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Short communication

The possible presence of natural β-D-glucosidase inhibitors in jujube leaf extract

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ABSTRACT

Isoquercitrin is a phenolic compound well-known for having greater health benefits than quercitin, its aglycone derivative, and other related glycosides. However, isoquercitrin is rarely found in nature. Here, we optimized the conditions for the enzymatic transformation of isoquercitrin from rutin that was extracted from jujube leaf using the hesperidinase, enzyme complex containing β-D-glucosidase and α-L-rhamnosidase. The maximum productivity (2.57 ± 0.16 mg/mL) was experimentally found under the following conditions: 47.3 °C, 52.16 h, and pH 5.31, which agreed well with the predicted value (2.65 mg/mL). However, the achievement of this maximum yield was due to the absence of β-D-glucosidase activity. Further investigations using a β-D-glucosidase assay and reaction measurements under various conditions revealed that the β-D-glucosidase activity was not blocked by denaturation or known inhibitory factors. Currently, there are no recognized β-D-glucosidase inhibitors present in the jujube leaf; however, our observations strongly suggest that an unidentified β-D-glucosidase inhibitor exists in jujube leaf extract.

1. Introduction

Isoquercitrin is a phenolic compound that is well-known for its health benefits, which include anti-proliferative effects, lipid peroxidation, oxidative-stress protection, and several others (Walle, 2004; Cermak, Landgraf, & Wolffram, 2003; You, Ahn, & Ji, 2010). Isoquercitrin was recently shown to exhibit greater bioactivity than quercetin or rutin (Valentová, Vrba, Bancířová, Ulrichová, & Křen, 2014; Paulke, Eckert, Schubert-Zsilavecz, & Wurglics, 2012). However, isoquercitrin is scarce in nature relative to its aglycone derivative quercitin and its glycoside derivatives such as rutin (Wang et al., 2012).

Rutin is abundantly present in various plants, including mulberry and buckwheat (Zhishen, Mengcheng, & Jianming, 1999; Jiang et al., 2007; Zhang et al., 2014a). Isoquercitrin can be produced by selectively hydrolyzing rhamnose from rutin, and a number of methods have been developed to de-rhamnosylate rutin. Methods for the production of isoquercitrin using this approach include acid hydrolysis, heating, biological transformation (Wang, Sun, Yu, Wu, & Guo, 2013; Weignerová, Marhol, Gerstorferová, & Křen, 2012), and enzymatic transformation, the last of which has become the favored method due to its economic merits and applicability to the food industry.

However, most natural α-L-rhamnosidase preparations result in additional β-glucosidase or rutinosidase activities, which undesirably transform isoquercitrin to quercetin or directly produce quercetin from rutin, respectively (Vila-Real, Alfaia, Bronze, Calado, & Ribeiro, 2011; Manzanares, Graaff, & Visser, 1997). Furthermore, the only two enzyme complexes that are commercially available for this purpose are hesperidinase and naringinase (Yadav, Yadav, Yadav, & Yadav, 2010), both of which exhibit β-glucosidase activity (You et al., 2010; da Silva et al., 2013). Currently, purified α-L-rhamnosidase is commercially unavailable (Yadav et al., 2010), and the use of recombinant enzymes precludes food-grade applications (Valentová et al., 2014).

There are two biotransformation strategies using the available commercial enzyme complexes that can enhance the isoquercitrin production yield. First, the introduction of enzyme inhibitors can

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selectively block unwanted β-D-glucosidase activity, resulting in improved selectivity of the α-L-rhamnosidase activity and enhanced yields of isoquercitrin. Currently the synthetic glucosidase inhibitors, nojirimycin and deoxynojirimycin, are available, although natural inhibitors have proved to be more difficult to obtain (Borges de Melo, da Silveira Gomes, & Carvalho, 2006). For food-grade applications, however, the use of these synthetic inhibitors is restricted (Shayman, 2010). The second option is to optimize the enzymatic reaction by improving kinetic control (You et al., 2010).

The jujube (*Ziziphus* spp.) leaf is a rich source of rutin (Zhang et al., 2014a). In contrast to its fruit, the jujube leaf is commonly considered to be waste, even though jujube leaf has long been a source of tea in China (Zhang et al., 2014b). Therefore, a method that effectively produces isoquercitrin from jujube leaf and is simultaneously applicable for food and pharmaceutical uses is of great economic interest. Thus, we tried to maximize the isoquercitrin yield from jujube leaf extracts through enzymatic transformation using the commercially available enzyme complex. Initially, we employed response surface methodology (RSM) in our kinetic investigations of the reaction, as the previous literature has reported that acknowledged β-D-glucosidase inhibitors were not present in jujube leaf. However, quercetin, an end product of the hesperidinase, was not produced under any condition as if the hesperidinase had no β-D-glucosidase activity. Therefore, the possible presence of β-D-glucosidase inhibitor in jujube leaf extract was investigated.

2. Materials and methods

2.1. Chemicals and reagents

Hesperidinase from *Aspergillus niger* (hesperidin-α 1,6-rhamnosidase, 3 U/g of hesperidinase activity, EC. 3.2.1.168) and rutin (quercetin-3-rutinoside trihydrate), along with the isoquercitrin (quercetin 3-β-D-glucoside) and quercetin (quercetin dihydrate) standards for HPLC analysis, were purchased from Sigma Aldrich (St. Louis, Mo., U.S.A.). Citric acid (0.1 M) (Yakuri Pure Chemicals Corp., Kyoto, Japan) and sodium phosphate dibasic anhydrous (0.2 M) (Duksan Pure Chemical Corp., Asan, Korea) were mixed for the preparation of McIlvaine buffer (pH 3.8), which was used to dissolve the chemicals.

2.2. Preparation of jujube leaf extract

Sun-dried jujube leaves, harvested in April 2012 in Boeun, South Korea, were stored at −80 °C. Frozen jujube leaves were pulverized using a household blender (HMF-1000, Hanil Electric, Seoul, Korea) and filtered with a fine mesh (150–300 μm). The pulverized samples were stored at −18 °C.

Extraction of the jujube leaves was performed using a 45% (w/v) ethanol solution for the food-grade extraction. A mixture of 0.5 g of pulverized jujube leaf per 10 mL of 45% ethanol was stirred at 45 °C for 15 min before filtering out solid debris with filter paper (filter paper No. 4, 110 mm ϕ, Whatman International Ltd., Maidstone, England) (Min, Lim, Ahn, & Choi, 2010). The extract was powdered by removal of solvent at 40 °C using a rotary vacuum evaporator (EYELA, N-N series, Tokyo, Japan). To use the same rutin concentration of 2.66 ± 0.25 mg/mL, the amount of powdered extract dissolved in McIlvaine buffer was controlled till the absorbance at 357 nm of the solution using a UV–visible spectrophotometer (UV-1700, Shimadzu Corp., Tokyo, Japan) reached 1.55. This value corresponds to the concentration of rutin of 2.66 mg/mL by HPLC quantification.

2.3. HPLC analysis

The flavonoid concentrations (i.e., those of rutin, isoquercitrin, and quercetin) were analyzed by HPLC (2695 Separations module, WATERS, Milford, MA, USA) coupled to a UV detector (996 Photodiode array detector, WATERS). A C-18 column (Hibar 250-4, LiChrospher 100 RP-18 endcapped (5 μm), Merck, Darmstadt, Germany) was used to separate and detect rutin, isoquercitrin and quercetin at 264, 352.6, and 369 nm, respectively. Before injecting each sample into the HPLC, the samples were diluted in methanol to dissolve crystallized flavonoids and were filtered through a 0.45-μm syringe filter (PVDF, 0.45 μm, Olimpeak, Barcelona, Spain). The mobile phase consisted of a 2.5% (v/v) acetic acid solution (solvent A) and acetonitrile (solvent B). Chromatography was performed at a flow rate of 1 mL/min, and the gradient settings were as follows; 0 min, 95% A and 5% B; 0–25 min, 75% A and 25% B; 25–40 min, 50% A and 50% B; 40–50 min, 20% A and 80% B; 50–55 min, 50% A and 50% B; 55–60 min, 95% A and 5% B; and 60–65 min, 95% A and 5% B.

2.4. Enzymatic biotransformation

All of the enzymatic biotransformation experiments were conducted in a temperature-controlled shaking water bath with agitation at 120 rpm. In this study, citric acid and sodium phosphate dibasic were used to control the pH of the reaction buffer. Three milliliters of jujube leaf extract were added to 150 μL of the enzyme-buffer solution to initiate the reaction. The reaction progress was measured at variable times, temperatures, and enzyme concentrations and at multiple pH values. The enzyme activity was terminated by placing the samples in a 70 °C water bath for 10 min (Soria & Ellenrieder, 2002).

2.5. Response surface methodology (RSM)

To optimize the reaction conditions for the production of isoquercitrin by hesperidinase, RSM was performed using the Box-Behnken design (BBD). Based on the experimental results, the reaction temperature (X_1, 40–60 °C), processing time (X_2, 24–72 h), and pH (X_3, pH 2–6) were selected as independent variables and the isoquercitrin concentration (mg/mL) was considered to be the responsive variable. The levels were coded as −1 (low), 0 (middle) or 1 (high). The following quadratic polynomial equation was used to fit the experimental data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the responsive variable; X_1, X_2, and X_3 are the independent variables; β_0 is the model intercept coefficient; β_1, β_2, and β_3 are the linear coefficients; β_{12}, β_{13}, and β_{23} are the interaction coefficients among the three factors; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. The experimental design and statistical analyses were performed using the statistical analysis system (SAS) (Version 9.3, Cary, NC, USA). For statistical assessments, ANOVA and *F*-tests were used. The statistical model was fit based on the R^2 coefficient. The quadratic models for the independent variables are shown as 3D and 2D contour plots.

2.6. Verification of β-glucosidase activity

β-Glucosidase activity of hesperidinase was verified based on investigated optimal condition. At pH 5.31 and 47.3 °C, 150 μL of hesperidinase from *A. niger* (0.3 U/mL) reacted with 3 mL of *p*-nitrophenyl β-D-glucopyranoside (5.5 μmol/mL) in a water bath. The starting substrate concentration matched the final

isoquercitrin concentration under the determined optimal conditions. The enzyme reaction was terminated by placing the samples into an equal volume of 400 mM sodium carbonate (JUNSEI Chemical Co., Ltd., Tokyo, Japan). The products were measured with a UV–visible spectrophotometer at 400 nm (Hernandez, Espinosa, Fernandez-Gonzalez, & Briones, 2003).

2.7. Metal ion analysis

The concentrations of the metal ions Cu^{2+}, Zn^{3+}, Mn^{2+}, Fe^{2+}, Al^{3+}, Li^{+}, Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, and Co^{2+} in the jujube leaf extract were determined using inductively coupled plasma emission spectrometry (ICP-OES) with an ICP 730 ES apparatus (Varian, Palo Alto, CA, USA).

3. Results and discussion

Rutin (quercetin-3-rutinoside) is a glycoside of quercetin that is glycosylated with rutinose. Rutinose is a disaccharide that is sequentially linked to two saccharides: glucose and rhamnose. Thus, using hesperidinase, rutin can be sequentially transformed into isoquercitrin and then quercetin via single-stage deglycosylation because hesperidinase is an enzyme complex that has α-L-rhamnosidase and β-D-glucosidase activities.

The response of each of the enzymatic activities of hesperidinase to conditional factors are independent; the α-L-rhamnosidase activity is optimal at pH 4 and 50 °C (Yadav et al., 2010), while the β-D-glucosidase activity is optimal at pH 3.8 and 40 °C. The dependence of the kinetics of each enzyme transformation on different conditional factors allows for the tuning of the reaction to allow for the accumulation of isoquercitrin despite the presence of the β-D-glucosidase function. However, it is unlikely that the conditions that maximize the α-L-rhamnosidase activity coincide with conditions that minimize the β-D-glucosidase activity. Thus, we sought to determine the conditions that are best able to maximize isoquercitrin accumulation and minimize quercetin production by capitalizing on the differences in the kinetic preferences of the two enzyme activities.

Employing RSM, the optimal temperature, pH, reaction time and interactions between these variables were assessed for the production of isoquercitrin. The following polynomial regression equation was obtained for describing the isoquercitrin yield (Y):

$$Y = -23.812263 + 0.787849X_1 + 0.100424X_2 + 1.963675X_3 - 0.007834X_1^2 - 0.000442X_1X_2 - 0.000832X_2^2 - 0.004469X_3X_1 + 0.001376X_3X_2 - 0.171669X_3^2 \quad (2)$$

Based on ANOVA, the p-value for the total regression was 0.0144. Additionally, the R^2 value for the regression was 0.9394. The linear and quadratic regressions were statically significant, whereas the cross-product regression was not. The temperature and pH had the greatest influence on isoquercitrin production.

Fig. 1 provides a visual interpretation of the interactions between each pair of variables for facilitating the determination of the optimal experimental conditions while holding the third variable constant at the zero level. As shown in Fig. 1a, the isoquercitrin yield rapidly rose upon increasing the pH (X_3) from 2 to 5.31 before showing a rapid decrease above pH 5.31. When the pH was held constant, the isoquercitrin yield also rose when the reaction time (X_2) was increased from 24 to 52.16 h, at which point the yield started to decrease. Fig. 1b shows that the isoquercitrin yield increased when the temperature (X_1) was raised from 40 to 47.3 °C, but above 47.3 °C, the reaction yield decreased. Fig. 1c shows the impact of varying the reaction times and temperatures on the yield when the pH was fixed at the zero level.

The suitability of this model for optimizing the isoquercitrin yield was tested with a verification assay. Based on the canonical analysis, the isoquercitrin yield peaked at a stationary point that indicated the following optimal combination of factors: 47.3 °C, 52.16 h, and pH 5.31. The predicted isoquercitrin yield under these conditions was 2.65 mg/mL, which proved to be comparable to the experimental results (2.57 ± 0.16 mg/mL). This value was similar to the initial rutin concentration (2.66 ± 0.25 mg/mL), implying a nearly complete conversation of rutin to isoquercitrin. Moreover, the conditions yielding the peak isoquercitrin yield are very similar to the optimal conditions for the *A. niger* α-L-rhamnosidase activity.

According to previous kinetic based approaches, these observations were unanticipated. Accordingly, we hypothesized that the expected β-D-glucosidase activity was blocked. Some known causes of enzyme inactivation are as follows: (1) environmental stress, (2) denaturation, and (3) inhibition of the enzyme.

Among many factors affecting enzyme activity, temperature, time, pH, substrate concentration, and enzyme concentration are typically considered to be important. To determine whether the apparent lack of β-D-glucosidase activity was caused by those environmental factors, additional experiments were conducted using hesperidinase from *A. niger* with various environmental conditions (Fig. 2). The accumulation of isoquercitrin from rutin and the subsequent accumulation of quercitrin from isoquercitrin were observed, confirming functional hesperidinase α-L-rhamnosidase and β-D-glucosidase activities, respectively.

When testing for environmental stress effects, the impact of temperature and pH were investigated using 0.3 U/mL of hesperidinase because this enzyme level is the acknowledged optimal

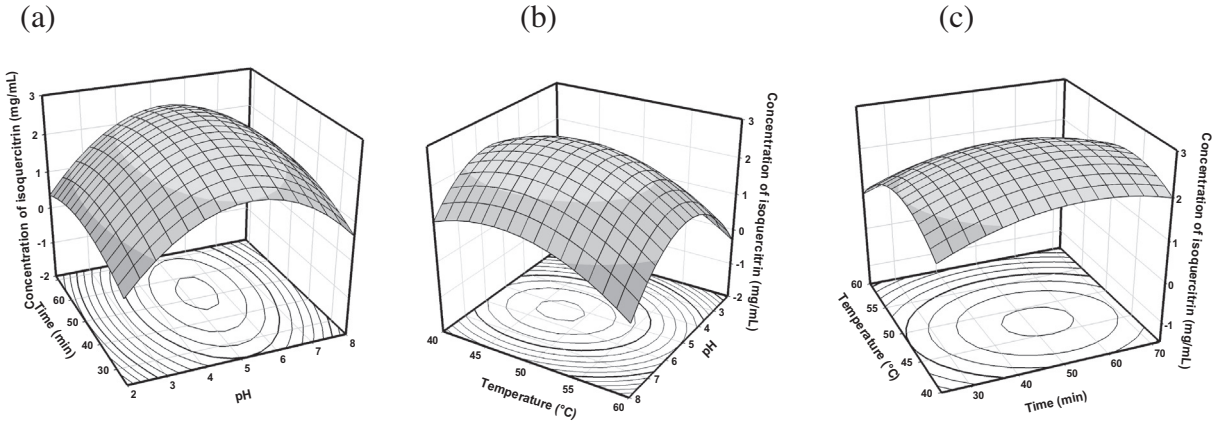

Fig. 1. Response surface plots and contour plots (a–c) showing the effects of (a) reaction time and pH, (b) reaction temperature and pH and (c) reaction time and temperature on the yield of isoquercitrin.

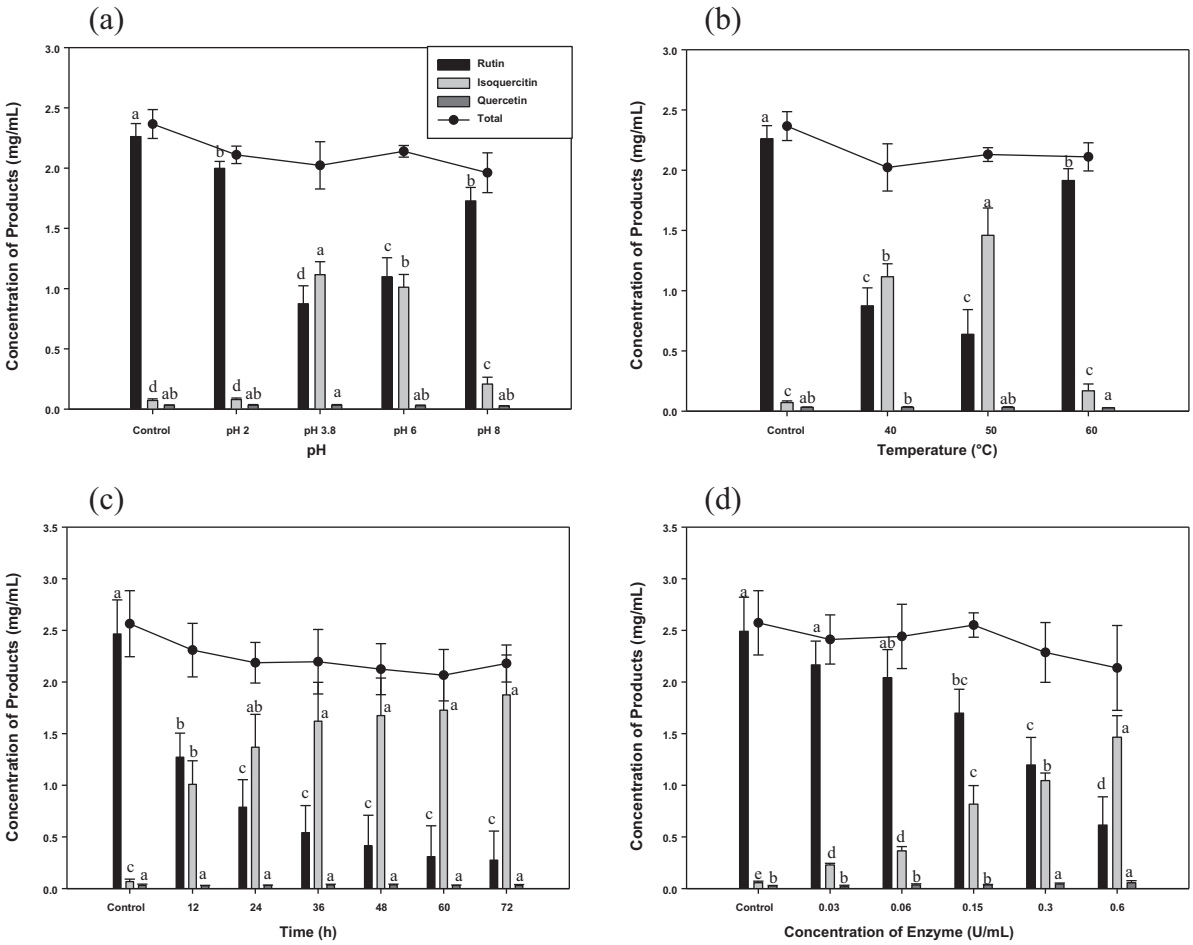

Fig. 2. Effect of (a) pH, (b) temperature, (c) treatment time and (d) the enzyme concentration of the reaction on the concentration of products. Different small alphabet letters indicate significantly different results as measured by the Tukey test.

condition for β-D-glucosidase activity. With regard to pH, the accumulation of isoquercitrin and quercitrin were analyzed after 24 h of enzymatic transformation under pH values of 2, 3.8, 6, and 8 at 40 °C. As shown in Fig. 2a, significant production of isoquercitrin was observed at pH 3.8 (1.12 ± 0.11 mg/mL) and pH 6 (1.01 ± 0.11 mg/mL). Nevertheless, the expected accumulation of quercetin was not observed at any pH.

Identical phenomena were also observed when the impact of temperature was evaluated after 24 h of enzymatic transformation at pH 3.8 at 40 °C, 50 °C and 60 °C (Fig. 2b). At all of the tested temperatures, the accumulation of isoquercitrin was observed as expected. However, the concentration of quercetin did not increase even at the optimal temperature for β-D-glucosidase activity in the presence of significant amounts of isoquercitrin. These observations are compatible with previous RSM results.

Considering possible kinetic suppression, the enzymatic biotransformation at pH 3.8 and 40 °C was followed for 72 h (Fig. 2c). The isoquercitrin yield first greatly increased before increasing more slowly after a sufficient time (36 h). The de-glycosylation rate for isoquercitrin was 90% after 90 min at 37 °C, as stated in a report from Day et al. The isoquercitrin concentration increased from 0.07 ± 0.02 to 1.88 ± 0.39 mg/mL. After 36 h, the conversion rate did not change significantly. However, β-D-glucosidase activity was not observed even after 72 h.

The effect of the enzyme concentration on enzyme activity was investigated by performing the reaction for 24 h, at pH 3.8 and 40 °C at various enzyme concentrations, including 0.03, 0.06, 0.15, 0.3, and 0.6 U/mL. As shown in Fig. 2d, increasing the enzyme concentration (0–0.6 U/mL) increased the isoquercitrin concentration from 0.06 ± 0.01 to 1.47 ± 0.21 mg/mL. In contrast, the quercetin concentration remained virtually unchanged. Despite the increase in enzyme concentration up to 0.6 mg/mL, which is equivalent to four times the concentration used by Wang et al. (2012), quercetin was not produced. Consequently, we concluded that environmental stress was not the reason for the unexpected inactivation of the β-D-glucosidase activity.

Denaturation of hesperidinase was not considered to be a likely cause for the lack of quercetin production because the α-L-rhamnosidase activity had been determined. However, selective denaturation of the β-D-glucosidase active site in the enzyme preparation may have occurred. The β-D-glucosidase assay was conducted at the accepted optimal conditions to verify the β-D-glucosidase activity of the enzyme using the substrate *p*-nitrophenyl β-D-glucopyranoside. The catalytic product resulted, indicating that hesperidinase had β-D-glucosidase activity (Fig. 3). Therefore, we concluded that neither environmental stress nor enzyme denaturation was the cause of the observed inactivation of β-D-glucosidase.

We recognized that some metal ions are capable of inhibiting enzyme activities. The presence of Ag^{+} or Hg^{2+} is known to quantitatively inhibit β-D-glucosidase when present at concentrations above 0.008 mM and 0.016 mM, respectively (Esen, 1992). Therefore, the presence of metal ions in the jujube leaf extract

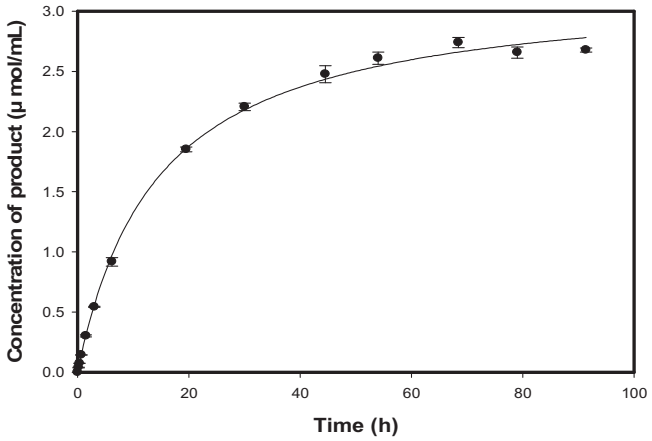

Fig. 3. The known optimal conditions for β-glucosidase activities, verifying the activity and substrate-inhibition capacities of the enzyme itself. The enzyme activity was tested at the optimal conditions (pH 5.31, 47.3 °C, 0.3 unit/mL of hesperidinase and 5.5 μmol/mL of substrate).

Table 1
Concentration of metal ions affecting the β-D-glucosidase activity in jujube leaf extract.

Metal ions	Concentration (mM)
Cu^{2+}	4.09×10^{-6}
Zn^{2+}	3.99×10^{-5}
Mn^{2+}	5.28×10^{-6}
Fe^{3+}	2.69×10^{-6}
Al^{3+}	1.11×10^{-6}
Li^{+}	ND
Ca^{2+}	2.44×10^{-4}
Na^{+}	5.35×10^{-5}
Mg^{2+}	2.27×10^{-3}
K^{+}	1.11×10^{-2}
Co^{2+}	ND
Ag^{+}	ND
Hg^{2+}	ND

was assessed using ICP-OES. The jujube leaf extract contained approximately 0.01 mM metal ions in total with no Ag^{+} and Hg^{2+}. This concentration of metal ions in the jujube leaf extract was judged too low to affect the β-D-glucosidase activity (Table 1).

Substrate inhibition was also considered. However, previous observations allowed us to exclude this potential source of inhibition. As shown in Fig. 2c, the concentration of isoquercitrin, the β-D-glucosidase substrate, increased from 0.07 ± 0.02 to 1.87 ± 0.38 mg/mL when the reaction was allowed to proceed for 72 h. If an excessively high concentration of the β-D-glucosidase substrate, isoquercitrin, was the reason for β-D-glucosidase inhibition, quercetin should have accumulated before increasing the isoquercitrin concentration. No accumulation of quercetin was observed; therefore, substrate inhibitory effects were unlikely.

The possibility of product inhibition was also checked. The jujube leaf extract contained about 2.5 mg/mL of glucose and other free sugars were under detection level. Moreover, glucose content was unchanged after enzyme treatment. Even though glucose can inhibit β-D-glucosidase activity by product inhibition, this amount of glucose was not enough to show inhibition (data not shown). Even with 250 mg/mL of glucose, the degree of inhibition was a lot less than that with jujube leaf extract.

We further considered the potential presence of additional substances that are capable of inhibiting the β-D-glucosidase activity. Well-known β-D-glucosidase inhibitors include ionic liquids (Wang et al., 2013) and chemicals, such as cyclophellitol and β-epoxide (Gloster, Madsen, & Davies, 2007). According to the research of Zhang et al. (2014a), jujube leaves do not contain these ionic liquids and chemicals. Hence, the presence of previously undetected β-D-glucosidase inhibitors in the jujube leaf was suspected.

By employing a commercial enzyme, production of isoquercitrin from jujube leaf is highly effective both because jujube leaf contains a high amount of rutin and because a maximum yield of enzymatic biotransformation can be achieved without any additives. In addition, the experimental results led us to believe that unknown β-D-glucosidase inhibitor(s) would exist in jujube leaf. If this suspicion is correct, the undiscovered inhibitor(s) is of natural origin and thus would be allowed in food-grade applications, particularly because jujube leaf has long been consumed as tea in China. Furthermore, identification of a potential β-D-glucosidase inhibitor could be used to elucidate the mechanisms of various biochemical reactions. Moreover, natural β-D-glucosidase inhibitors are potential candidates for treating lysosomal storage disorders, such as Gaucher disease. For these reasons, we hope that this report will stimulate further research into potential natural β-D-glucosidase inhibitors in jujube leaves.

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