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Characterization of a rutin-hydrolyzing enzyme with β-glucosidase activity from tartary buckwheat (*Fagopyrum tartaricum*) seeds

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Abstract

The rutin-hydrolyzing enzyme (RHEs) catalyse hydrolysis of rutin to quercetin. In this study, a RHE purified from natural tartary buckwheat flour (FtRHE) exhibited β-glucosidase activity. It was highly active on cello-oligosaccharides and p-nitrophenyl-β-D-glucopyranoside (pNPG). FtRHE exhibited optimum β-glucosidase activity at 40 °C and pH 4.0, which was elevated by the presence of Ca^{2+}, suggesting that Ca^{2+} may act as a co-factor for its activation. The K_m and V_{max} of FtRHE were 0.22 mM and 310.48 U/mg, respectively, when pNPG was the substrate. FtRHE retained only 6.6% activity in the presence of 1.5 M glucose, indicating that glucose acted as its inhibitor. This study demonstrates β-glucosidase activity of a RHE from natural tartary buckwheat flour and provides better understanding of its role in glycoside metabolism, establishing a basis for further investigations.

Keywords: rutin-hydrolyzing enzyme; β-glucosidase; *Fagopyrum tataricum*; enzymatic hydrolysis; flavonol 3-glucosidase.

Practical Application: This research has practical applicability at several levels. It reveals the β-glucoside hydrolysis activity of rutin-hydrolyzing enzyme in tartary buckwheat, providing a new direction for further study of the biological value this plant. It also reveals its biological function in glycoside metabolism of tartary buckwheat and its potential use in the conversion of cello-oligosaccharides to glucose. In addition, it provides a new idea for effectively inhibiting glycoside hydrolysis in the process of Tartary Buckwheat food processing by inhibiting the activity of FtRHE.

1 Introduction

Lignocellulosic wastes, considered abundant renewable resources and ideal feed stock for biofuel production, are mainly composed of cellulose, hemicellulose, and lignin. Glycoside hydrolases (GHs) hydrolyze cellulosic and hemicellulosic fractions of plant biomass. Cellulose from forests and agricultural wastes can be degraded to cellodextrin and cellobiose or glucose. The last step in cellulose hydrolysis requires β-glucosidases (β-D-glucopyranoside glucohydrolases, E.C.3.2.1.21), which are enzymes that remove the nonreducing terminal β-D-glucosyl residue from glucoconjugates, including glucosides, 1-O-glucosyl esters, and oligosaccharides (Gao et al., 2013). Owing to the abundance of different types of compounds containing nonreducing terminal glucosides in plants, beta-glucosidases may possess several functions such as lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release in plants (Cairns & Esen, 2010). β-glucosidases play a key role in cellulose degradation by removing the inhibitory cellobiose. In addition, β- glucosidases can synthesize compounds such as oligosaccharides, glycoconjugates, and alkylglucosides owing to their transglycosylation activity (Brimer et al., 1998; Pal et al., 2010). This indicates that they may be involved in glucoconjugate synthesis in plants (Franková & Fry, 2013). Indeed, acyl-glucose-dependent transglucosidases that function in anthocyanin synthesis are closely related to β-glucosidases (Matsuba et al., 2010; Nishizaki et al., 2013) .

Genomic sequences have revealed that each plant harbors many putative isoenzymes of β-glucosidase. β-Glucosidases have been categorized into glycoside hydrolase families based on their amino acid sequence, namely, GH1, GH2, GH3, GH5, GH9, GH30, and GH116, with those from plants belonging to the GH1, GH3, GH5, and GH116 families (Lombard et al., 2014; Opassiri et al., 2006; Suthangkornkul et al., 2016; Drula et al., 2022). Among these, GH1 is the most well-characterized group of enzymes. The GH1 β-glucosidases consist of a single domain of glycohydro1 (pfam00232) with an α/β barrel topology. Furthermore, β- glucosidases are used in various biotechnological processes, such as the hydrolysis of isoflavone glucosides (Li et al., 2012), ethanol fuel production from agricultural residues (Liu et al., 2012), and aromatic compound release from flavorless precursors (Romo-Sánchez et al., 2014).

Tartary buckwheat (*Fagopyrum tataricum* Moench) is an annual dicotyledonous crop of *Fagopyrum* Mill, the seed of which is rich in primary nutrients, such as starch, fatty acid (linoleic acid), seed proteins, and the flavonoid rutin (approximately 0.8-1.7% dry weight) (Fabjan et al., 2003; Zielińska et al., 2012). In fact, tartary buckwheat contains more rutin than common buckwheat (*Fagopyrum esculentum* Moench), which has been utilized as a rutin-rich material for numerous food products (Kim et al., 2007). Large amounts of quercetin can be generated

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in tartary buckwheat products as a result of rutin degradation in food processing, which may contribute to the bitter taste (Vogrinčič et al., 2010). The rutin hydrolyzing enzyme (RHE) in tartary buckwheat seeds catalyzes the conversion of rutin into quercetin and rutinose. Suzuki et al. (2002) reported that flavonol 3-glucosidase (f3g) consists of two isozymes, and their characteristics are similar to those of RHE, although their molecular weights and kinetic constants are different. F3g and RHE in tartary buckwheat seeds may perform physiological roles in seed ripening such as screening of ultraviolet light and/or production of anti-fungal agents (Suzuki et al., 2002).

Previously, we have purified a rutin-hydrolyzing enzyme (FtRHE) from tartary buckwheat seeds using anion exchange and size exclusion chromatography, and studied its specific catalytic activity toward rutin (Cui & Wang, 2012). At the same time, we demonstrated that quercetin is the product of FtRHE hydrolysis using 1H nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-electrospray ionization-mass spectroscopy (LC-ESI-MS)/MS. Recently, we observed that purified FtRHE also possesses β-glucosidase activity, which is 54-62% similar to the activities of β-glucosidases from other plants. However, studies evaluating the β-glucosidase characteristics of rutin-hydrolyzing enzymes and the properties of highly purified RHEs from plants are limited. In this study, we investigated the β-glucosidase activity of FtRHE to understand its biological function in glycoside metabolism of tartary buckwheat and evaluate its potential use in the conversion of cello-oligosaccharides to glucose.

2 Materials and methods

2.1 Materials and chemicals

Tartary buckwheat seeds (Yunqiao No. 1) was provided by the Institute of Biotechnology and Germplasm Resources, Yunnan Academy of Agricultural Sciences (Kunming, Yunnan, China). Esculin and p-nitrophenyl-β-D-glucopyranoside (pNPG) were purchased from Sangon Biotech (Shanghai) Co. Ltd. Methanol (HPLC grade) was obtained from Sinopharm Group Shanxi Co. Ltd. All other chemicals and reagents used were of analytical grade.

2.2 Preparation and purification of FtRHE

The native FtRHE was extracted according to an established method (Cui & Wang, 2012).

2.3 Enzyme activity assay

RHE was assayed as described previously (Cui & Wang, 2012). β-Glucosidase activity was determined by measuring pNPG hydrolysis. The reaction mixture contained 100 μL of 10 mM pNPG in 20 mM ammonium acetate buffer (pH 5.0) and 100 μL FtRHE. After incubation at 40 °C for 15 min, the reaction was stopped by adding 200 μL ice-cold 1 M Na_2CO_3. The amount of released p-nitrophenol was measured using a spectrophotometer at 405 nm. The enzyme assays were performed in triplicates. Enzyme- and substrate-free controls were also incubated and measured under the same conditions. One unit (U) of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmol p-nitrophenol per minute per milliliter under the assay conditions.

2.4 Native-polyacrylamide Gel Electrophoresis (PAGE) and Sodium Dodecyl Sulfate (SDS)-PAGE analysis

SDS-PAGE was performed using a 12.5% (w/v) separating gel. Proteins in the gel were stained using Coomassie Brilliant Blue R-250, and the molecular mass was estimated after comparison with a medium range molecular mass protein standard (Sangon Biotech, Shanghai, China).

After non-denaturing PAGE, the gel was stained using 0.25% Coomassie Brilliant Blue staining solution for 30 min and was subsequently destained until it was clear.

Based on the well-established reaction of β-glucosidase with esculin (6,7- dihydroxycoumarin-β-glucoside), esculin was used as the substrate for screening strains producing β-glucosidase from several organisms. β-Glucosidase was detected in chromatographic fractions using PAGE (Zhao et al., 2008). For esculin staining, the gel was soaked in 200 mM acetate-sodium acetate buffer solution, pH 5.0, for 10 min, followed by incubation with 200 mM acetate-sodium acetate buffer (pH 5.0) with 0.1% of esculin and 0.3% of ferric chloride at 40 °C. After the appearance of black bands, the gel was soaked in 10% glucose solution.

2.5 Effect of temperature and pH on FtRHE

The optimum temperature and pH of the enzyme were determined by reacting the enzyme with pNPG for 15 min in the temperature range of 30-100 °C and pH range of 2.0-11.0, respectively. The effects of pH on β-glucosidase activity was tested using 20 mM glycine-HCl buffer (pH 2.0-3.0), sodium acetate buffer (pH 4.0-5.0), sodium phosphate buffer (pH 6.0-8.0), or glycine-NaOH buffer (pH 8.5-11.0) at 40 °C. The enzyme activity observed at the optimum temperature or pH was used to calculate the relative percentage of enzyme activity at varying temperature and pH values. Temperature stability was determined by incubating the enzyme for 30 min without pNPG at 30-70 °C and measuring the residual activity.

2.6 Effect of metal ions and chemical reagents on FtRHE

Enzyme activity was evaluated by reacting the enzyme under standard assay conditions with pNPG supplemented with various metal ions ($CaCl_2$, NaCl, KCl, $MgCl_2$, $FeCl_3$, $NiCl_2$, $CoCl_2$, NH_4Cl, $ZnCl_2$, and $MnCl_2$) or chemical reagents (urea, SDS, EDTA) (Table 1).

2.7 Determination of kinetic parameters

The apparent Michaelis-Menten constant (Km) and maximum velocity (Vmax) of the purified FtRHE were assessed by measuring the rate of hydrolysis of pNPG at various concentrations (0.01-5.0 mM) at 40 °C for 15 min in ammonium acetate buffer (20 mM, pH 5.0). The enzymatic kinetic parameters were determined from the Michaelis-Menten function using the SigmaPlot software version 12.0 (Systat Software, Chicago, USA).

2.8 Substrate hydrolysis of FtRHE

To investigate the substrate specificity of FtRHE, four different disaccharides and three chemicals, which possess various types of glycosidic bonds, were used. The substrate group consisted of terrestrial biomass-derived disaccharides, namely cellobiose (formed with two units of D-glucose with a β-1,4-glycosidic linkage), lactose (formed with D-galactose and D-glucose units with a β-1,4-glycosidic linkage), sucrose (formed with D-glucose and D-fructose units with an α-1,2-glycosidic linkage), and maltose (formed with two D-glucose units with an α-1,4-glycosidic linkage) (all purchased from Sigma). To screen the substrate specificity of FtRHE, 100 µL 1% (w/v) of these compounds (Table 2) was reacted with 10 µL of 1 U purified enzyme. All reactions were performed in ammonium acetate buffer (20 mM, pH 5.0), at 40 °C for 15 min, unless otherwise specified. Reactions were stopped by boiling for 5 min, and the samples were prepared and analyzed using gas chromatography (GC)-MS.

GC-MS analysis was performed on GC-MS 3200 (East & West Analytical Instruments, Beijing, China), using an Equity-5 (30 m × 0.25 mm × 0.25 µm) quartz capillary column (Sigma-Aldrich LLC., St. Louis, USA). The sample was inserted in the splitless mode using helium as carrier gas. Initially the oven temperature was fixed at 100 °C, followed by increase to 280 °C at a rate of 10 °C per minute after 1 min. The temperature was maintained at 280 °C for 25 min. Electron impact ionization (EI) was used as an ionization source for the GC/MS analysis at 70 eV. GC-MS analysis was performed according to the GC parameters described previously. Data was acquired in full scan mode from 45-850 m/z in 0.5 sec scan time.

Table 1. Effects of metal ions and chemicals on FtRHE activity.

Chemicals	Relative activity (%)	
	2.5 mM	5 mM
Control	100.00 ± 0.02	100.00 ± 0.02
$CoCl_2$	100.63 ± 0.03	89.25 ± 0.02
$MgCl_2$	86.07 ± 0.03	76.64 ± 0.04
$CuCl_2$	35.98 ± 0.00	26.01 ± 0.01
$CrCl_3$	84.32 ± 0.00	78.88 ± 0.01
$ZnCl_2$	80.42 ± 0.01	67.41 ± 0.01
KCl	105.26 ± 0.00	99.44 ± 0.01
$NiCl_2$	92.13 ± 0.01	83.43 ± 0.01
$FeCl_3$	50.39 ± 0.02	39.25 ± 0.01
NH_4Cl	94.21 ± 0.01	87.19 ± 0.00
NaCl	88.02 ± 0.00	59.87 ± 0.00
$CaCl_2$	102.98 ± 0.01	119.29 ± 0.02
$MnCl_2$	68.60 ± 0.00	63.26 ± 0.01
Urea	96.75 ± 0.01	93.37 ± 0.01
SDS	79.29 ± 0.02	63.43 ± 0.01
EDTA	98.79 ± 0.01	96.37 ± 0.02

Table 2. Hydrolysis of various substrates by FtRHE.

Substrate [concentration, 1% (w/v)]	Linkage of the glycosyl group	Product formation
Cellobiose	β(1 → 4)Glc	glucose
CMC-Na	β(1 → 4)Glc	-
Xylan	β(1 → 4)xyl	-
Lactose	β(1 → 4)Gal	glucose,galactose
Salicin	β-salicyl alcohol glucoside	-
Sucrose	α(1 → 2)Fru	-
Maltose	α(1 → 4)Glc	-

2.9 Effect of glucose on FtRHE

The effect of glucose on purified FtRHE was evaluated by reacting pNPG in the presence of various concentrations of glucose using the standard assay procedure. The relative activity (%) was defined as the relative value to the activity of a control without glucose or mannitol.

3 Results

3.1 Purification of FtRHE

The purified fractions of FtRHE eluted from DEAE FF column and Sephacryl™ S-100 column were pooled and analyzed using SDS-PAGE. A single band corresponding to a molecular mass of approximately 60 kDa was observed (Figure 1A). According to native-PAGE and staining, purified FtRHE showed a single band after esculin staining, which was identified to be a β-glucosidase (Figure 1B).

3.2 Effects of optimum temperature and pH on FtRHE

The temperature and pH stability of FtRHE were determined using pNPG as the substrate. Purified FtRHE exhibited maximum activity at 40 °C and pH 4.0, while its relative activity was higher than 50% of the maximum activity at pH ranging from 2.0 to

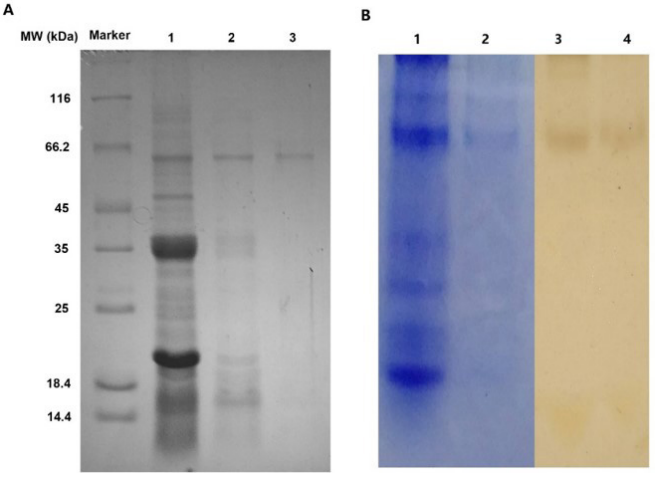

Figure 1. SDS-PAGE analysis of purified fractions of FtRHE (A). Lanes: 1, crude protein; 2, fraction of proteins eluted from DEAE FF column; 3, the purified fraction of FtRHE eluted from SephacrylTM S-100 column. Native-PAGE analysis of crude enzyme and purified FtRHE (B). Lanes: 1, crude enzyme; 2, purified FtRHE (stained by Coomassie brilliant blue R-250); 3, crude enzyme (stained by Esculin); 4, purified FtRHE (stained by Esculin).

4.5 and temperature ranging from 30 to 50 °C (Figure 2A-2B). The optimum temperature required for the activity of FtRHE was similar to β-glucosidases from *Trichoderma harzianum* (Florindo et al., 2018), *Prunus armeniaca* (Bhalla et al., 2017) and *Brassica oleracea* (Bešić et al., 2017), which was lower than that from *Anoxybacillus sp.* (Chan et al., 2016), *Sechium edule* (Mateos et al., 2015), *Nasutitermes takasagoensis* (Uchima et al., 2012)and *Thermoanaerobacterium thermosaccharolyticum* (Pei et al., 2012) (Table 3). The optimum pH was comparable to β-glucosidase from *Sechium edule* (Mateos et al., 2015). The activity half-life of FtRHE at 50 °C was 1.5 h (Figure 2C).

Although the optimum temperature required for FtRHE activity was 40 °C, its stability drastically reduced with time. FtRHE lost its activity completely after preincubating in the absence of pNPG for 30 min at 70 °C (Figure 2C). This indicated that pNPG may stabilize FtRHE at high temperatures. Therefore, for subsequent analysis, the reaction was performed at 40 °C.

3.3 Effect of metal ions and reagents on purified FtRHE

The effect of various metal ions (2.5 or 5 mM) on FtRHE activity was investigated (Table 1). Ca^{2+} elevated β-glucosidase activity by 19.19%, suggesting that the enzyme may require Ca^{2+} as a co-factor. Reports show that Ca^{2+} improves the activity of multiple GH enzymes, including β-glucosidase from *T. thermosaccharolyticum DSM 571* and *Anoxybacillus* sp. DT3-1(Chan et al., 2016; Pei et al., 2012). However, the same

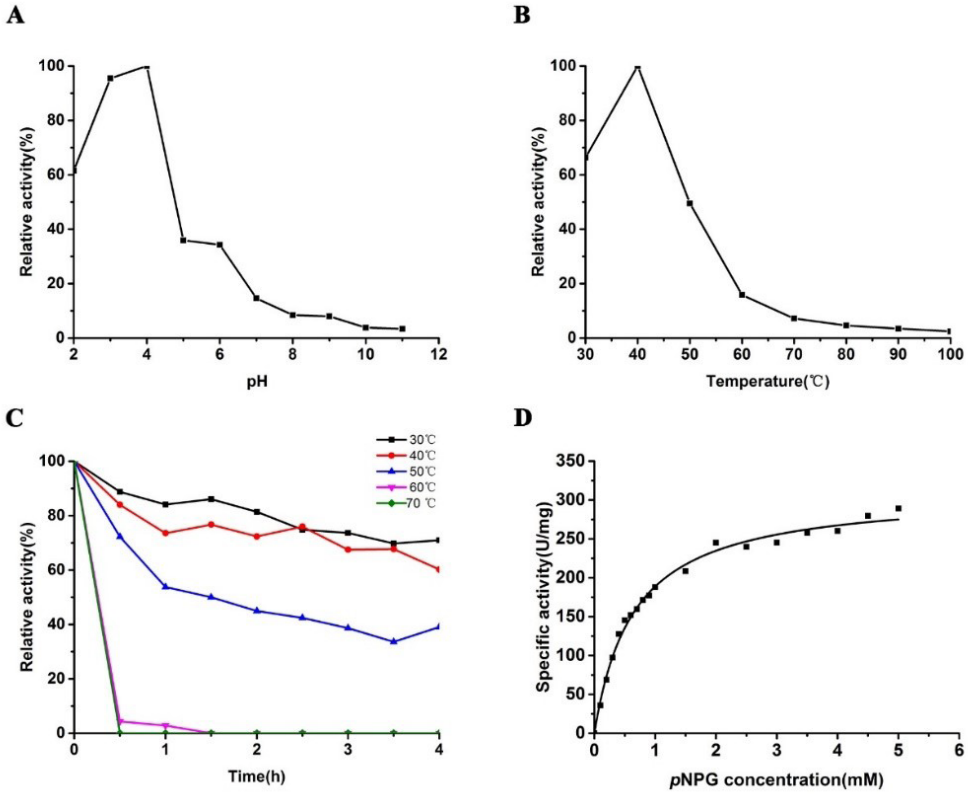

Figure 2. Characterization of FtRHE. Effects of pH (A) and temperature (B) on FtRHE activity and stability (C), nonlinear Michaealis-Menten plot of FtRHE using pNPG as substrate (D).

Table 3. Characteristics of FtRHE compared with BGL from other organisms.

Source	MM (kDa)	T_{opt} (°C)	pH_{opt}	K_m [a] (mM)	V_{max} (U^b/mg)	Reference
Fagopyrum tatarucum	60	40	4.0	0.22	310.48	This study
Trichoderma harzianum	53	40	6.0	0.97	29.3	Florindo et al., 2018
Prunus armeniaca	66	35	6.0	0.158	131.6	Bhalla et al., 2017
Brassica oleracea	50	35	6.0	0.755	604	Bešić et al., 2017
Anoxybacillus sp.	53	70	8.5	0.22	923.7	Chan et al., 2016
Sechium edule	58	50	4.0	4.88	104.1	Mateos et al., 2015
Nasutitermes takasagoensis	60	65	5.5	0.67	8	Uchima et al., 2012
Thermoanaerobacterium thermosaccharolyticum	52	70	6.4	0.62	64	Pei et al., 2012

a. The K_m was determined by measuring the rate of hydrolysis using pNPG as substrate. b. One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol p-nitrophenol per minute per milliliter under the assay conditions. [Topt] refers to the optimum temperature.

concentration of Fe^{3+} and Cu^{2+} inhibited the activity by 61% and 74%, respectively. Salts, such as NaCl, KCl, and NH_4Cl, showed negligible effect on its activity. Enzymatic activity was also reduced in the presence of Mg^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+}, and Mn^{2+} at either concentration (Table 1). The addition of 2.5 or 5 mM urea did not significantly affect its activity. FtRHE retained approximately 95% of its original activity in the same molarity (2.5 and 5 mM) of EDTA, indicating that FtRHE is not a metalloprotein.

3.4 Kinetic parameters

Using the Michaelis-Menten equation, the K_m of FtRHE was determined to be 0.22 mM and V_{max} as 310.48 U/mg when pNPG was used as the substrate (Figure 2D). These values could not be compared to those of the closest taxa shown in Table 3 because of lack of information regarding these β-glucosidases and their homologues.

3.5 Substrate specificity

FtRHE showed substrate-cleaving activity (Table 2). Although cellobiose is the main substrate for β-glucosidase, FtRHE was unable to completely hydrolyze the dimeric compound to glucose monomer using 1 U of purified FtRHE at 40 °C for 30 min (Table 2). It also showed low hydrolyzing activity towards lactose. Negligible hydrolysis was detected when maltose and sucrose were used as substrates. The maximum length of substrate (degree of polymerization) required by FtRHE is not clear as we have not reacted the enzyme with any linear cello-oligosaccharides larger than cellobiose. Interestingly, FtRHE was able to efficiently convert rutin and isoquercitrin to rutinose and glucose. Similar to most β-glucosidases, FtRHE was not reactive towards complex substrates, including carboxymethylcellulose sodium salt (CMC-Na) and xylan. No sugars were liberated from these complexes even when the reaction time was increased to 24 h. Based on substrate specificity, β-glucosidases are divided into three groups: (i) broad range β-glucosidase, (ii) cellobiase, and (iii) aryl-β-glucosidase (Chan et al., 2016). Collectively, based on the hydrolyzing ability of FtRHE, the enzyme can be assumed to belong to broad range β-glucosidases.

When FtRHE was incubated with certain flavonoid glycosides such as rutin as substrate, we observed that FtRHE exhibited glycosidase activity towards the flavonoid glycoside. The reaction product of rutin (molecular mass 610.51) was analyzed using LC-MS (Figure 3C), which shows decrease of 308 molecular mass corresponding to the removal of a rutinose molecule. When the reaction products of the glycoside was analyzed using high pressure liquid chromatography (HPLC), rutin showed a peak that had the same retention time and the UV-spectra as that of quercetin (Figure 3A-3B). These results qualitatively suggested that FtRHE can hydrolyze natural substrate of flavonoid glycoside.

3.6 Effect of glucose on FtRHE activity

FtRHE retained 68.8% and 50.6% relative activity in 0.05 and 0.1 M glucose, respectively, when pNPG was used as the substrate. FtRHE retained 6.6% relative activity in 1.5 M glucose (Figure 4). Glucose tolerance of FtRHE was lower than that of the β-glucosidase from *T. thermosaccharolyticum* DSM 571 and

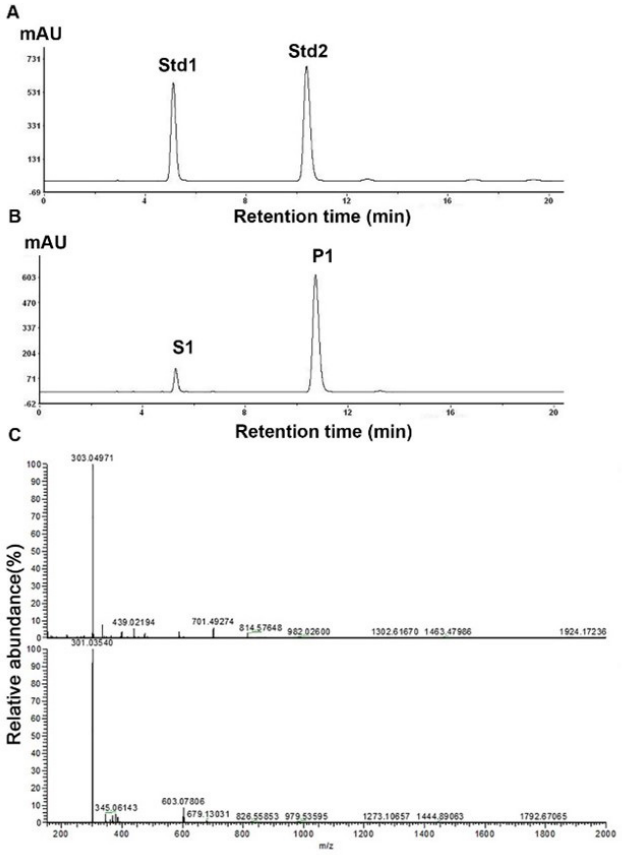

Figure 3. HPLC chromatograms of FtRHE assay mixture with (A) Std1: rutin; Std2: quercetin; (B) S1: rutin; P1: quercetin; (C) Positive and negative ion mass spectrum of P1.

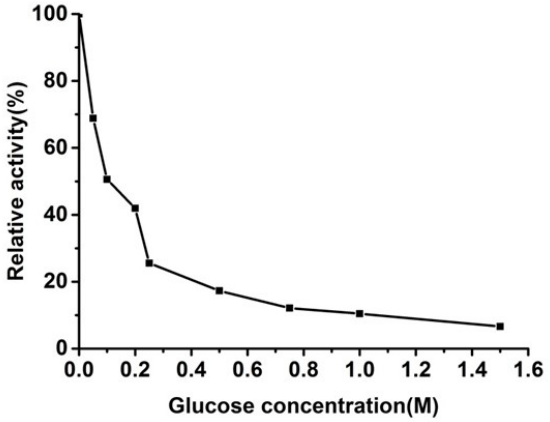

Figure 4. The effects of glucose on FtRHE activity towards pNPG.

Aspergillus niger ASKU28, which were known to be glucose-tolerant β-glucosidases (Pei et al., 2012; Thongpoo et al., 2014).

4 Discussion

Buckwheat is an important crop that is used as both medicine and food, and flavonoids are one of its main medicinal

components. Among buckwheat species, tartary buckwheat is a particularly rich source of rutin, containing approximately 100-fold higher concentrations of rutin in seeds than that of common buckwheat (Suzuki et al., 2014). The rutin-hydrolyzing enzyme (RHE) catalyzes hydrolysis of rutin to quercetin. Enzymological characteristics of a RHE purified from tartary buckwheat (FtRHE) were measured in this study, including optimum temperature, pH, metal ions and reagents on β-glucosidase activity and kinetic parameters (V_{max} and K_m). FtRHE exhibited optimum β-glucosidase activity at 40 °C and pH 4.0. A similar result was obtained in a β-glucosidase from Jincheng oranges (Ren et al., 2015). In contrast, the maximum activity of β-glucosidase from *Nasutitermes takasagoensis* (Uchima et al., 2012) and Sicilian blood oranges (Barbagallo et al., 2007) was observed at 65 °C. Cellulose hydrolysis is usually performed under mild conditions at 45-50 °C (Duff & Murray, 1996). Owing to its stability at these temperature and pH, the cellulose-degrading property of FtRHE can be potentially used for industrial saccharification processes, for example, in textiles, pulp, and paper industries, and in waste treatment (Kuhad et al., 2011). FtRHE was significantly activated by Ca^{2+}, while Zn^{2+}, Cu^{2+}, Fe^{3+} and Mn^{2+} had an inhibitory influence on enzyme activity. Nevertheless, Mn^{2+} is known to be an activator of β-glucosidases from Jincheng oranges (Ren et al., 2015) and *T. thermosaccharolyticum DSM 571* (Pei et al., 2012). As the copper-rutin complex was the most efficient scavenger of oxygen radicals under both in vitro and ex vivo conditions (Afanas'eva et al., 2001), the fact that β-glucosidase activity of FtRHE were inhibited strongly by copper ions was supposed to preserve the effect of the rutin-copper complex. The K_m and V_{max} of FtRHE were 0.22 mM and 310.48 U/mg, respectively, using pNPG as substrate. The K_m of FtRHE was lower than those β-glucosidases of other plant species, including *Brassica oleracea* (Bešić et al., 2017) and *Sechium edule* (Mateos et al., 2015), with the exception of *Prunus armeniaca* (Bhalla et al., 2017). FtRHE was able to efficiently hydrolyze rutin and isoquercitrin as a kind of rutinase or flavonol 3-glucosidase (Figure 3). It had a broad substrate specificity since rutin and isoquercitrin were substrates while pNPG was also substrates as well as cellobiose and lactose. This was contrast to the opinion that RHEs from tartary buckwheat had a rather narrow substrate specificity (Yasuda et al., 1992). Kalinová et al. (2018) found RHE could also be active on other compounds present in tartary buckwheat as piceid, kaempferol-3-D-glucoside and also kaempferol-3-O-rutinoside. This supported our results. FtRHE exhibited rutinase and β-glucosidase activity, which might be involved in the rutin catabolic pathway to transform 3-glycosylated flavonols. But the α-L-rhamnosidase activity of FtRHE have not yet been tested. More work should be done to confirm this subject.

Current reports have focused on the characteristics of RHEs or β-glucosidases from plants (Zhang et al., 2017; Zhou et al., 2017). However, knowledge on encoded gene of FtRHE are limited. Previously, we have isolated FtRHE using extraction with 20 mmol/L acetate buffer, ammonium acetate fractionation, anion exchange chromatography, and size exclusion chromatography (Cui & Wang, 2012). Based on the DNA sequences obtained from the transcriptome of tartary buckwheat seeds, the gene encoding FtRHE was found to be an open reading frame of 1,539 bases, encoding 512 amino acids (Zhuanhua, 2018). This was the first report revealing the complete primary structure of FtRHE. Multiple sequence alignments of FtRHE with other β-glucosidase-like enzymes revealed high similarity and the presence of conserved sequence elements. FtRHE contains the LNEP (amino acid 198-201) and TENG (411-414) motifs that are characteristic motifs of GH1 β-glucosidases. The two conserved carboxylic acid catalytic residues (Glu200 and Glu412) are located at β-strands 4 and 14, and act as the catalytic acid–base and nucleophile, respectively (Nijikken et al., 2007). The overall three-dimensional predicted structure of FtRHE resembles the typical (β/α)8-barrel structure observed in other structurally characterized GH1 family member, with the active site pocket located in the barrel. The protein sequence showed 54.4%~66.7% identity to homologous sequences present in the genomes of *Putranjiva roxburghii* (Kar et al., 2018), *Camellia sinensis* (Zhou et al., 2017) and other plants. Many of these homologous proteins have been characterized, although few were identified as RHEs. We expect that the present study on the biological function of FtRHE with respect to glycoside metabolism will be useful for the cultivation of high rutin buckwheat varieties using molecular methods and provides instructions in buckwheat food processing, establishing a basis for further investigations.

Abbreviations

RHE: rutin-hydrolyzing enzyme. FtRHE: native rutin-hydrolyzing enzyme. BGL: β-glucosidase. f3g: flavonol 3-glucosidase. CMC-Na: carboxymethylcellulose sodium salt. GH: glycoside hydrolase. HPLC: high-performance liquid chromatography. SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis. MW: molecular weight. pNPG: p-nitrophenyl-β-D-glucopyranoside.

Conflict of interest

All of the authors have read and approved the submitted manuscript. And the authors declare that there are no conflicts of interest.

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