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ORIGINAL ARTICLE

Convenient Preparation of 2″-O-Rhamnosyl Icariside II, a Rare Secondary Flavonol Glycoside, by Recyclable and Integrated Biphase Enzymatic Hydrolysis

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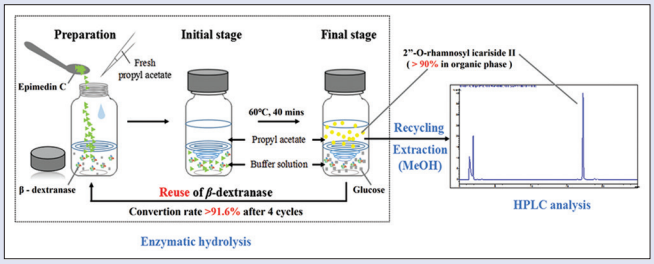
ABSTRACT

Background: 2″-O-rhamnosyl icariside II, a rare secondary flavonol glycoside in Epimedii Folium (EF), has much better *in vivo* bioactivities than its original glycoside epimedin C. Its preparation methods, such as acidic hydrolysis, are of low efficiency, with by-products generated. **Objective:** The objective of this study was to establish a novel catalysis system for convenient preparation of this compound based on the recyclable and integrated biphase enzymatic hydrolysis. **Materials and Methods:** β-dextranase was selected from five commercial enzymes due to the best catalysis performance. After optimization of conditions, the biphase system was constructed with propyl acetate and HAc-NaAc buffer (pH 4.5) (3:2, v/v) containing β-dextranase/epimedin C (1:2, w/w), and the hydrolysis was performed at 60°C for 40 min. **Results:** Epimedin C was completely hydrolyzed to 2″-O-rhamnosyl icariside II, and 93.38% of the product has been transferred into organic phase; moreover, a high conversion rate had been achieved at 91.69% even after the enzyme solution was used for four cycles. In addition, the procedure was much simplified compared with conventional enzymatic hydrolysis. **Conclusion:** The newly proposed strategy is an efficient and promising approach for the preparation of 2″-O-rhamnosyl icariside II in industrial application.

Key words: 2″-O-rhamnosyl icariside II, biphase enzymatic hydrolysis, Epimedii Folium, epimedin C, reusability

SUMMARY

- A novel recyclable and integrated catalysis system was established to prepare a rare flavonoid 2″-O-rhamnosyl icariside II for the first time
- The conversion of epimedin C and extraction of 2″-O-rhamnosyl icariside II were realized in an integrated step by the efficient and convenient approach
- The β-dextranase solution remained about 92% of its initial activity after repeatedly uses for four cycles.



Abbreviations used: EF: Epimedii Folium; ACN: Acetonitrile; MeOH: Methanol; PTFE: Polytetrafluoroethylene; SD: Standard deviation; ILO-ICSC: International Labor Organization-The International Chemical Safety Cards; HMDB: Human Metabolome Database; HSDB: Hazardous Substances Data Bank.

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INTRODUCTION

Epimedii Folium (EF) is the dried leaves of four *Epimedium* plants including *E. brevicornum* Maxim., *E. sagittatum* (Sieb. et Zucc.) Maxim., *E. pubescens* Maxim., and *E. koreanum* Nakai.[1] The herb is one of the most recognized traditional herbal medicines for the treatment of cardiovascular diseases, bone loss, and impotence in Asia region. In previous studies, EF has demonstrated lots of pharmacological and biological activities, such as anti-osteoporosis,[2,3] anti-Alzheimer's disease,[4] anti-inflammatory, promotion of sexual function,[5] and anti-aging.[6] Moreover, it has been revealed that its major bioactive constituents are flavonol glycosides with an isopentene group at C-8 position of ring A, such as icariin, epimedoside A, epimedin B, epimedin C, and baohuoside I.[7-12] Epimedin C, one of the principal 8-prenylflavonoids in EF, is well known to promote the proliferation of osteoblast-like cells and has represented the corresponding clinical efficacy of this herb to a certain extent.[13-15] However, few epimedin C molecules could pass through small intestine due to its great hydrophilicity of three sugar moieties, and they were hardly absorbed into blood,[16] which has largely restricted its potential medicinal applications. 2″-O-rhamnosyl icariside II [Figure 1], the product of specific hydrolysis of glycosidic bond (-OR_2) at C-7 position on epimedin C,[17] could be readily transported into plasma and plays an important bioactive role *in vivo*.[18] It is a rare secondary flavonol glycoside in EF with relatively higher price than its original glycoside (epimedin C) due to its trace quantities in raw material. Thus, it is promising to develop this flavonoid

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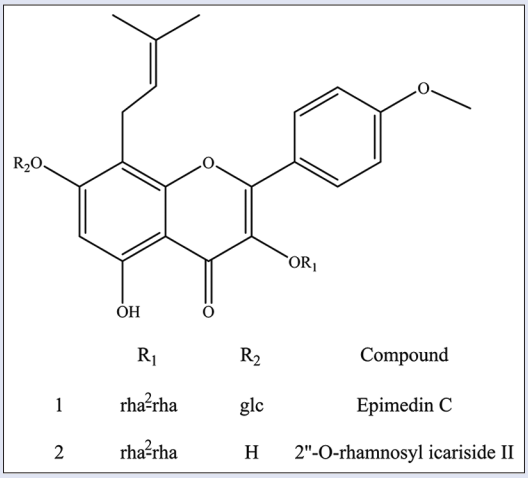

Figure 1: Chemical structures of epimedin C (1) and 2”-O-rhamnosyl icariside II (2). glc: glucose; rha: rhamnose

to be a new molecular entity, and the convenient preparation of this rare component is a crucial task.

In previous investigation, a lot of efforts have been put to obtain 2”-O-rhamnosyl icariside II. However, the methods based on the chemical hydrolysis often bring some adverse effects on the stability of 2”-O-rhamnosyl icariside II and usually form a number of by-products due to the hardly controlled catalysis in the course of hydrolysis. In addition, the highest yield was achieved at 97.7% when biotransformation was employed to convert epimedin C to 2”-O-rhamnosyl icariside II, but it is also not preferred at the industrial level because of its time-consuming and inconvenient procedures.[18] Alternatively, the mild enzymatic hydrolytic process has been proved to be effective and predominant in the applications for the production of many secondary glycosides or their aglycones nowadays.[19-22]

In this study, we aimed to establish a novel recyclable and integrated catalysis system for convenient preparation of 2”-O-rhamnosyl icariside II based on the enzymatic hydrolysis. At first, the enzyme showing the best performance on hydrolysis of epimedin C was selected among five commercially available enzymes, followed by the optimization of the hydrolysis conditions sequentially. Then, enzymatic hydrolysis in an aqueous organic two-phase system was carried out, and the key technique was developed to achieve a procedure of more efficiency and higher convenience for the preparation of this rare flavonol glycoside. Furthermore, the separated enzyme solution and the recycled organic solvent were continuously used in the newly proposed system to investigate its reusability.

MATERIALS AND METHODS

Materials and chemicals

Epimedin C (Batch No.: 16123007; purity ≥98.0% by high-performance liquid chromatography-ultraviolet [HPLC-UV]) was purchased from Chengdu Pufei De Biotech Co., Ltd. (Sichuan Province, China). Acetonitrile (ACN) was of HPLC grade and obtained from OmniGene LLC (United States), and methanol (MeOH) of analytical grade was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ethyl acetate, propyl acetate, vinyl acetate, ethyl propionate, isopropyl ether, and methyl tert-butyl ether of analytical grade were all the products from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). *β*-glucosidase (activity: 100 U/g) and *β*-dextranase (activity: ≥20000 U/g) were bought from Jinsui Biotech Co., Ltd. (Shanghai, China) and Jiangsu Ruiyang Biotech Co., Ltd. (Wuxi, China). Cellulase (activity: ≥15000 U/g) and glucoamylase (activity: ≥100000 U/g) were provided by Sinopharm Chemical Reagent Co., Ltd. and Duly Biotech Co., Ltd. (Nanjing, China). Naringinase (activity: 100000 U/g) was supplied by Henan Baikang Chemical Products Co., Ltd. (Shangqiu, China).

Enzymatic hydrolysis

Screening of enzymes

In the first stage, five commercial enzymes including *β*-glucosidase, *β*-dextranase, cellulase, naringinase, and glucoamylase were investigated for efficient hydrolysis of epimedin C to 2”-O-rhamnosyl icariside II. In details, 100 μg of epimedin C and each of these enzymes (12.5–800 μg) was dissolved in 2 mL of 0.20 M HAc-NaAc buffer (pH 4.5) and incubated at 60°C for 1 h. Then, an equal volume of MeOH was added into the hydrolysate to extract 2”-O-rhamnosyl icariside II after hydrolysis, and the resulting solution was subjected to filtration through a 0.20-μm polytetrafluoroethylene (PTFE) membrane syringe before subsequent HPLC-UV analysis. The conversion rate of epimedin C was set as an index for the selection of enzymes demonstrating the best catalysis performance among the five enzymes.

Optimization of enzymatic hydrolysis conditions

The effects of five factors, including the enzyme/epimedin C ratio, hydrolysis duration, reaction temperature, pH of buffer, and metal ion, on the conversion rate of epimedin C were investigated by a single-factor experiment. Initially, the hydrolysis efficiency of *β*-dextranase was measured, while different enzyme/epimedin C ratios were employed for the enzymatic hydrolysis to acquire the optimal level. In details, 100 μg of epimedin C was incubated with *β*-dextranase (12.5–600 μg) in 2 mL of 0.20 M HAc-NaAc buffer (pH 4.5) at 60°C for 1 h. Moreover, the duration of enzymatic hydrolysis (10–60 min) was investigated, while the substrate was hydrolyzed by 50 μg of the enzyme. Then, the reaction temperatures (30°C ~ 80°C) were compared, while hydrolysis was performed in the buffer (pH 4.5) for 40 min. Furthermore, the hydrolysis was conducted at 60°C for 40 min in different buffer solutions of pH values ranging from 3 to 8 individually. The control was performed without *β*-dextranase under the same reaction conditions to prepare a blank solution. In addition, the influence of metal ions on the *β*-dextranase activity was also investigated under the previously optimized conditions. The enzyme was reacted with epimedin C in the presence of 1-mM metal ions, and the conversion rate of epimedin C was compared with that of control without any addition of metal ions by calculation of the relative conversion rate. All experiments were performed in triplicate, the mean and standard deviation (SD) were calculated, and the data were presented as mean ± SD.

Establishment of the integrated enzymatic catalysis system

An integrated enzymatic catalysis system was newly developed based on the conventional biphase hydrolysis[23-25] in this study, and the aims were to improve the convenience of production of 2”-O-rhamnosyl icariside II and recycle the enzyme solution and organic solvent for repeatedly uses in this application. As illustrated in Figure 2, both epimedin C and *β*-dextranase were added and dissolved into the bottom aqueous phase (0.20-M HAc-NaAc buffer, pH 4.5) and further covered with a water-immiscible organic solvent to form a two-phase status. In this catalysis system, the glucose on the R_2O-position of epimedin C was removed by enzymatic hydrolysis; meanwhile, the resulting 2”-O-rhamnosyl icariside II in the aqueous solution could be immediately transferred into the top organic phase since the less polar secondary flavonol glycoside has a much higher partition coefficient than its original glycoside. Then, 2”-O-rhamnosyl icariside II could be easily obtained after the distillation of the volatile organic solvent under

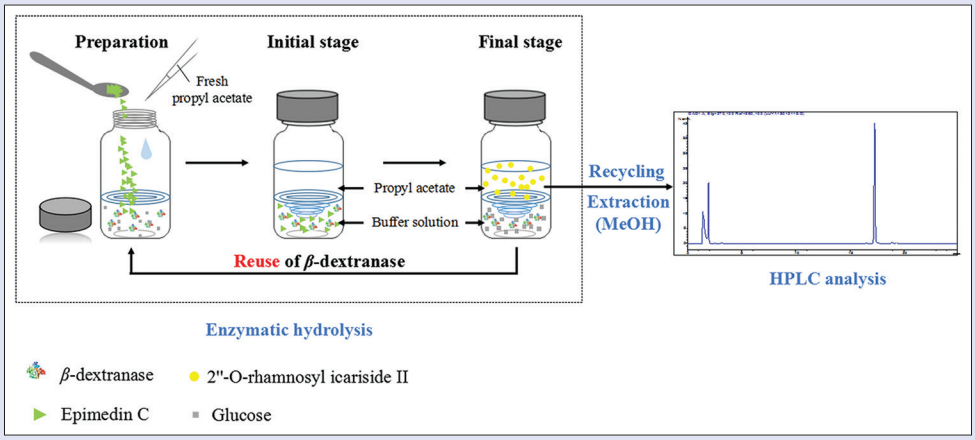

Figure 2: Diagram of biphase enzymatic hydrolysis of epimedin C

reduced pressure. After reacting 40 min, we only took 200-μL both organic solvent and buffer solution from biphase system for further HPLC quantitative analysis. To evaluate the efficiency, residual epimedin C was quantified by HPLC-UV analysis to calculate the conversion rate. The 200-μL organic solvent was dried and redissolved with methanol for HPLC analysis. The 200-μL buffer solution was then mixed with methanol, and the resulting solution was subjected to filtration before subsequent HPLC analysis. The rest of organic (ca. 6 mL) in biphase hydrolysis system was distilled under reduced pressure to prepare 2"-O-rhamnosyl icariside II.

Organic solvent played a crucial role in the newly proposed enzymatic catalysis system, which was closely related to hydrolysis performance of enzyme solution, extraction efficiency of 2"-O-rhamnosyl icariside II, and convenience improvement in the application. Therefore, six solvents, including ethyl acetate, propyl acetate, vinyl acetate, ethyl propionate, isopropyl ether, and methyl tert-butyl ether were compared in terms of their effects on the conversion rate of epimedin C and the transfer rate of 2"-O-rhamnosyl icariside II in organic phase. To select the most ideal organic solvent for the novel hydrolysis scheme, the biphase system composed of 6-mL buffer solution and another 6-mL organic solvent was established. The concentrations of epimedin C and *β*-dextranase in buffer were 0.5 mg/mL and 0.25 mg/mL, respectively. The reaction solution was gently stirred at 60°C for 4 h, nevertheless 50°C for methyl tert-butyl ether due to its low boiling point. Finally, epimedin C and 2"-O-rhamnosyl icariside II in those two phases were determined by HPLC-UV.

Optimization of conditions for enzymatic hydrolysis

The hydrolysis duration (30 min ~ 4 h) was examined for integrated biphase enzymatic hydrolysis. Moreover, in the propyl acetate buffer system (1:1, v/v), 0.5-mg/mL epimedin B and 0.25-mg/mL *β*-dextranase were reacted at 60°C. In addition, the volume ratio of organic solvent to buffer was also optimized to achieve the highest transfer rate of 2"-O-rhamnosyl icariside II from the aqueous phase. Finally, the enzyme solution and organic solvent were recycled for repeatedly uses in the newly proposed biphase system to investigate its reusability.

High-performance liquid chromatography-ultraviolet analysis of epimedin C and 2"-O-rhamnosyl icariside II

The determination of epimedin C and 2"-O-rhamnosyl icariside II was conducted using an Agilent 1100 HPLC instrument equipped with a G1322A Degasser, G1312A BinPump, G1313A ALS Autosampler, G1316A Thermostatted Column Compartment, and G1315A Photodiode Array Detector. The data and chromatograms were collected for processing via ChemStation Software (Rev. A.07.01 [682]). All sample solutions were separated on a ZORBAX SB-C18 column (150 mm L. × 4.6 mm I. D., 5 MM). The column temperature was kept at 40°C throughout the analysis, and the mobile phases consisted of ACN (A) and ultrapure water (B). Gradient elution was performed at a flow rate of 1.0 mL/min, and the time programs were as follows: 0–8 min, A (28%) and B (72%); 17 min, A (50%) and B (50%); 21 min, A (28%) and B (72%); and 21–25 min, A (28%) and B (72%). The UV detection wavelength was set at 270 nm, and injection volume was 20 μL.[26-27]

Epimedin C (10 mg) or 2"-O-rhamnosyl icariside II (10 mg) was precisely weighed and then dissolved in MeOH and scaled to 50 mL as stock-solution mixture. Then, a series of dilutions were performed to prepare standard solutions with concentrations ranging from 1.563–200 μg/mL. Before any injection, all solutions were filtered through a 0.20-μm PTFE membrane syringe. To determine linearity, the standard solutions were analyzed, and the peak area (*Y*) versus the concentration (*X*) of either epimedin C or 2"-O-rhamnosyl icariside II was then plotted for calibration curves by ordinary linear regression using Microsoft Office Excel 2003.

Calculations

The concentrations of residual epimedin C and resulting 2"-O-rhamnosyl icariside II in both organic phase and buffer solution were calculated according to the plotted calibration curves. Then, the conversion rate of epimedin C in conventional enzymatic hydrolysis (I_1%) and the novel strategy (I_2%) as well as the transfer rate of 2"-O-rhamnosyl icariside II (*B*%) in organic phase were calculated according to the following equations:

$$I_1(\%) = \frac{C_{kb}V_b - C_{rb}V_b}{C_{kb}V_b} \times 100\%$$

$$I_2(\%) = \frac{(C_{ko}V_o + C_{kb}V_b) - (C_{ro}V_o + C_{rb}V_b)}{C_{ko}V_o + C_{kb}V_b} \times 100\%$$

Where C_{ko} and C_{kb} were the concentrations of epimedin C in organic phase and buffer of blank solution without enzymes added, respectively, in mg/mL; C_{ro} and C_{rb} were the concentrations of residual epimedin C in the organic phase and buffer of sample solution with enzymes added, respectively, in mg/mL; and V_o and V_b were the volumes of organic phase and buffer, in mL.

Where C_o and C_b were the concentrations of 2"-O-rhamnosyl icariside II in the organic phase and buffer of the biphase system, in mg/mL, and V_o and V_b were the volumes of organic phase and buffer, in mL.

Identification of 2"-O-rhamnosyl icariside II

After integrated biphase enzymatic hydrolysis, propyl acetate was recycled below 50°C under reduced pressure by a rotovap. The resulting yellow powder was obtained and analyzed by ESI-MS (Thermo LXQ, USA), ^{1}H-NMR, and ^{13}C-NMR (Bruker Avance II 400 MHz, USA) for the confirmation of chemical structure with desired 2"-O-rhamnosyl icariside II.

RESULTS AND DISCUSSION

High-performance liquid chromatography-ultraviolet chromatograms and standard curves

HPLC chromatograms of epimedin C, 2"-O-rhamnosyl icariside II, and sample solution are shown in Figure 3. Standard curves of two references were drawn as $Y = 6853.4237X–10.8710$ ($R^2 = 0.9997$) and $Y = 11279.1323X–9.0287$ ($R^2 = 0.9999$), respectively, indicating good linear relationships in the concentration ranging from 1.563 to 200.0 μg/mL.

Screening of enzymes for integrated biphase hydrolysis

The most commonly used biocatalysts for the hydrolysis of flavonol glycosides, β-glucosidase, β-dextranase and cellulase[19-20] as well as two available enzymes, namely glucoamylase and naringinase in our laboratory were assayed in this study. The HPLC-UV chromatograms of sample solution after conventional hydrolysis of epimedin C by the five commercial enzymes under the same conditions are shown in Figure 4a. While the concentration of enzyme was 400 μg/mL, epimedin C had been completely hydrolyzed by β-glucosidase, β-dextranase, or cellulase, but only a little or none was hydrolyzed by either glucoamylase or naringinase. These results were in agreement with our previous study, in which a higher conversion rate of icariin, another flavonol glycosides in EF, was obtained via β-glucosidase, β-dextranase, and cellulase compared with that of glucoamylase or naringinase.[19]

As shown in Figure 4b, the hydrolysis ability of cellulase was much weaker than β-glucosidase and β-dextranase, as only 73.0% of epimedin C had been converted when the enzyme solution was diluted to 25.0 μg/mL. In addition, β-glucosidase and β-dextranase exhibited similar efficiency to hydrolyze epimedin C; however, while the concentration was further decreased to 12.5 μg/mL, >90% of epimedin C was still converted by β-dextranase, compared to 76.7% achieved by the other. As reported, β-glucosidases, β-dextranase, or cellulase can catalyze the hydrolysis of glucosidic linkages of various disaccharides, oligosaccharides, or glycosides.[28-33] The three enzymes have exhibited a difference in their hydrolytic efficiency of epimedin C in our research, which could be caused by the various affinities with this specific substrate under the conditions.

As a consequence, β-dextranase was eventually selected from five commercial enzymes due to the best catalysis performance.

Optimization of integrated enzymatic hydrolysis conditions

Enzyme/epimedin C ratio

The effect of the enzyme/epimedin C ratio on hydrolysis was investigated, and the result was shown in Figure 4c. The conversion rate of epimedin C increased from approximately 60% to 100% when the enzyme/epimedin

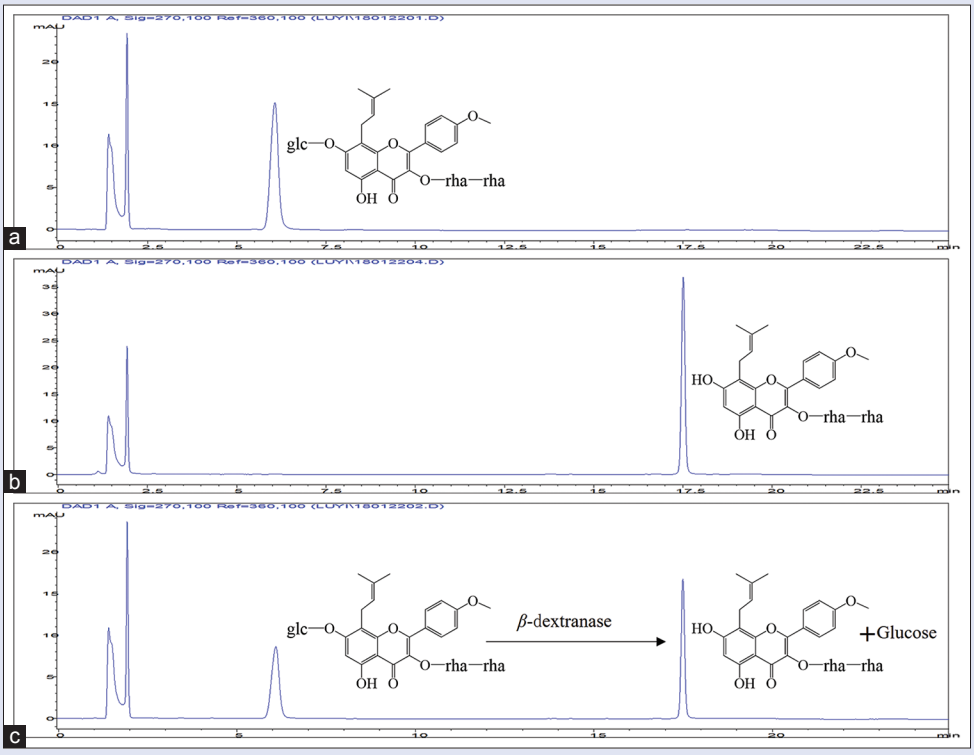

Figure 3: High-performance liquid chromatography-ultraviolet chromatograms. (a) epimedin C. (b) 2"-O-rhamnosyl icariside II. (c) sample solution after catalysis reaction

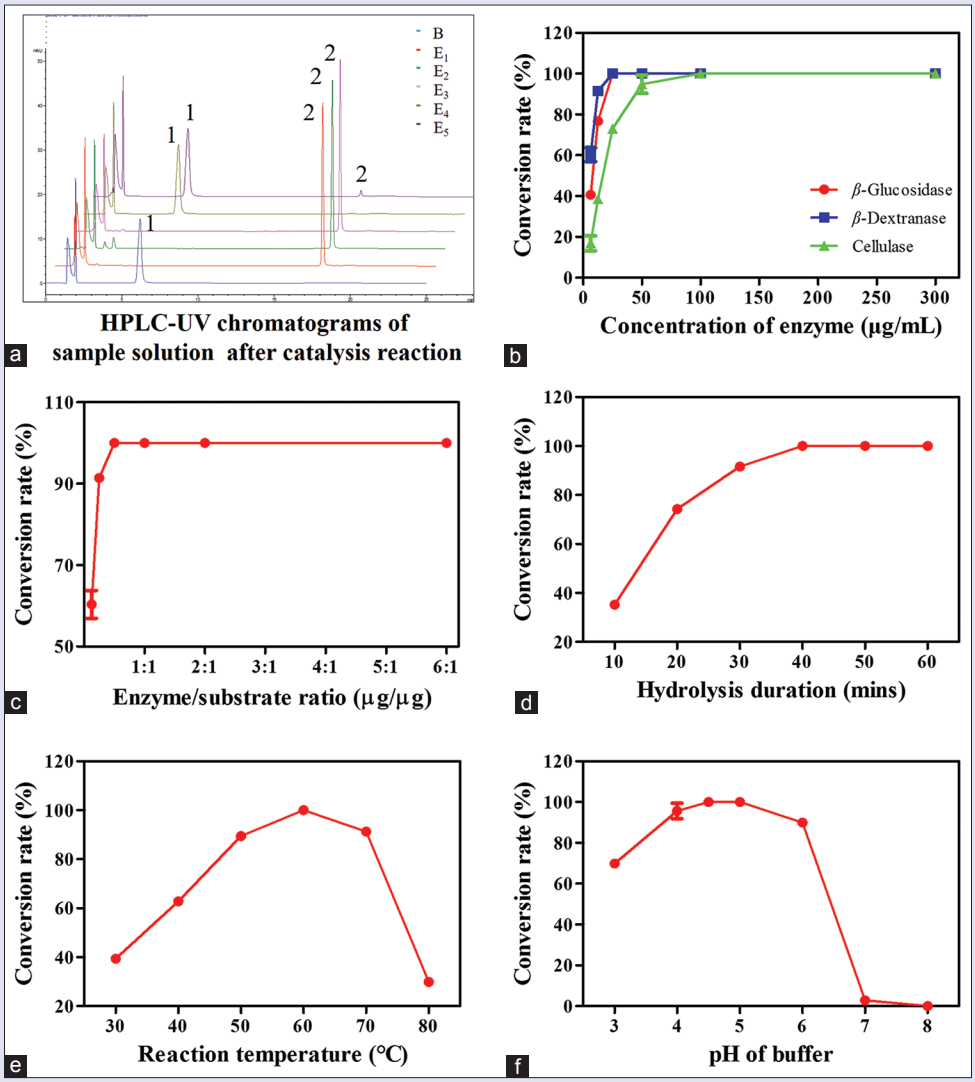

Figure 4: Effects of the (a): enzyme, 400 μg/mL, B: No enzyme, E1: β-glucosidase, E2: β-dextranase, E3: Cellulase, E4: Naringinase, E5: Glucoamylase, 1: Epimedin C, 2: 2"-O-rhamnosyl icariside II. (b) Enzyme concentration. (c) Enzyme/epimedin C ratio. (d) Hydrolysis duration. (e) Reaction temperature. (f) pH of the buffer, on the conversion rate of epimedin C

C ratio increased from 1:8 (μg/μg, w/w) to 1:2 (μg/μg, w/w), and the rate still remained at 100% when the enzyme/epimedin C ratio was 1:1 or higher. Therefore, an enzyme/epimedin C ratio of 1:2 was chosen for the following enzymatic hydrolysis:

Hydrolysis duration

Hydrolysis durations (10–60 min) were investigated to optimize the hydrolysis reaction conditions. As shown in Figure 4d, the conversion rate of epimedin C was dramatically increased to 74.3% within the initial 20 min, followed by a moderate increase to 100% in the next 20 min. Hence, 40 min was chosen as the most appropriate duration to ensure complete hydrolysis.

According to the enzyme kinetics, the fastest speed occurred in the initial period of enzymatic hydrolysis; then, the rate decreased gradually because of the reduced amount of substrate or impaired enzyme activity. In addition, feedback inhibition of the product likely stimulates the reverse reaction and further reduces the speed of enzymatic hydrolysis. The concentration of glucose in buffer continuously increased with the hydrolysis lasts, which could have led to an inhibitory effect on the catalysis of *β*-dextranase.

Reaction temperature

The conversion rate of epimedin C was compared at different reaction temperatures (30°C ~ 80°C), after hydrolysis at pH 4.5 for 40 min. From Figure 4e, it can be seen that the most suitable temperature of enzymatic hydrolysis was 60°C, at which all of the epimedin C has been converted within 40 min. However, the conversion rate rapidly decreased when the reaction temperature was lower than 50°C or higher than 70°C. To achieve complete hydrolysis, 60°C was selected for the catalysis in the subsequent experiments.

pH of buffer

Figure 4f illustrates the effect of pH of buffer on the hydrolysis performance of *β*-dextranase after constant incubation for 40 min. In general, >80% of epimedin C was converted to 2"-O-rhamnosyl icariside II in the pH range of 4.0–6.0; however, the conversion rate dramatically declined to <5% at pH 7.0 or above. Considering the greater robustness in the performance of *β*-dextranase in more acidic condition, buffer at pH 4.5 was chosen for enzymatic hydrolysis with the highest conversion rate achieved at 100%.

Metal ions

Some metal ions have been demonstrated to promote or inhibit β-dextranase activity at different concentrations.[34,35] The effect of several cations (1 mM) on converting epimedin C to 2"-O-rhamnosyl icariside II via β-dextranase was investigated, and the conversion rate of epimedin C without any extra cations was considered as 100%. As shown in Table 1, robust inhibition was observed in the presence of Ag+. The cation Cu^{2+} exerted no advance effects with regard to the enzyme activity. Moreover, Ca^{2+}, K^{+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, Ba^{2+}, Mn^{2+}, and Al^{3+} did not show capability to enhance activity significantly. Thus, no metal ions were added intentionally into enzymatic solutions in further experiments.

Table 1: Effects of metal ions on the enzymatic hydrolysis of epimedin C (*n*=3)

Metal ions[a]	Relative conversion rate[b] (%)
Control	100
Ca^{2+}	103.8±1.1
K^{+}	105.4±0.7
Ag^{+}	59.7±1.3
Mg^{2+}	103.9±0.7
Fe^{2+}	103.3±1.2
Fe^{3+}	100.8±0.1
Cu^{2+}	93.6±0.4
Co^{2+}	100.7±0.5
Zn^{2+}	102.1±0.8
Ba^{2+}	103.9±0.8
Mn^{2+}	103.6±0.6
Al^{3+}	102.9±0.3

[a]The concentration of metal ions used in hydrolysis was 1 mM; [b]The conversion rate without metal ions was considered as 100%

Table 2: Relevant characteristics of investigated organic solvents

Organic solvents	Solubility in water 20°C (g/L)	Log P[c]	Boiling point[c] (°C)
Ethyl acetate	87[a]	0.73	77.1
Vinyl acetate	20[a]	0.73	72.8
Methyl tert-butyl ether	42[a]	0.94	55
Ethyl propionate	19.2[b]	1.21	99.2
Propyl acetate	18.9[c]	1.24	101.3
Isopropyl ether	8.8[c]	1.52	68.5

[a]Data retrieved from the ICSC (ILO-ICSC: http://www.ilo.org/dyn/icsc/showcard.home); [b]Data retrieved from (HMDB: http://www.hmdb.ca); [c]Data retrieved from (HSDB: http://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm). ILO: International Labor Organization; ICSC: International Chemical Safety Cards; HMDB: Human Metabolome Database; HSDB: Hazardous Substances Data Bank

Integrated enzymatic catalysis

Construction of the integrated system

A number of less polar solvents including ethyl acetate, propyl acetate, vinyl acetate, ethyl propionate, methyl tert-butyl ether, and isopropyl ether[36] were examined as the organic phases in the integrated system for the hydrolysis reaction by β-dextranase. Relevant characteristics of these solvents are summarized in Table 2.

In Figure 5a, the conversion rate of epimedin C over hydrolysis durations in the integrated biphasic system consisting of various organic solvents was shown. In the initial stage (0–1 h), the conversion rate increased gradually and then maintained at 100% (97% for ethyl acetate) after 1 h. Moreover, the conversion of epimedin C in methyl tert-butyl ether/buffer biphasic system was much lower than the others. This effect could have been resulted from the relatively low reaction temperature (50°C) as well as the serious deactivation of β-dextranase caused by methyl tert-butyl ether. After being hydrolyzed at 60°C for 40 min, epimedin C can be converted completely if propyl acetate, vinyl acetate, or isopropyl ether was employed. Meanwhile, the conversion rates of epimedin C were 90.2% and 97.2% for ethyl acetate and ethyl propionate, respectively. This implied that the solvent molecules dissolved in the aqueous phase could have led to a significant negative impact on the activity of β-dextranase.

The transfer rate of 2"-O-rhamnosyl icariside II over hydrolysis durations in the integrated biphasic system consisting of various organic solvents was also investigated. As shown in Figure 5b, the transfer rate rose within the first 1 h and has been stable from 1 h onward. Compared with other solvents, the transfer rate of 2"-O-rhamnosyl icariside II in isopropyl ether was much lower (<20%) owing to the significant difference in polarity between the solvent and 2"-O-rhamnosyl icariside II. Furthermore, there was no obvious distinction in the transfer rate of 2"-O-rhamnosyl icariside II after reaction for 1 h between methyl tert-butyl ether and vinyl acetate. Meanwhile, the transfer rate of 2"-O-rhamnosyl icariside II in ethyl acetate, propyl acetate, and ethyl propionate was all above 95% and higher than that in those two solvents.

Based on the results of the above investigates, propyl acetate was consequently selected as the most proper organic solvent for the integrated biphasic system since it has exhibited minimal environmental impact to maintain the activity of β-dextranase and maximal extraction of 2"-O-rhamnosyl icariside II. After being hydrolyzed for 40 min, the

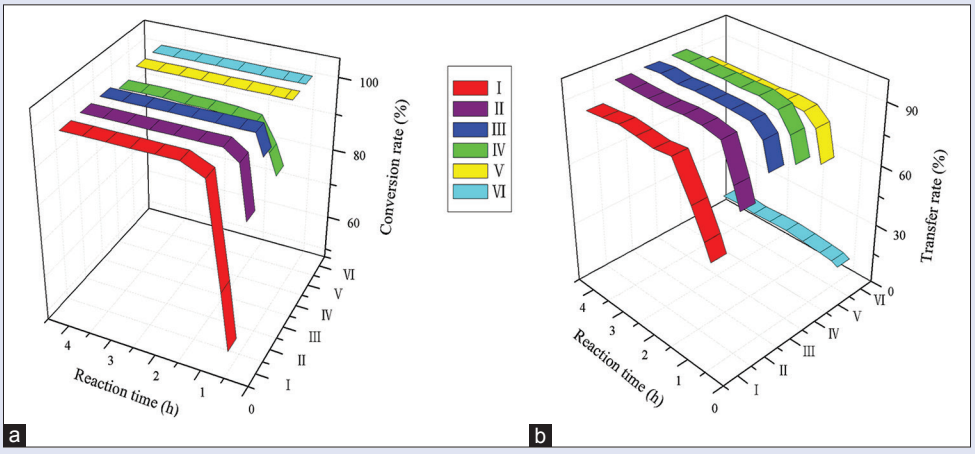

Figure 5: Conversion rate of epimedin C (a) and transfer rate of 2"-O-rhamnosyl icariside II in organic phase (b) in biphasic system with different organic solvents. I: methyl tert-butyl ether; II: ethyl propionate; III: propyl acetate; IV: ethyl acetate; V: vinyl acetate; VI: isopropyl ether

conversion rate of epimedin C and the transfer rate of 2"-O-rhamnosyl icariside II in propyl acetate were 100% and 90.2%, respectively. Accordingly, 40 min was chosen for the integrated process.

Optimization for integrated biphase enzymatic hydrolysis

To further improve the transfer rate of 2"-O-rhamnosyl icariside II in the integrated biphase system, the volume ratio of propyl acetate to buffer was investigated. As shown in Table 3, the highest transfer rate of 2"-O-rhamnosyl icariside II was obtained when the volume ratio of propyl acetate to buffer was 3:2. Compared to others, the conversion rate of epimedin C decreased to 93.24% in the biphase enzymatic system with 2:1 volume ratio. The lower conversion rate of epimedin C was attributed to the extraction of epimedin C by the larger volume of propyl acetate and unavailable contact of epimedin C in the top phase and *β*-dextranase. According to the results, the optimal volume ratio was set as 3:2.

Reusability of β-dextranase

To explore the reusability of the enzyme solution containing commercial *β*-dextranase for the conversion of epimedin C to 2"-O-rhamnosyl icariside II, the hydrolysis of epimedin C was conducted for ten cycles using the bottom buffer solution. As shown in Figure 6, the conversion rate of epimedin C was kept above 90% even after the buffer has been used for four cycles, and the *β*-dextranase showed an excellent stability against the reaction and remained almost 70% of its initial activity after seven cycles. The decrease in conversion rate may be caused by the deactivation on enzyme by the organic solvent, inhibition effect on enzymatic hydrolysis by more and more glucose in the buffer, and the enzyme's denaturation under heating for a long time.

Identification of 2"-O-rhamnosyl icariside II

ESI-MS and NMR were applied to identify the prepared product from integrated enzymatic hydrolysis, and the chemical formula of this compound was determined as $C_{33}H_{40}O_{14}$. The positive mode ESI-MS spectrum is shown in Figure 7.

Table 3: Effects of volume ratio on the transfer rate of 2"-O-rhamnosyl icariside II in propyl acetate

Propyl acetate/buffer (mL/mL)	Conversion rate (%)	Transfer rate (%)
1:2	100	91.36
1:1	100	90.21
3:2	100	93.38
2:1	93.24	90.89

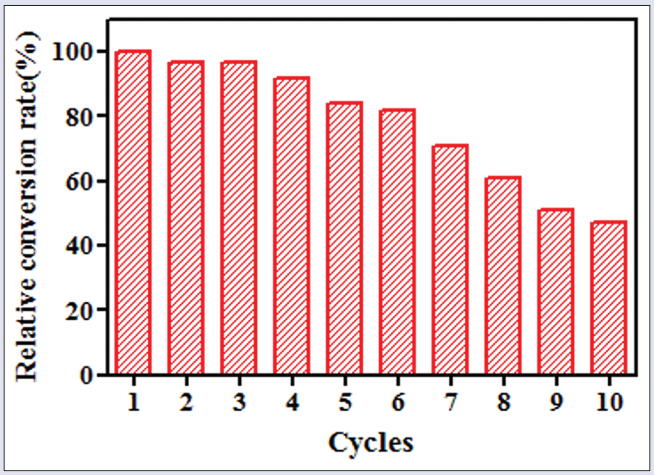

Figure 6: The reusability of β-dextranase in an integrated biphasic system

ESI$^+$-MS: m/z 699 [M + K] +, 661 [M + H] +, 515 [M + H-Rha] +, 369 [M + H-Rha-Rha] +; ^{1}H-NMR (400 MHz, DMSO-d_6) *δ*: 12.58 (1H, s, 5-OH), 7.86 (2H, d, J = 8.9 Hz, 2', 6'-H), 7.12 (2H, d, J = 9.0 Hz, 3', 5'-H), 6.32 (1H, s, 6-H), 5.37 (1H, s, rha-1'-H), 5.17 (1H, t, 2"-H), 4.89 (1H, s, rha-1-H), 3.85 (3H, s, 4'-OCH_3), 1.67 (3H, s, 5"-H), 1.62 (3H, s, 4"-H), 0.81 (3H, d, J = 5.7 Hz, rha-6-H); ^{13}C-NMR (101 MHz, DMSO-d6) *δ*: 178.34 (C-4), 162.31 (C-7), 161.77 (C-5), 159.34 (C-4'), 157.10 (C-2), 154.23 (C-9), 134.83 (C-3), 131.45 (C-3"), 130.86 (C-2', 6'), 122.75 (C-2"), 114.51 (C-3', 5'), 106.44 (C-8), 104.54 (C-10), 102.07 (rha-C-1'), 101.16 (rha-C-1), 98.85 (C-6), 76.00 (rha-C-2), 72.40 (rha-C-4'), 71.81 (rha-C-4), 71.11 (rha-C-3'), 70.95 (rha-C-2'), 70.72 (rha-C-3), 70.60 (rha-C-5'), 69.27 (rha-C-5), 55.93 (4'-OCH_3), 25.86 (C-5"), 21.63 (C-1"), 18.22 (C-4"), 18.06 (rha-C-6'), 17.94 (rha-C-6). These spectral data are consistent with reported studies.[16-18]

To sum up, the results showed that the developed system realized the optimization of β-dextranase-mediated catalytic conversion to pure 2"-O-rhamnosyl Icariside II from epimedin C efficiently and conveniently. The purity of epimedin C in this study is higher than or equal to 98% by HPLC-UV, which means there were few or no impurities induced by substrates. Besides, a propyl acetate and HAc-NaAc buffer biphase enzymatic hydrolysis system was established. In this catalysis system, the glucose on the R2O-position of epimedin C was removed by enzymatic hydrolysis. Because of the polarity of glucose and the properties of enzymes, the glucose and β-dextranase remained in buffer solution. Due to β-dextranase' high substrate specificity, there were no by-products hydrolyzed from epimedin C. The resulting 2"-O-rhamnosyl icariside II in the aqueous solution could be immediately transferred into the top organic phase since the less polar secondary flavonol glycoside has a much higher partition coefficient than its original glycoside. Therefore, the only substance in the top organic phase was 2"-O-rhamnosyl icariside II indicating the 100% purity of the product [Figure 8].

It is worth emphasizing that the novel technology is much lower cost than other conventional method and biotransformation-based preparation. In conventional enzymatic hydrolysis system, organic solvent is added into the enzymatic hydrolysate to extract 2"-O-rhamnosyl icariside II, and therefore, *β*-dextranase was denatured. Compared with traditional enzymatic hydrolysis system, biphase catalysis system can reuse the *β*-glucosidase and organic solvent. The methods based on the chemical hydrolysis often bring some adverse effects on the stability of 2"-O-rhamnosyl icariside II and usually form a number of byproducts such as baohuoside I and anhydroicaritin due to the hardly controlled catalysis in the course of hydrolysis. Thus, the products should be extracted by organic solvent and isolated and purified by column chromatography to obtain 2"-O-rhamnosyl icariside II which costs more than this novel biphase enzymatic hydrolysis system. In addition, for the biotransformation-based preparation, the biotransformation

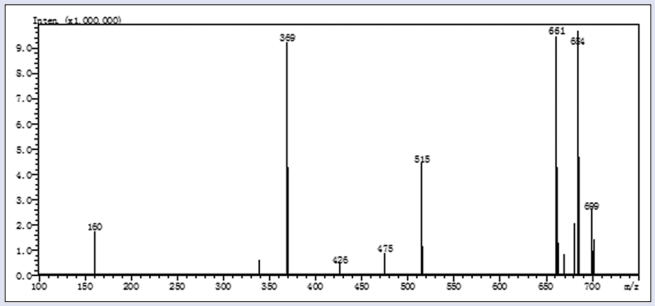

Figure 7: ESI$^+$-MS spectrum of 2"-O-rhamnosyl icariside II

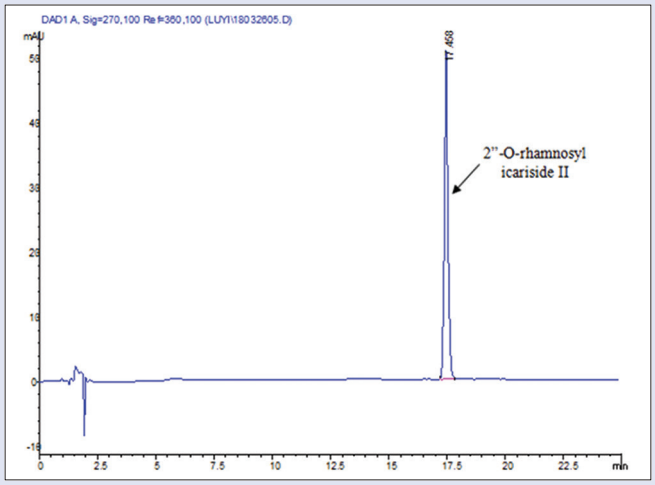

Figure 8: High-performance liquid chromatography-ultraviolet chromatograms of organic solvent after catalysis reaction

costs 5 days. While after 40 min, the epimedin C can be converted into 2"-O-rhamnosyl icariside II completely in biphase hydrolysis system which saved a lot of energy. The metabolites should be extracted by EtOAc and isolated and purified by an ODS column[37] to obtain 2"-O-rhamnosyl icariside II which also costs more than this novel biphase enzymatic hydrolysis system.

CONCLUSION

In this study, a novel recyclable and integrated catalysis system based on the biphase enzymatic hydrolysis was established for the efficient and convenient preparation of a rare secondary flavonol glycoside 2"-O-rhamnosyl icariside II from its original form epimedin C. Compared with conventional enzymatic hydrolysis, this newly constructed system had an apparent advantage in terms of the favorable reusability of enzyme and organic solvent due to the convenience of their recycling. In addition, the conversion of epimedin C and extraction of 2"-O-rhamnosyl icariside II were realized in an integrated step, largely facilitating the holistic reaction and eliminating further tedious chromatography separation. In summary, this novel system was demonstrated to have high efficiency and convenience, suggesting the feasibility and potential to produce other important compounds including secondary glycosides or aglycones from natural products.

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Conflicts of interest

There are no conflicts of interest.

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