Enzyme and Microbial Technology 64–65 (2014) 38–43

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Production of rubusoside from stevioside by using a thermostable lactase from *Thermus thermophilus* and solubility enhancement of liquiritin and teniposide

Thi Thanh Hanh Nguyen[a], Seung-Jin Jung[b], Hee-Kyoung Kang[c], Young-Min Kim[d], Young-Hwan Moon[e], Misook Kim[f], Doman Kim[a,g,*]

[a] Institutes of Green Bio Science & Technology, Seoul National University, Gangwon-do 232-916, Republic of Korea
[b] Korea Atomic Energy Research Institute Advanced Radiation Technology Institute, Jeollabuk-do 500-185, Republic of Korea
[c] Research Center for Proteinaceous Materials (RCPM), Chosun University, Gwang-Ju 501-759, Republic of Korea
[d] Infection Control Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeollabuk-do 580-185, Republic of Korea
[e] Audubon Sugar Institute, Louisiana State University Agricultural Center, Gabriel, LA 70776, USA
[f] Department of Food Science and Nutrition, Dankook University, Gyeonggi-do 448-701, Republic of Korea
[g] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea

ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form 3 July 2014
Accepted 4 July 2014
Available online 11 July 2014

Keywords:
Rubusoside
Thermus thermophilus
Immobilized lactase
Liquiritin
Teniposide

ABSTRACT

Solubility is an important factor for achieving the desired plasma level of drug for pharmacological response. About 40% of drugs are not soluble in water in practice and therefore are slowly absorbed, which results in insufficient and uneven bioavailability and GI toxicity. Rubusoside (Ru) is a sweetener component in herbal tea and was discovered to enhance the solubility of a number of pharmaceutically and medicinally important compounds, including anticancer compounds. In this study, thirty-one hydrolyzing enzymes were screened for the conversion of stevioside (Ste) to Ru. Recombinant lactase from *Thermus thermophiles* which was expressed in *Escherichia coli* converted stevioside to rubusoside as a main product. Immobilized lactase was prepared and used for the production of rubusoside; twelve reaction cycles were repeated with 95.4% of Ste hydrolysis and 49 g L^{-1} of Ru was produced. The optimum rubusoside synthesis yield was 86% at 200 g L^{-1}, 1200 U lactase. The purified 10% rubusoside solution showed increased water solubility of liquiritin from 0.98 mg mL^{-1} to 4.70 ± 0.12 mg mL^{-1} and 0 mg mL^{-1} to 3.42 ± 0.11 mg mL^{-1} in the case of teniposide.

1. Introduction

The major challenge with the design of oral dosage is its poor bioavailability. The oral bioavailability depends on several factors including aqueous solubility, drug permeability, dissolution rate, first pass metabolism, presystemic metabolism, and susceptibility to efflux mechanism. The most frequent causes of low oral bioavailability are poor solubility and low permeability [1]. Seventy percent of new drug candidates have shown poor aqueous solubility in recent years [2]. Poorly water soluble drugs having slow drug absorption, leading to inadequate and variable bioavailability, gastrointestinal mucosal toxicity and delaying the drug's clinical development [1,3]. A common approach to ensure the water solubility of drugs is to use techniques such as micronization, self-emulsification, cyclodextrin complexation, co-crystallization, super critical fluid technology, and solubilization by changing the pH, salt formation, co-solvents, melt granulation, solid dispersion, or liposomal/niosomal formulations [4]. Among the bioactive compounds obtained from plant sources, it has been discovered that some steviol glycosides such as stevioside (Ste), rebaudioside A, and rubusoside possess solubilizing properties. Rubusoside (Ru) showed solubility enhancement for curcumin from 61 μg mL^{-1} to 2.318 mg mL^{-1} in 1–10% Ru (w/v) [5] and for etoposide from 80.85 μg to 8458 μg mL^{-1} in 0.1–8.5% of Ru [6].

Rubusoside (13-O-β-glucosyl-19-O-β-D-glucosyl-steviol) is the main component of the leaves of *Rubus suavissimus* S. Lee (Rosaceae), known as tiancha in Chinese or Chinese sweet tea, which is widely grown in southwestern China. Ru has a slightly bitter aftertaste, but it is 115-fold sweeter than sucrose at a concentration of 0.025% [7]. In addition to its use as a sweetener, Chinese

* Corresponding author at: Institutes of Green Bio Science & Technology, Seoul National University, Gangwon-do 232-916, Republic of Korea. Tel.: +82 33 339 5720; fax: +82 33 339 5716.
E-mail address: kimdm@snu.ac.kr (D. Kim).

http://dx.doi.org/10.1016/j.enzmictec.2014.07.001
0141-0229/

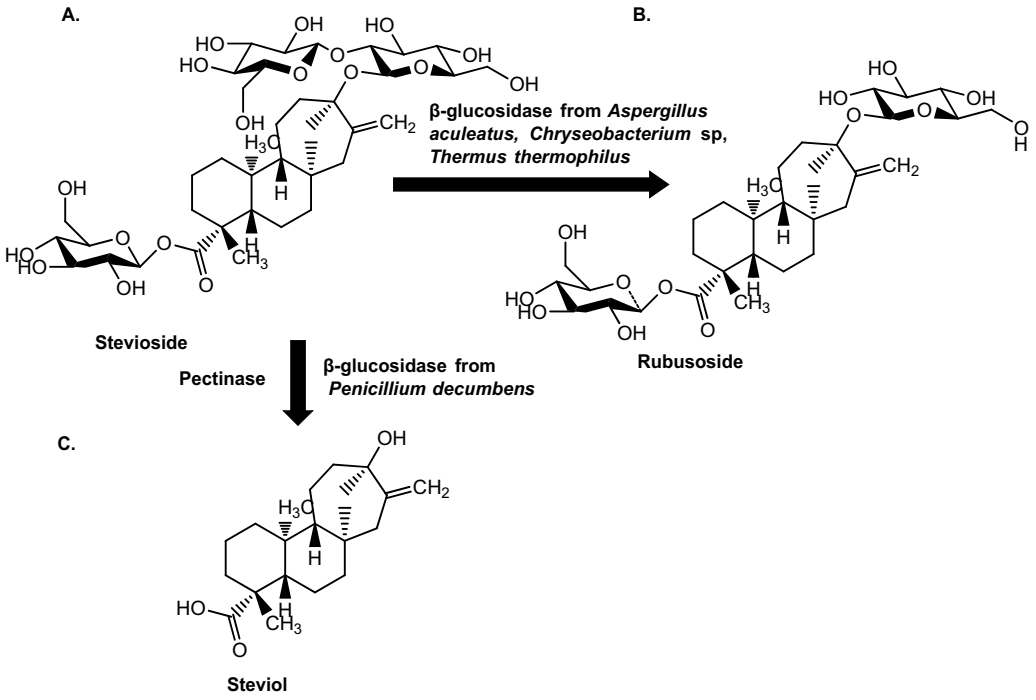

Fig. 1. Chemical structure of stevioside (Ste, A), rubusoside (Ru, B), and steviol (C).

sweet leaf has been used to treat various diseases such as hypertension, diabetes, atherosclerosis, maintaining healthy kidneys, relieve coughs [8]. However, the tea plant grows only in Southern China with variable yearly yields depending on local climate [9] and large scale purification of Ru is complicated [7,10]. Ste is isolated and extracted from the *Stevia rebaudiana* (bertoni) plant commercially cultivated in Japan, Singapore, Taiwan, South Korea, China, Israel, India, Brazil, Australia, and Paraguay [11]. Ste has three β-glycosidic bonds (β-linked sophorose, β-1,2-D-glucopyranosyl on C13 and an ester β-glucosidic linkage on the C19 carboxyl group). Thus, selective cleavage of β-1,2-glucosidic linkage of sophorosyl moiety at C13 of Ste can produce Ru. However, the hydrolysis of sequential glucosyl units of Ste may produce steviol, isosteviol, steviolmonoside, steviolbioside or their mixtures [9]. Previous studies on the hydrolysis of Ste have been reported that β-glucosidases from *Aspergillus aculeatus* and β-galactosidase from *Aspergillus* sp. were able to catalyze the conversion Ste to produce Ru at 63 °C with the productivity of 52% (118.9 g L^{-1} Ru from 225.36 g L^{-1} Ste) [7] and at 60 °C with the product yield of 91.4% from 10 g L^{-1} Ste by using 0.8 kU g^{-1} Ste [9] (Fig. 1A and B), respectively, and β-glucosidase from *Penicillium decumbens* was able to hydrolysis Ste to steviol (Fig. 1C) [12]. Liquiritin is one of the major constituents of *Glycyrrhizae radix* [13], which comprises flavonoids of 2-phenylchromo as a major constituent [14]. Liquiritin demonstrated an antidepressant effect on chronic stress in depressed rats and mice [15,16] and anti-viral activity [17]. Liquiritin showed solubility in water 0.98 mg mL^{-1}. Teniposide is a semisynthetic podophyllotoxin derivative that is a natural product found in the root of the American mandrake (*Podophyllum peltatum*) and showed resistance against a variety of solid tumors, leukemias, lymphoma, and neuroblastoma [18,19]. Due to its poor water solubility, teniposide is currently supplied as a nonaqueous formulation [20].

In this study, in order to find enzymes for more efficient conversion of Ste to Ru, thirty commercial enzymes were tested, having the mixed activities of pectinase, cellulases, hemicellulases, α-galactosidase, β-galactosidase and/or β-glucanase, along with a purified recombinant lactase. We found that crude pectinases from *Aspergillus niger* (Sumizyme SPC, sumilact L, validase AGS), naringinase from *Penicillium* spp. (Cellulase Kn), and recombinant lactase from *Thermus thermophilus* could all convert Ste to Ru as a main product. Among these, the recombinant lactase from *T. thermophilus* showed the highest Ru productivity. Therefore, it was chosen for immobilization on sodium alginate beads, and used to prepare Ru. Ru was then purified, and its ability to solubilize liquiritin and teniposide was examined.

2. Materials and methods

2.1. Enzyme and chemicals

The thirty-one hydrolyzing commercial enzymes with optimum pH, temperature, and sources are provided in Table S1. Stevioside (Fig. 1A) (≥90%, HPLC) was purchased from the Qufu Shengren Pharmaceuticals Company (Shandong, China). Rubusoside standard (Fig. 1B) (≥95%) was kindly supplied by Dr. Young-Min Kim at the Korea Research Institute of Bioscience and Biotechnology (Jeonbuk, Korea). o-Nitrophenyl β-D-galactopyranoside and other chemicals were purchased from Sigma. Recombinant lactase (β-GLYPI) of *T. thermophilus* was prepared by expression in *E. coli* BL21(DE3)pLyS.

2.2. Preparation of recombinant β-GLYPI

pGEM-T easy vector bearing the β-glycosidase gene, β-GLYPI, was synthesized by Bioneer (Daejaen, Korea) based on the amino acid sequence of β-GLY (GenBank Accession No. AY130254.1, Fig. S1) of *T. thermophilus*. The *β-glypi* gene (1.3 kb) was ligated into the *Xho*I/*Eco*RI-digested pRSETB vector. pRSETB-β-GLYPI was transformed and expressed in *E. coli* BL21(DE3)pLysS. *E. coli* BL21(DE3)pLysS cells harboring the β-GLYPI-pRSETB plasmid were grown in LB medium (1 L), supplemented with 50 μg mL^{-1} ampicillin at 37 °C until absorbance at 600 nm reached approximately 0.5. Then, recombinant protein expression was induced by adding 1 mM IPTG at 18 °C for 12 h. The cells were collected by centrifugation (8000 × *g* for 10 min at 4 °C), resuspended in 50 mL of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0), and disrupted by sonication (Ultrasonic processor 250, Sonics and Materials, Inc., Newtown, CT, USA; output 4, duty cycle 50% for 30 s of sonication, 25 repeats on ice). The cell lysate was centrifuged for 15 min at 12,000 × *g* and the supernatant was immersed in a 70 °C water bath for 30 min. Heat-denatured proteins from *E. coli* were removed by centrifugation for 15 min at 12,000 × *g*. The clarified supernatant was applied to a Ni-agarose column (Qiagen, Valencia, CA, USA) that had been equilibrated with 50 mL of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). After loading the sample, the column was washed with 50 mL of washing buffer (20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0), then eluted with 40 mL of elution buffer (250 mM imidazole, 300 mM NaCl,

50 mM NaH_2PO_4, pH 8.0). Among the eluted fractions, the fraction containing the highest protein concentration was collected and applied to a Centriplus column (Amicon, USA) at 3000 × *g* for 120 min for desalting and concentrating the enzyme. The protein concentration was determined by the Bradford method, with crystalline bovine serum albumin (Sigma–Aldrich) as the standard.

The galactosidase activity was determined using o-nitrophenyl β-D-galactopyranoside as a substrate. The increase in the absorbance at 420 nM caused by the release of o-nitrophenol was measured to calculate the galactosidase activity. The reaction mixture containing Tris–HCl (40 mM, pH 7.0), enzyme solution, and o-nitrophenyl β-D-galactopyranoside (20 mM) was reacted at 70 °C for 10 min, and then quenched with Na_2CO_3 (1 M). One unit (U) of galactosidase activity is defined as the amount of enzyme required to release 1 μM o-nitrophenol per min under the above reaction conditions.

2.3. Screening of enzymatic hydrolysis of stevioside to rubusoside

Thirty different commercial enzymes with mixed enzyme activities (Table S1) and recombinant lactase were screened to identify an enzyme capable of converting Ste to Ru. Each enzyme was incubated with 0.5% (w/v) Ste at the optimal pH and temperature (Table S1), and the hydrolysis products were analyzed by TLC using pre-coated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) developed in a solvent system consisting of acetonitrile: water [85:15 (v/v)]. The plates were visualized by dipping into a solvent mixture of 0.5% (w/v) N-(1-Naphthy)ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol and heating at 120 °C for 10 min. The amount of Ste remaining and Ru formed were analyzed using integrated density values (IDV) by employing the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA) with standard Ste and Ru. The conversion ratio of Ste and Ru was calculated as follows:

$$\text{Conversion ratio of Ste} = \frac{[\text{Amount of Ste used} - \text{Ste left}] \times 100}{\text{initial Ste amount in reaction digest}};$$

$$\text{Conversion ratio of Ru} = \frac{[\text{Amount of Ru formed}] \times 100}{\text{initial Ste amount in reaction digest}}$$

2.4. Enzyme immobilization

Recombinant β-galactosidase of *T. thermophilus* (6 kU) was mixed with 20 mL of 3% (w/v) sodium alginate solution to give a final 300 U mL^{-1} of sodium alginate bead. The mixture obtained was extruded drop-wise through a mini-pump variable flow (Fisher Scientific, Waltham, MA, USA) into a 40 mM Tris–HCl buffer solution at pH 7.0 containing 2% (w/v) $CaCl_2 \cdot 2H_2O$ with gentle stirring for 30 min to obtain a bead size of 3 mm. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water, rewashed with 40 mM Tris–HCl buffer (pH 7.0) containing 2% (w/v) $CaCl_2 \cdot 2H_2O$ solution for stabilizing, and washed with distilled water, then kept at 4 °C for further studies.

The reaction mixture containing 20 mL of 1% (w/v) Ste solution in Tris–HCl buffers (40 mM, pH 7.0) and 10 mL alginate beads were incubated at 70 °C in a water bath. After the enzymatic reaction had proceeded for 24 h, the Ru produced was checked by TLC. The beads were reused for 12 reaction cycles. The free enzyme was kept at 70 °C and the thermo stability was compared with the immobilized enzyme.

2.5. Purification of rubusoside

For the purification of Ru, 20 mL (100 g mL^{-1}) of Ste reaction digest was applied to Reveleris® Amino 80 g Flash Cartridges (Grace Discovery Science, Shanghai, China) and Ru was detected by an evaporative light scattering detector (ELSD). A mixture of acetonitrile and water was used as an eluent with a gradient from 95:5 (v/v) to 50:50 (v/v) of acetonitrile: water at a flow rate of 60 mL min^{-1} and room temperature (Fig. S2). The purification was performed after 42 min. The purified Ru fractions were pools and freeze-dried for NMR determination or for further study.

Approximately 12 mg of purified Ru was dissolved and evaporated three times with D_2O, and then placed into 3-mm nuclear magnetic resonance (NMR) tubes. NMR spectrum was obtained using a Unity Inova 500 spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz for ^1H and 125 MHz for ^{13}C at 25 °C. Spectra of homo nuclear correlation spectroscopy (COSY), hetero nuclear single quantum coherence (HSQC), and hetero nuclear multiple bond correlation (HMBC), were analyzed to ascertain the linkages. The NMR data of Ru is shown in Table S2.

2.6. Effect of Ste concentration for enzyme reaction

The reaction mixture containing 1200 U lactase and different concentrations of Ste from 50 to 400 g L^{-1} in Tris–HCl buffers (40 mM, pH 7.0) were incubated at 70 °C in a water bath. After the enzymatic reaction had proceeded for 9 h, the reaction products were checked using TLC. The amount of Ste remaining and the Ru formed were analyzed using the integrated density values (IDV) in the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA) with standard Ste and Ru.

2.7. Preparation of liquiritin- and teniposide–rubusoside solution

Preparation of liquiritin–rubusoside and teniposide–rubusoside solution was prepared with the modification described by Zhang et al. [6]. 20 mg Ru and 2 mg liquiritin or 20 mg Ru and 2 mg teniposide were weighted and mixed at the ratio of 10:1 (w/w). Then, 1 mL of absolute ethanol was added to each mixture in a tightly sealed tube, vortexed, and warmed in a water bath for 5 min to form a clear ethanol solution. The mixture solution was centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to another Eppendorf tube. The ethanol was evaporated. The resulting liquiritin–Ru and teniposide–Ru powder were dissolved with 0.01 mL water and centrifuged at 12,000 rpm for 10 min. The concentration of each compound in the supernatant was analyzed using thin layer chromatography with one ascent of acetonitrile/water 85:15 (v/v). Liquiritin and teniposide on TLC plate were visualized under UV 254 nm, and were also visualized by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-naphthy)ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol and heating at 120 °C for 10 min. The amount of liquiritin and teniposide solubilized in the Ru solution was calculated using the AlphaEaseFC 4.0 program. The standard solution in DMSO was the preparation for liquiritin and teniposide with a concentration ranging from 0.1% to 2.0%.

3. Results and discussion

3.1. Expression of recombinant lactase β-GLYPI (rβ-GLYPI)

The β-glycosidase gene, *β-glypi*, based on the amino acid sequence of β-GLY (GenBank Accession No. AY130254.1, Fig. S1) of *T. thermophilus* was constructed using a custom gene synthesis service and cloned into a pGEMT vector. The *β-glypi* (1.3 kb) was isolated from the vector pGEMT-β-GLYPI by cutting with *Xho*I/*Eco*RI, and was inserted into the pRSETB. The rβ-GLYPI protein was expressed in *E. coli* BL21(De3)pLysS by using 1.0 mM IPTG induction at 18 °C for 12 h and was purified in a single Ni-NTA chromatograph. The molecular mass of the purified enzyme on SDS-PAGE was about 53 kDa, including 4 kDa of His-tag (Fig. S3). The purified rβ-GLYPI has a specific activity of 66.8 U mg^{-1} corresponding to a 12.4-fold purification with 56.7% recovery. The purified enzymes were pooled and lyophilized for further study.

3.2. Screening for Ste hydrolyzing enzyme to produce Ru

The primary screening of enzymes that could convert Ste to Ru was carried out using 0.5% Ste. Most of the commercial enzymes with pectinase activity as the dominant enzyme activity converted Ste to produce steviol, as previously reported [9]. The commercial Sumizyme SPC enzyme from *A. niger* showed 89% conversion of Ste, producing Ru with an efficiency of about 59% of the Ste used (Fig. 2). The commercial Sumilact L and Validase AGS enzymes from *A. niger* showed 60% and 63% conversion of Ste, with 35% and 56% production of Ru, respectively. Naringinase from *Penicillium* sp. (Cellulase Kn) also showed a conversion of 52% for Ste, producing 51% Ru. The highest conversion was obtained using recombinant lactase (rB-GLYPI) from *T. thermophilus* expressed in *E. coli* BL21(DE3)pLyS; 98% conversion of Ste was used and 92% of theoretical synthesis as Ru (Fig. 2). This enzyme was selected for further immobilization.

3.3. Production of Ru using immobilized rB-GLYPI lactase

Twelve sequential reaction cycles using immobilized enzymes in alginate beads were carried out with 10 g L^{-1} Ste. Since immobilized enzyme were used in twelve cycles, the yield of free and immobilized enzymes were calculated as the average yield of twelve cycles. As shown in Table 1, the amount of Ste remaining, the Ste conversion percentage, and the amount of Ru synthesis were 9.62 g L^{-1}, 96.2%, and 3.97 g L^{-1}, respectively, for free enzymes, and 9.54 g L^{-1}, 95.4%, and 4.91 g L^{-1}, respectively, for immobilized lactase.

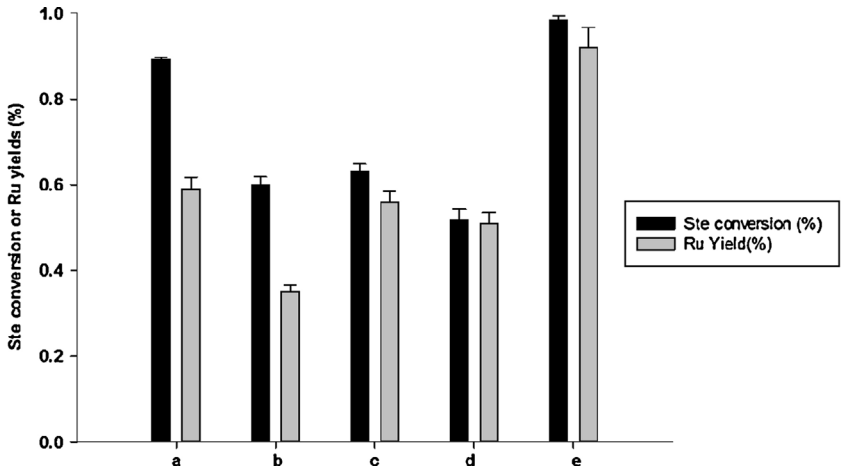

Fig. 2. Conversion of Stevioside (Ste) to Rubusoside (Ru) using various enzymes. Lane a: Sumizyme SPC; lane b: Sumilact L; lane c: Validase AGS; lane d: Cellulase Kn; lane d: purified recombinant lactase.

3.4. Effect of Ste and lactase concentration to reaction

The effect of high Ste concentration on Ru production was investigated. The lactase activity was fixed at 1200 U, while Ste concentration was varied from 50 to 400 g L^{-1} at pH 7.0 at 70 °C (Table 2). All Ste was hydrolyzed at the initial Ste concentration of 100 g L^{-1} in the reaction digest, and the amount of Ru synthesized per 1000 U lactase per h was increased from 2.58 g L^{-1} to 14.44 g L^{-1}

Table 1
Comparison of conversion % from Ste to Ru using free or immobilized lactase.

	Free lactase (FR)	Immobilized lactase (IM)[1]	Ratio (IM/FR)
Amount of Ste hydrolysis (g L^{-1})[a]	9.62	9.54	0.99
Ste conversion (%)	96.2	95.4	
Ru synthesized (g L^{-1})[a]	4.00	4.91	1.24

[−1] Reaction condition was of 1% (w/v) Ste solution in Tris–HCl buffers (40 mM, pH 7.0) and 10 mL alginate beads were incubated at 70 °C in a water bath.
[a] Average data for twelve reaction cycles.

Table 2
Amount of Ste left and production of Ru based on Ste concentration (%w/w).

Initial Ste amount in reaction digest (g L^{-1})	Amount of Ste hydrolysis/Ru synthesis after 9 hr reaction (g L^{-1})	Conversion (%) Ste/A[a] Ru/A[a]	Yield of Ru synthesis[b] (%)	Ru synthesized/1000 U lactase/h (g L^{-1})
50				
Ste	49.2	98		
Ru	27.9	56	71	2.6
80				
Ste	79.9	100		
Ru	35.6	44	56	3.3
100				
Ste	99.9	100		
Ru	47.3	50	63	4.4
120				
Ste	112.8	94		
Ru	58.0	48	64	5.4
150				
Ste	133.8	89		
Ru	71.9	48	67	6.7
200				
Ste	169.9	85		
Ru	116.4	58	86	10.8
250				
Ste	211.6	85		
Ru	133.3	53	79	12.3
300				
Ste	243.5	81		
Ru	152.4	51	78	14.1
400				
Ste	260.5	65		
Ru	155.9	39	75	14.4

Ste: used Ste amount after 9 h reaction; Ru: synthesized amount of Ru after 9 h reaction.
[a] A: initial Ste amount in reaction digest (g L^{-1}).
[b] Yield of Ru synthesis: [(amount of Ru synthesized)/(theoretical Ru which can be synthesized from used amount of Ste)].

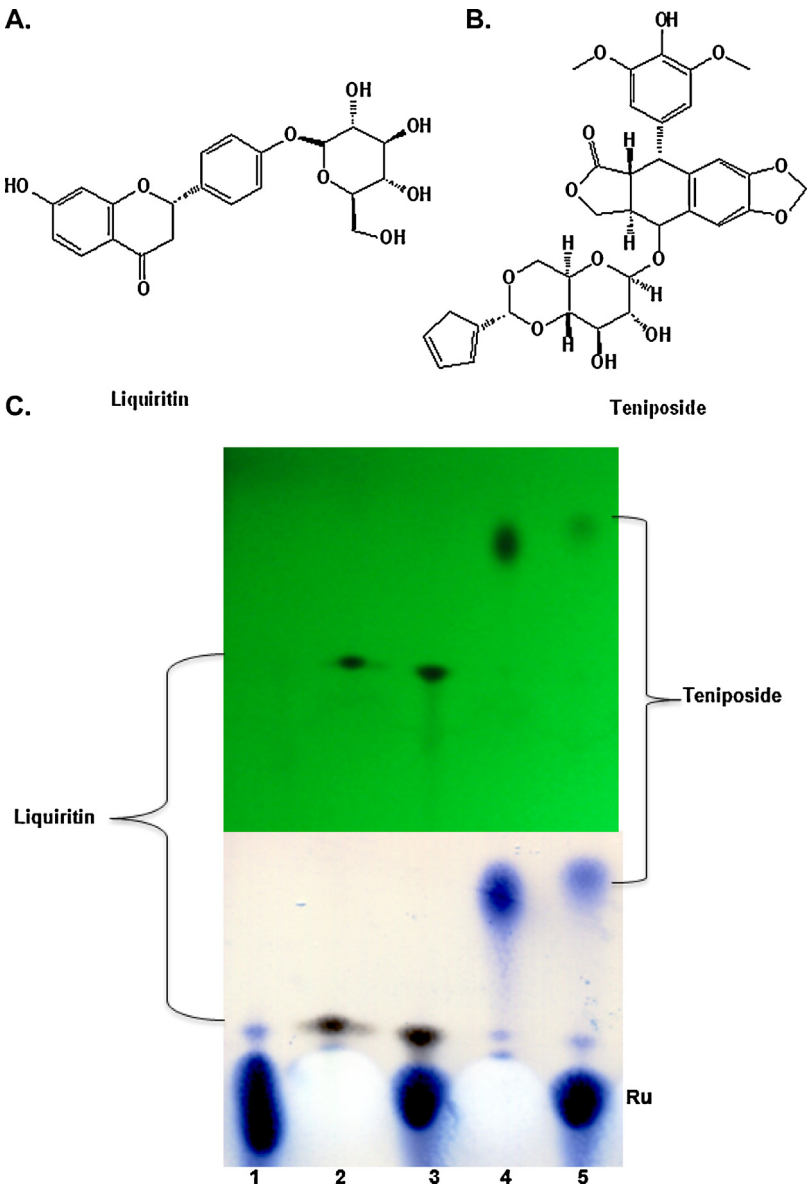

Fig. 3. Chemical structure of liquiritin (A) and teniposide (B) and solubility of liquiritin and teniposide in water solutions reconstituted from liquiritin–Ru (Li–Ru), and teniposide–Ru (Te–Ru) powder (C). Lane 1: Ru; lane 2: liquiritin in DMSO; lane 3: liquiritin in Ru; lane 4: teniposide in DMSO; lane 5: teniposide in Ru.

when the initial Ste amount was increased from 50 to 400 g L^{-1} Ste, and the maximum Ru synthesis percentage was reached at 86% with 200 g L^{-1} initial Ste concentration in the reaction digest. At 100 g L^{-1}, 100% of Ste was hydrolyzed and the maximum of Ru conversion percentage reached 200 g L^{-1} Ste.

3.5. Solubility enhancement of liquiritin and teniposide using Ru

Liquiritin ($C_{21}H_{22}O_9$, M.W 418.39) {7-hydroxy-4′-glucosyloxyflavone} is the main constituent of licorice, which is one of the most popular medicinal herbs used in traditional Chinese medicine (Fig. 3). The liquiritin was mixed with Ru at a ratio of 1:10 (w/w) and the solubility of liquiritin was 4.70 ± 0.12 mg mL^{-1} (Fig. 3). Teniposide ($C_{32}H_{32}O_{13}S$, M.W. 656.66) {4′-demethylepipodophyllotoxin 9-[4,6-O-(R)-2-thenylidene-β-D-glucopyranoside]} is a cancer drug with the trade name of VUMON® and is a semisynthetic derivative of podophyllotoxin (Fig. 3). The teniposide was mixed with Ru at a ratio of 10:1 (w/w) (10% Ru with 1% of teniposide in the final) and was calculated at 3.42 ± 0.11 mg mL^{-1} (Fig. 2).

4. Discussions

Among the thirty commercial enzymes with mixed enzyme activities, four enzymes demonstrated the conversion of Ste to Ru. Three of them were from *A. niger*, which has widely been used in industry for the production of organic acids and enzymes, and is an eminent source for the production of these glycosidases [21,22]. Different molecular forms of β-galactosidase activity in *A. niger* have been reported [23,24]. Naringinase is an enzyme complex used in deglycosylation, which has a high potential in the food and pharmaceutical industries. Naringinase provides both α-L-rhamnosidase and β-D-glucosidase activities [25]. The mixed enzyme activities, present in Sumizyme SPC, Sumilact L, validase AGS, and cellulase Kn, could facilitate conversion of Ste to Ru.

The enzyme β-galactosidase (EC.3.2.1.23), most commonly known as lactase which hydrolyses lactose into its monomers that is glucose and galactose, is one of the most important enzymes used in food processing. In this study, the recombinant lactase from *T. thermophilus* was found for the first time to convert Ste to Ru. For large scale production of Ru, the enzyme can be added

directly to Ste reaction, but after complete reactions the enzyme can be deactivated by heat treatment and enzyme cannot be reused, thus the resulting operation is not cost effective. To overcome this problem, immobilized lactase is used for the production Ru from Ste. Lactase immobilization provides considerable advantages with the possibility of continuous processing, reuse of the enzyme and reduction of auto-digestion. The immobilization procedure on alginate beads is not only inexpensive but also very easily carried out under mild conditions, giving it considerable potential for industrial applications [26,27]. The operational stability of immobilized enzymes is one of the most important factors affecting the utilization of an immobilized enzyme system. The free enzymes could hydrolyze Ste slightly stronger than immobilized lactase, but the Ru yield of immobilized lactase was still higher 1.2 times than that of free enzymes. Thus, the results indicated that immobilized enzyme increased thermal stability with higher efficiency of hydrolysis compared to that using free enzyme. On the other side, lactase from *T. thermophilus* was retain its activity at 70 °C instead of 60 °C of β-glucosidase from *A. aculeatus* [7] or at 63 °C of β-galactosidase from *Aspergillus* sp. [9] for prolonged periods, and the process is less prone to microbial contamination due to higher operating temperature. And by comparison the amount of Ru synthesis/1000 U/h, lactase from *T. thermophilus* was showed 23.2 and 56.8 times higher than the reports of 0.19 g L^{-1} Ru synthesis/1000 U/h of β-galactosidase from *Aspergillus* sp. [9].

There were previous reports that Ru can be enhance the solubility of chemical compounds [5,6] so the Ru product from Ste and immobilized lactase enzyme was used for solubility of liquiritin and teniposide. Liquiritin, the main constituent of licorice, which has solubility in 0.98 mg mL^{-1} of water was increase of 4.8 times solubility in the presence of 10% (w/v) of Ru. Teniposide differs from etoposide. It is a lipophilic compound and insoluble in water or ether. Due to its poor water solubility, teniposide is currently supplied as a nonaqueous formulation and thus precipitates from the intravenous solution when diluted with other fluids for infusions. However, this formulation causes severe side effects such as hypersensitivity, hypertension, and cardiovascular collapse [20]. Many techniques have been applied to improve the formulation of teniposide such as mixed micelles as proliposomes [20], self-assembled nano-carriers for further oral chemotherapy [28] or formulated with PVP-b-PDLLA [poly(N-vinulpyrrolidone)-block-poly(D,L-lactide)] [3]. The teniposide was showed solubility at 3.42 ± 0.11 mg mL^{-1} in the presence of 10% (w/v) of Ru.

Acknowledgements

This work was partially supported by the National Research Foundation of Korea (NRF) grant, funded by the Korea government (MEST) (No. NRF-2012R1A2A2A01045995).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2014.07.001.

References

[1] Savjani KT, Gajjar AK, Savjani JK. Drug solubility: importance and enhancement techniques. ISRN Pharm 2012;2012:195727.
[2] Kawabata Y, Wada K, Nakatani M, Yamada S, Onoue S. Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. Int J Pharmaceut 2011;420:1–10.
[3] Le Garrec D, Gori S, Luo L, Lessard D, Smith DC, Yessine MA, et al. Poly(N-vinylpyrrolidone)-block-poly(D,L-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: *in vitro* and *in vivo* evaluation. J Control Release 2004;99:83–101.
[4] Kumar S, Bhargava D, Thakkar A, Arora S. Drug carrier systems for solubility enhancement of BCS class II drugs: a critical review. Crit Rev Ther Drug Carrier Syst 2013;30:217–56.
[5] Zhang F, Koh GY, Jeansonne DP, Hollingsworth J, Russo PS, Vicente G, et al. A novel solubility-enhanced curcumin formulation showing stability and maintenance of anticancer activity. J Pharm Sci 2011;100:2778–89.
[6] Zhang F, Koh GY, Hollingsworth J, Russo PS, Stout RW, Liu Z. Reformulation of etoposide with solubility-enhancing rubusoside. Int J Pharm 2012;434:453–9.
[7] Ko JA, Kim YM, Ryu YB, Jeong HJ, Park TS, Park SJ, et al. Mass production of rubusoside using a novel stevioside-specific beta-glucosidase from *Aspergillus aculeatus*. J Agric Food Chem 2012;60:6210–6.
[8] Koh GY, Chou G, Liu Z. Purification of a water extract of Chinese sweet tea plant (*Rubus suavissimus* S. Lee) by alcohol precipitation. J Agric Food Chem 2009;57:5000–6.
[9] Wan HD, Tao GJ, Kim D, Xia YM. Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with beta-galactosidase from *Aspergillus* sp. J Mol Catal B – Enzym 2012;82:12–7.
[10] Sugimoto N, Sato K, Liu HM, Kikuchi H, Yamazaki T, Maitani T. Analysis of rubusoside and related compounds in tenryocha extract sweetener. S Shokuhin eiseigaku zasshi 2002;43:250–3.
[11] Upreti M, Strassburger K, Chen YL, Wu SX, Prakash I. Solubility enhancement of steviol glycosides and characterization of their inclusion complexes with gamma-cyclodextrin. Int J Mol Sci 2011;12:7529–53.
[12] Ko JA, Ryu YB, Kwon HJ, Jeong HJ, Park SJ, Kim CY, et al. Characterization of a novel steviol-producing beta-glucosidase from *Penicillium decumbens* and optimal production of the steviol. Appl Microbiol Biotechnol 2013;97:8151–61.
[13] Wang ZY, Nixon DW. Licorice and cancer. Nutr Cancer 2001;39:1–11.
[14] Okamura N, Miki H, Orii H, Masaoka Y, Yamashita S, Kobayashi H, et al. Simultaneous high-performance liquid chromatographic determination of puerarin, daidzin, paeoniflorin, liquiritin, cinnamic acid, cinnamaldehyde and glycyrrhizin in Kampo medicines. J Pharm Biomed Anal 1999;19:603–12.
[15] Zhao Z, Wang W, Guo H, Zhou D. Antidepressant-like effect of liquiritin from *Glycyrrhiza uralensis* in chronic variable stress induced depression model rats. Behav Brain Res 2008;194:108–13.
[16] Wang W, Hu X, Zhao Z, Liu P, Hu Y, Zhou J, et al. Antidepressant-like effects of liquiritin and isoliquiritin from *Glycyrrhiza uralensis* in the forced swimming test and tail suspension test in mice. Prog Neuro-Psychopharmacol Biol Psychiatry 2008;32:1179–84.
[17] Hatano T, Yasuhara T, Miyamoto K, Okuda T. Anti-human immunodeficiency virus phenolics from licorice. Chem Pharm Bull (Tokyo) 1988;36:2286–8.
[18] Boas J, Rasmussen D, Hansen OP, Engelholm SA, Dombernowsky P. Phase II study of teniposide in advanced breast cancer. Cancer Chemother Pharmacol 1990;25:463–4.
[19] Bork E, Hansen M, Dombernowsky P, Hansen SW, Pedersen AG, Hansen HH. Teniposide (VM-26), an overlooked highly active agent in small-cell lung cancer. Results of a phase II trial in untreated patients. J Clin Oncol 1986;4:524–7.
[20] Alkan-Onyuksel H, Son K. Mixed micelles as proliposomes for the solubilization of teniposide. Pharm Res 1992;9:1556–62.
[21] Bahl OP, Agrawal KML. Glycosidases of *Aspergillus niger*. Purification and characterization of α- and β-galactosidases and β-N-acetylglucosaminidase. J Biol Chem 1969;244:2970–8.
[22] Adya S, Elbein AD. Glycoprotein enzymes secreted by *Aspergillus niger*: Purification and properties of α-galactosidase. J Bacteriol 1977;129:850–6.
[23] Widmer F, Leuba JL. β-Galactosidase from *Aspergillus niger*. Separation and characterization of three multiple forms. Eur J Biol Chem 1979;100:559–67.
[24] Greenberg NA, Mahoney RR. Rapid purification of β-galactosidase (*Aspergillus niger*) from a commercial preparation. J Food Sci 1981;46:684–7.
[25] Real HJV, Alfaia AJ, Calado ART, Ribeiro MHL. High pressure-temperature effects on enzymatic activity: Naringin bioconversion. Food Chem 2007;102:565–70.
[26] Arya SK, Srivastava SK. Kinetics of immobilized cyclodextrin gluconotransferase produced by *Bacillus macerans* ATCC 8244. Enzyme Microb Tech 2006;39:507–10.
[27] Goel A, Sharma RK, Tandon HKL. A comparison of different polymeric gels for entrapment of cells of *Streptococcus thermophilus* containing beta-galactosidase. J Food Sci Tech Mys 2006;43:526–31.
[28] Zhang Z, Ma L, Jiang S, Liu Z, Huang J, Chen L, et al. A self-assembled nanocarrier loading teniposide improves the oral delivery and drug concentration in tumor. J Control Release 2013;166:30–7.