaken for 7 h at 37 °C for cell growth. Protein expression was induced for overnight at 16 °C by addition of 0.5 mM IPTG. The cells were harvested by centrifugation at 3500 rpm for 30 min and readied for screening.

The cells in each well were resuspended in 100 µL 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5), and then 22.5 µL purified TnBgl1A-DM (final concentration, 10 µg/mL) and 27.5 µL rutin (final concentration, 1.0 mM) were added. The reaction was performed by shaking for 2 h at 37 °C in 150 rpm, and then incubating for 20 min at 80 °C. The reaction was stopped by adding 150 µL the 100% methanol, and the mixture was centrifuged at 3500 rpm for 30 min. Then the supernatants of 15 µL from each well were added into an UV-transparent plate containing 285 µL 50 mM sodium carbonate (pH 10.0). After incubating for 30 min at room temperature, the absorption values at 320 nm and 400 nm were measured using a microplate reader Synergy Mx (BioTek, Vermont, USA). The sequence of better mutants were confirmed by DNA Sanger sequencing (Sangon Biotech, Shanghai, China).

Biotransformation of rutin by BtRha78A wild type and the mutant V338S

The reaction mixture of 500 µL contained 465 µL 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5), 25 µL rutin (20 mM, in 100% methanol), and 10 µL purified enzymes (2 mg/mL) or whole cells (400 mg/mL) of BtRha78A wild type or the mutant V338S. After incubating for 2 h at 37 °C, the reaction was stopped by adding the 100% methanol of 500 µL, and the mixture was centrifuged at 13,000 rpm for 5 min to remove the denatured enzymes or cell debris. When whole cells were used, cells sedimentation was prevented by shaking at 200 rpm. The supernatant was filtered through a 0.22-µm filter for HPLC. The reactions replacing purified enzymes or whole cells with 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 8.0) were used as controls.

High performance liquid chromatography (HPLC)

HPLC was carried out by using Waters 1525 binary pump and Waters 2487 dual λ absorbance detector (Massachusetts, USA) on a reverse-phase ZORBAX SB-C18 column (4.6 × 150 mm, particle size 5 µm, Agilent, California, USA) at room temperature. The rutin and quercetin were separated by using 0.5% (v/v) acetic acid (A) and 100% acetonitrile (B) as the mobile phase with the ratio of 30% B for 10 min at a flow rate of 1.0 mL/min. However, the rutin and isoquercetin were separated by using 0.5% (v/v)

acetic acid (A) and 100% acetonitrile (B) as the mobile phase for 30 min at a flow rate of 1.0 mL/min with a linear gradient starting from 18% B at 0 min to 11% B at 18 min. UV chromatograms were recorded at 260 nm. The quantification of rutin and isoquercetin were performed based on their standard curves (Fig. S1). The conversion rate was calculated using the following equation:

Conversion rate (%)

$$= (\text{isoquercetin concentration} / \text{initial rutin concentration}) \times 100.$$

Results and discussion

UV-visible spectral properties of the flavonoids rutin, isoquercetin, and quercetin

The hydrolytic activity of α -L-rhamnosidase on rutin was quantified by measuring the generated isoquercetin. To establish a simple spectrophotometric method for screening a large number of α -L-rhamnosidase samples in 96-well microtiter plates by a microplate reader, the UV-visible spectra of the substrate rutin, the product isoquercetin, and their aglycone quercetin were performed by full wavelength scanning at different conditions and 25 °C after incubating for 30 min at room temperature. It has been suggested that these results (Fig. S2, Fig. 1b) were consistent with the previous study (Weiz et al. 2017). Rutin and isoquercetin displayed similar spectral characteristics under the 100% methanol (Fig. S2a) and acidic pH 6.5 (Fig. S2b). Rutin and isoquercetin showed two maximum absorbance peaks at the wavelengths of 260 nm and 356 nm, while quercetin exhibited two maximum absorbance peaks at the wavelengths of 255 nm and 369 nm (Fig. S2). There were no significant difference for the spectra of three flavonoids in the 100% methanol and acidic pH 6.5. These two wavelengths were often used for detecting the three flavonoids by HPLC.

On the other hand, in alkaline pH 10.0, the two maximum absorbance peaks of rutin and isoquercetin shifted towards longer wavelengths, 270 nm and 400 nm, respectively (Fig. 1b). However, quercetin showed the maximum absorbance only at the wavelength of 320 nm in alkaline pH 10.0 (Fig. 1b), which might be due to quercetin autoxidation. The new maximum absorbance peak at 320 nm created by quercetin autoxidation in alkaline pH 10.0 could be considered as the characteristic peak of quercetin, because rutin and isoquercetin had low absorption intensity at 320 nm. Meanwhile, quercetin showed low absorption intensity at 270 nm and 400 nm (Fig. 1b). Therefore, it has been indicated that high absorption intensity at 320 nm responded to quercetin of high concentration and high intensity at 270 nm and 400 nm agreed with rutin

and isoquercetin of high concentration in the mixture of the three flavonoids.

Establishment of spectrophotometric method for activity screening of α -L-rhamnosidases

α -L-Rhamnosidases might hydrolyze rutin into isoquercetin. The activity of α -L-rhamnosidase on rutin could not be directly measured by UV-visible spectroscopy owing to similar spectra of rutin with isoquercetin. Nevertheless, the generated isoquercetin will be completely converted into quercetin, if β -D-glucosidase activity is present along with α -L-rhamnosidase activity (Fig. 1a). The thermostable β -D-glucosidase TnBgl1A from *Thermotoga neapolitana* might catalyze the hydrolysis of a series of quercetin glucosides including isoquercetin, quercetin-4'-glucoside and quercetin-3,4'-diglucoside at high temperature and in acidic pH (Turner et al. 2006; Lindahl et al. 2010), and the double mutant N221S/P342L (TnBgl1A-DM) displayed increased catalytic efficiency on isoquercetin (Lindahl et al. 2010; Kulkarni et al. 2017; Khan et al. 2011). The condon-optimized gene of TnBgl1A-DM had been successfully cloned into the vector pET-28a by POE-PCR (Fig. S3a, b, c). TnBgl1A-DM had been over-expressed in *E. coli* BL21(DE3) and purified into homogeneity by analysis of SDS-PAGE (Fig. S3d).

To validate whether the quercetin of high concentration responded to high absorption intensity at 320 nm and the rutin of high concentration was in agreement with high intensity at 270 nm and 400 nm in the enzymatic reactions, the α -L-rhamnosidase BtRha78A from *Bacteroides thetaiotaomicron* VPI-5482 (Li et al. 2018a) was used for preparing the enzymatic reactions in biotransformation rutin into quercetin by combining with the β -D-glucosidase TnBgl1A-DM. With simplification of the previous method, the quercetin was not necessary for the separation with rutin by liquid-liquid extraction with ethyl acetate after enzymatic reactions in our novel method. The results showed that with the increase of reaction time and enzyme concentration the absorption intensity at 320 nm gradually increased, but the absorption value at 400 nm gradually decreased, which suggested that the quercetin was gradually produced and the substrate rutin was gradually hydrolyzed (Fig. 2). Anyway, the wavelength at 270 nm was not considered for detecting the rutin. It has been concluded that this spectrophotometric method might be applied for relative quantification of rutin and quercetin among different reaction mixtures and could be used for quickly probing the hydrolytic process of rutin into isoquercetin.

The relative activities of nine different α -L-rhamnosidases (Table 1) including four Rha78s from human fecal metagenome, three Rha78s from *Bacteroides thetaiotaomicron* VPI-5482, and two Rha78s from *Pedobacter heparinus* were

Fig. 2 Hydrolytic process of the α -L-rhamnosidase BtRha78A on rutin in various reaction times (a) and enzyme concentrations (b) measured by the spectrophotometric method coupled with the β -D-

glucosidase TnBgl1A-DM. All reactions were performed in triplicate, and error bars represent the standard deviations of mean

evaluated by this spectrophotometric method using whole cells. The absorption values at 320 nm and 400 nm (Fig. 3a) showed that HFM-RhaA possessed the highest activity on rutin, HFM-RhaC, BtRha78A, and BtRha78D might hydrolyze rutin moderately, PhRha78G, HFM-Rha78, and PhRha78E only could biotransform rutin into isoquercetin slightly, and BtRha78E and HFM-RhaB could hardly. This was consistent with the result from HPLC method (Fig. 3b). No quercetin was indeed detected in the enzymatic reactions of BtRha78E and HFM-RhaB by HPLC. Therefore, this spectrophotometric method could be used for activity screening of different α -L-rhamnosidases on rutin.

High-throughput screening for the α -L-rhamnosidase mutants with improved activity

Sequence alignment showed that the general acid motif was conserved in Rha78s subfamily I (Fig. 4a). Alanine-scanning mutagenesis suggested that the conserved residues Asp330, Arg334, Trp339, Asp342 along the general acid Asp335 locating at the general acid motif (Asp330–Asp342) of BtRha78A interacted with the ligand Tris and played important roles in enzyme catalysis (Li et al. 2018a), and the other eight residues from the general acid motif regulated the catalytic activity on *p*NPR (Fig. 4b). The mutant V338A exhibited higher activity than wild type, and the residue Val338 in BtRha78A was different from the other homologous Rha78s (Fig. 4a). Therefore, the substitution of the residue Val338 might affect the activity of BtRha78A on rutin, and the mutant library for site-directed saturation mutagenesis at the residue Val338 had been successfully constructed using

Fig. 3 Relative hydrolytic activity of nine bacterial α -L-rhamnosidases on rutin measured by the spectrophotometric method (a) and HPLC method (b) coupled with the β -D-glucosidase TnBgl1A-DM. All reactions were performed in triplicate, and error bars represent the standard deviations of mean

Fig. 4 The residue Val338 locating at the general acid motif and selected for site-directed saturation mutagenesis. **a** Sequence alignment of the α -L-rhamnosidase BtRha78A with homologous bacterial Rha78s at the general acid motif. The initial sequence alignment is generated by the software DNAMAN 7, and then illustrated with the server ESPript 3.0 (Robert and Gouet 2014). Strictly conserved residues are highlighted with red-shaded boxes, and moderately conserved residues are highlighted with yellow-shaded boxed. The general acid Asp335 is marked with gray circle. The residue Val338 for site-directed saturation mutagenesis is labeled by gray triangle. **b** Relative activity of eight alanine-scanning mutants at general acid motif in BtRha78A on pNPR. WT indicates the wild-type BtRha78A. All reactions were performed in triplicate, and error bars represent the standard deviations of mean

whole-plasmid PCR (Fig. S4) in order to verify the potential of this spectrophotometric method for high-throughput screening of a number of α -L-rhamnosidase mutants in 96-well microtiter plates measured by a microplate reader.

180 mutants in two 96-well plates were picked for first screening by this spectrophotometric method, and 15 mutants were selected into the second screening based on higher absorbance at 320 nm and lower absorbance at 400 nm using the microplate reader. Subsequently, four mutants were obtained from 15 mutants by culturing 5 mL $2 \times$ YT medium in the tubes for second screening. As a result, two of the four mutants exhibited better hydrolytic abilities than wild type determined by HPLC. Both mutants with improved activities were mutated into the serine at the residue Val338 with the mutagenesis of GTA into TCG by DNA Sanger sequencing.

Fig. 5 SDS-PAGE (15%) analysis for purified BtRha78A wild type and the mutant V338S. Lane 1, protein marker; lane 2, the purified wild type; lane 3, the purified mutant V338S. Molecular mass of recombinant BtRha78A containing the N-terminal (His)₆ tag is about 86.1 kDa

Biotransformation of rutin using the better mutant

The BtRha78A wild type and the mutant V338S were over-expressed in *E. coli* BL21(DE3) and purified into homogeneity with Ni-NTA affinity chromatography (Fig. 5). There were no obvious differences in soluble expression between BtRha78A wild type and the mutant V338S by analysis of SDS-PAGE (Fig. S5). We had investigated the biotransformation of rutin into isoquercetin by BtRha78A wild type and the mutant V338S using whole cells and purified enzymes. The conversion rate of the mutant V338S reached to 88.5% and 84.3% and increased by 21.7% and 16.8% than wild type using whole cell and purified enzyme, respectively (Fig. 6).

Conclusions

In this work, based on spectral differences between rutin and its aglycone quercetin in alkaline pH 10.0, a novel and simple spectrophotometric method coupled with a highly active β -D-glucosidase has been developed for high-throughput screening of α -L-rhamnosidase activity on rutin. With this novel spectrophotometric method, the relative activities of nine different α -L-rhamnosidases on rutin were compared. High-throughput screening of the mutants library for site-directed saturation mutagenesis at the residue Val338 in the α -L-rhamnosidase BtRha78A was carried out by this novel spectrophotometric method, and the mutant V338S with improved activity was obtained. The conversion rate of

Fig. 6 Biotransformation of rutin into isoquercetin by BtRha78A wild type and the mutant V338S using whole cells (8 mg/mL) and purified enzymes (40 μ g/mL) for 2 h at 37 °C in 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.5). All reactions were performed in triplicate, and error bars represent the standard deviations of mean

the mutant V338S on rutin increased by 21.7% using whole cells. In comparison to previous method and traditional HPLC method, without the liquid–liquid extraction with ethyl acetate after enzymatic reactions and using whole cells for enzymatic reaction simplified the screening procedure. Because of simplicity and rapidity, this novel spectrophotometric method coupled with the β -D-glucosidase would be applicable in high-throughput screening for a number of α -L-rhamnosidases and the mutants in semi-rational design and directed evolution.

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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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