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Compound K producing from the enzymatic conversion of gypenoside by naringinase

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ABSTRACT

Compound K is a type of protopanaxadiol-type ginsenosides (PPDs) that has strong bioactivities due to fewer glycosyls. However, compound K is not present in raw and unprocessed ginseng. Some PPDs have the same structure with gypenosides, and could be obtained from *Gynostemma pentaphyllum*. The enzymolysis of PPD-type gypenosides of *G. pentaphyllum* by naringinase has been reported for the first time in this research. In addition, isolation and identification of enzymolysis end product, and the optimization of enzymolysis parameters were investigated. The results showed that compound K was produced from the enzymolysis of PPD-type gypenosides by naringinase, and could be isolated and purificated by HP-20 macroporous resin and C-18 column chromatography. The optimum enzymolysis conditions determined by the response surface methodology (RSM) are pH 4.1, 50 °C, and 71 h, with a yield of 65.44 ± 4.52% for compound K. These results demonstrated that enzymolysis could be a promising method for producing compound K from the biotransformation of PPD-type gypenosides of *G. pentaphyllum*.

1. Introduction

Ginsenosides are the main active component of *Panax* species, which are divided into protopanaxadiol-type (PPD)-, propanaxatriol-, octolillol-, and oleanic acid-type ginsenosides according to the different structure of aglycones (Mi et al., 2012; Shukla et al., 1992; Sun et al., 2010, 2011). Among them, PPDs, as the main group of ginsenosides, have been considered the main active ingredient in ginseng. PPDs share a dammarane-type triterpene structure, the *β*-OH at C-3 and/or C-20 of attached glycosyl residues (Shi et al., 2011). Differences in type, quantity and attachment position of glycosyls provide diversity in structure and function of ginsenosides (Qi et al., 2011). Furthermore, it has been found that ginsenosides with less glycosyls show stronger activities and better bioavailability (Chen et al., 2014).

Compound K (Fig. S1), *i.e.*, 20-O-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, is one of PPDs with strong bioactivities due to fewer glycosyls, can be produced from enzymolysis by removing the glycosyls from other PPDs, after oral administration of ginseng (Gao et al., 2011; Zhong et al., 2016). Compound K has been considered as the ingredient that really exerts the pharmacological activities *in vivo* (Akao et al., 2011). Compared with transformed precursors, compound K presents the characteristics of easier absorption and a variety of pharmaceutical functions including anti-cancer, antitumor, anti-inflammation and neuroprotection. However, compound K is not present in raw and unprocessed ginseng (Chen et al., 2013; Li et al., 2014; Oh and Kim, 2016; Park et al., 2011; Yao et al., 2018). Previous studies have shown that *Panax quinquefolius*, *Panax ginseng,* and *Panax notoginseng* are the three most commonly used ginseng herbs of *Araliaceae* family for extracting

Abbreviations: ANOVA, Analysis of variance; *G. pentaphyllum*, *Gynostemma pentaphyllum*; gyp TN-1, Gynosaponin TN-1; gyp XLVI, Gypenoside XLVI; HPLC, High performance liquid chromatography; MPLC, Medium pressure liquid chromatography; NMR, Nuclear magnetic resonance; PPD, Protopanaxadiol; PPDs, Protopanaxadiol-type ginsenosides; RSM, Response surface methodology; C-18, Reverse phase; SD, Standard deviation; TLC, Thin layer chromatography

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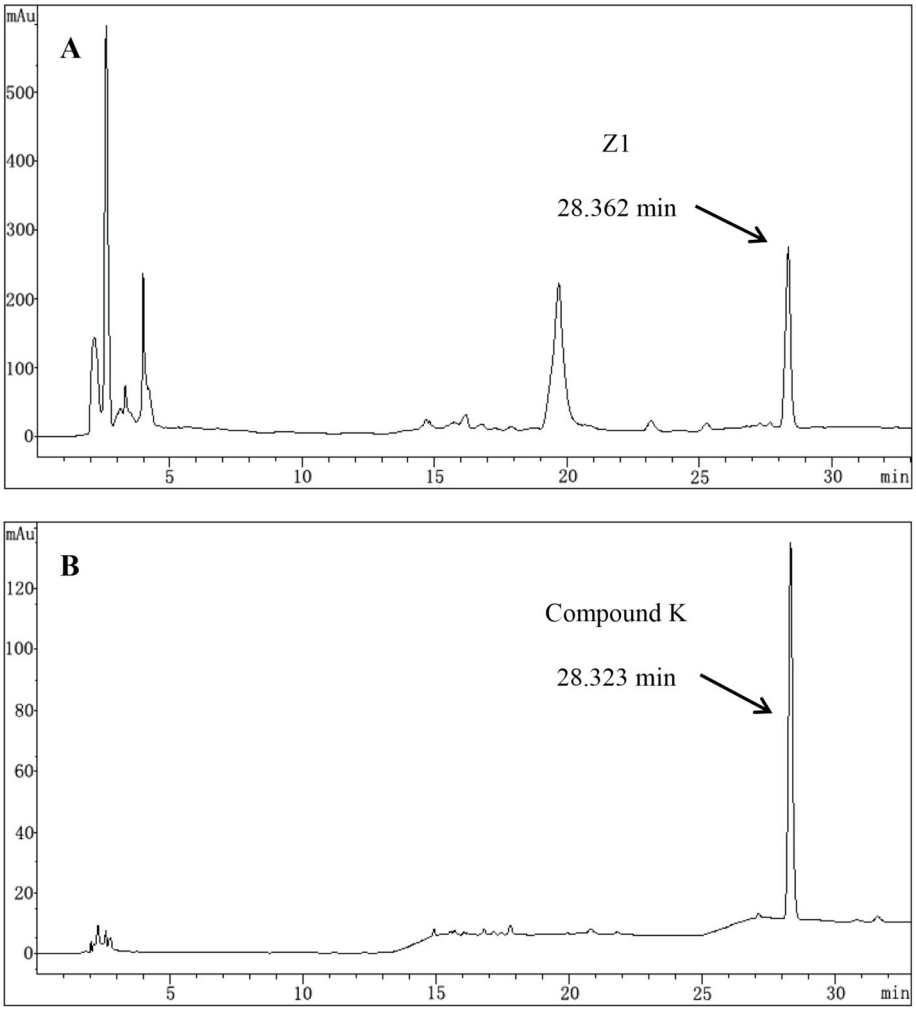

Fig. 1. The results of HPLC analysis for component in enzymolysis products (A) and comparison with compound K standard (B).

PPDs to prepare compound K (Chi et al., 2005; Jiang et al., 2004; Zhong et al., 2016). Nevertheless, this approach of preparation of compound K is uneconomical and inapplicable for the industry. Thus, there remains a need for alternative approaches to prepare compound K, such as more efficient enzymolysis method.

Gynostemma pentaphyllum (*G. pentaphyllum*) has been known as the only plant that contains ginsenosides apart from *Araliaceae* family, and it is widely distributed in China (He et al., 2013; Liou et al., 2010; Zheng et al., 2018a,b). Gypenosides are the main active ingredients in *G. pentaphyllum* (Alhasani et al., 2018; Zheng et al., 2018a,b). Particularly, the structures of gypenoside-III, IV, VIII, and XII are the same as ginsenoside-Rb_1, Rb_3, Rd, and F_2, respectively, the content of which in the elite strain of *G. pentaphyllum* are higher than that in ginseng (Shen et al., 2008). Additionally, the transformation pathway among PPDs has been verified as: ginsenoside-Rb_3→ginsenoside-Rd→ginsenoside-F_2→compound K→20(*S*)-PPD (Liu et al., 2015). Theoretically, the gypenoside-III, IV, VIII, or XII can be enriched from elite *G. pentaphyllum* strains, then efficiently transformed into compound K by modificating the structure and controlling the transformation pathway.

Previously, we found that *G. pentaphyllum* from Jinggangshan in Jiangxi Province of China was rich in ginsenoside-Rb_3 (gypenoside-IV). Compared to compound K, ginsenoside-Rb_3 is attached with one rhamnose at C-20, and two glucoses at C-3. Naringinase, a mixture of rhamnosidase and glucosidase, has been widely used in the food industry to remove bitter substances, which has a potential effect on hydrolysis of the rhamanopyranosyls and glucosyls. Gypenoside XLVI (gyp XLVI) is one of the chief dammarane-type triterpenoid saponins from *G. pentaphallum* with glucosyls at C-3 and C-20 positions (Zheng et al., 2018a,b). Our previous study showed that gynosaponin TN-1 (gyp TN-1) could be produced from the bioconversion of gyp XLVI by naringinase, with the optimum conditions at pH 4.2, 47.3 °C and 60 h. Additionally, compared to gyp XLVI, gyp TN-1 displayed higher inhibitory activities on human hepatoma cells SMMC7721 and Bel7402 (Zheng et al., 2018a,b). However, little literature is available on the compound K producing from the enzymatic conversion of gypenoside by naringinase.

In this study, raw materials of *G. pentaphyllum* from Jinggangshan in Jiangxi Province of China were used to extract the PPD-type gypenosides, and transformed into compound K by naringinase. The structure of the extract was identified by nuclear magnetic resonance (NMR). The enzymolysis conditions were optimized, including pH, temperature, and time. The aim of the present study was to develop an effective method to convert the gypenosides into compound K, thereby obtaining high bioactive ginsenosides at lower price.

2. Materials and methods

2.1. Materials

G. pentaphyllum, artificially cultivated for about one year old, was obtained from Jinggangshan in Jiangxi Province of China and stored in Fujian Institute of Subtropical Botany, Xiamen, China. After drying, the

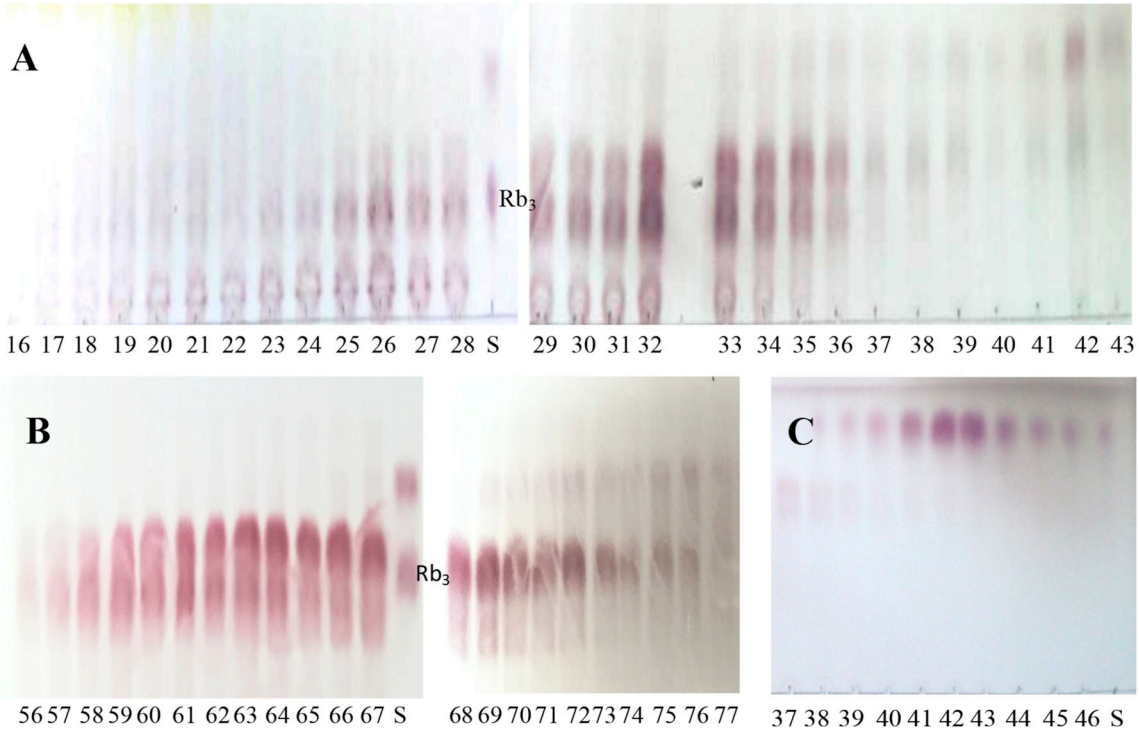

Fig. 2. The results of TLC analysis for HP-20 macroporous resin separated-gypenoside eluent (S: Rb_3 standard) (A), C-18 column separated-gypenoside eluent (S: Rb_3 standard) (B), and the eluent of enzymolysis products from protopanaxadiol-type gypenosides via naringinase (S: compound K standard) (C).

samples were ground by a cutting mill before passing through 24-mesh sieve to obtain powders. It was then stored at room temperature for further use.

Ginsenoside-Rb_3 and compound K standards were obtained from Solarbio Co. Ltd. (Beijing, China). Naringinase (390 U/g), acetonitrile and methanol (chromatographic grade) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals and reagents were in analytical grade. Ultrapure water (Millipore, Bedford, MA, USA) was used for all preparations.

HP-20 macroporous resin was bought from DIAION (Japan) and C-18 reverse phase column was purchase from Merck KGaA (Darmstadt, Germany).

2.2. Preparation of total gypenosides

G. pentaphyllum powders (500 g) were extracted with 75% ethanol (1: 8, *w/v*) for 30 min thrice, and sonicated by a KQ5200 DE ultrasonic device (Kunshan Ultrosonic Instrument Co., Ltd, China) at 45 °C. The ultrasound power density and frequency were 100 W and 40 kHz. The extract was filtered and evaporated at 40 °C to dryness for obtaining the crud gypenosides. The crud gypenosides were dissolved in water, followed by adding petroleum ether (1:3, *w/v*) and extracting 3 times. The subnatants were collected, added with *n*-butanol (1:3, *w/v*), and extracted 3 times again and the supernatants were collected. All the *n*-butanol supernatants were pooled and evaporated to dryness under vacuum at 40 °C, after which the residue was dissolved in 10 mL of distilled water and freeze-dried to obtain total gypenosides powders.

2.3. Preparation of PPD-type gypenosides

2.3.1. Isolation of PPD-type gypenosides by HP-20 macroporous resin

Initially, 250 g of HP-20 macroporous resin stuffings were poured into a glass column, which was previously activated with 1 L of ethyl alcohol, followed by adding water to equilibrize and form a layer of around 1 cm above the adsorbent. Gypenosides powders (10.28 g) were dispersed in 100 mL water and poured slowly into the column, then gradient eluting was conducted with water (1 L), 30% (*v/v*, 2 L), 50% (*v/v*, 2 L), 70% (*v/v*, 2 L), 90% (*v/v*, 2 L) and 100% (*v/v*, 2 L) of ethanol-water with flow rate at 10 mL/min and gathering 200 mL/tube. All eluents were analyzed by the thin-layer chromatography (TLC) method, the ginsenoside-Rb_3 standard was used as a reference. The eluents of the component nearing ginsenoside-Rb_3 were collected and evaporated to dryness under vacuum at 40 °C for obtaining the PPD-type gypenosides residues.

2.3.2. Purification of PPD-type gypenosides by C-18 column

The PPD-type gypenosides residues were further purificated by BUCHI medium pressure liquid chromatography (MPLC) system (BUCHI, Uppsala, Sweden) using a C-18 column. An amount (3.85 g) of the residues obtained previously were dissolved in 5 mL 20% (*v/v*) methanol solution, then gradient eluting was conducted with water (1 L), 50% (*v/v*, 2 L), 55% (*v/v*, 2 L), 60% (*v/v*, 2 L), 65% (*v/v*, 2 L), and 100% (*v/v*, 2 L) of methanol-water with flow rate at 20 mL/min and 15 mL/tube. All eluents were analyzed, collected, and evaporated as above, and the purer PPD-type gypenosides were obtained.

2.4. TLC method

TLC method was used to analyze the component and content of saponins in samples. The TLC chromatogram was developed in a glass chromatographic chamber (20 × 10 × 18 cm); the chromatographic chamber was covered with a glass lid, and 3 mL of chloroform-methanol-formic acid (7:2:2, *v/v/v*) was used as developing solvents to saturate for 30–40 min at room temperature (25 ± 5 °C) before use. R_f value ranging from 0.2 to 0.8 was considered as the potential index for identifying the best separation compound for developing solvents. R_f value was calculated with the following formula:

$$R_f = D_s / D_c$$

where R_f is the retardation factor, D_s is the sample migration distance (from the start line to the center of the chromatogram), and D_c is the carrier solution migration distance on TLC.

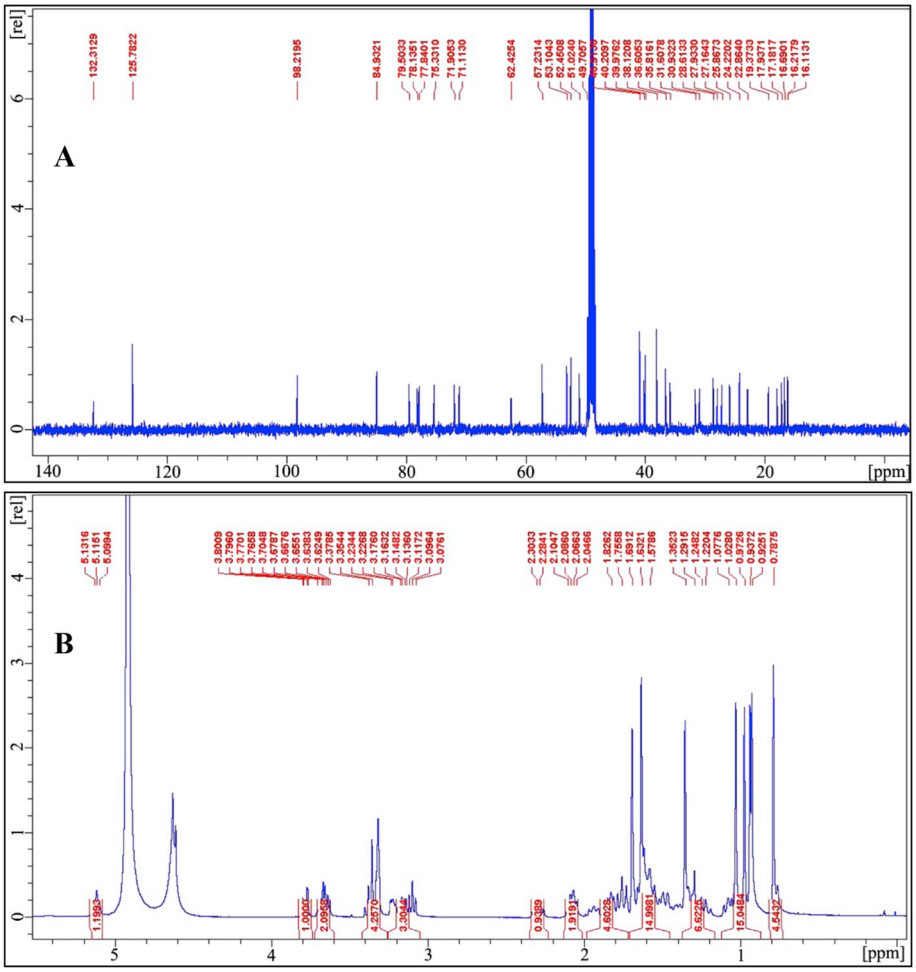

Fig. 3. The results of ^{13}C-NMR (A) and ^{1}H-NMR (B) analysis for hydrolyzate Z_1.

According to the formula, the best separation solvent was *n*-butanol-ethyl acetate -water (4:1:5, *v/v/v*). Ginsenoside-Rb_3 standard solutions (2 mg/mL, 2 μL), or sample solutions (10 μL) were applied on silica gel 60 F254 TLC plates (10 × 20 cm, Merck, Germany) as 5 mm bands, 10 mm from the lower edge, 7 mm from the left and right edge, and 6 mm apart, with glass capillaries (0.3 × 100 mm). Plates were air-dried for 5 min, then transfered to the chromatographic chamber, and expanded until the rising solvent-expansion front moved about three quarters of the plate length from the origin. After solvent expansion, plates were removed, dried in air for 5 min, and dipped in color-expansion agent (sulfuric acid: ethanol, 5:95, *v/v*) for 2 s, then dried in a stream of warm air, heated at 110 °C for 2 min, and then visualized.

2.5. Preparation and identification of enzymolysis products

2.5.1. Enzymolysis of PPD-type gypenosides by naringinase

The PPD-type gypenosides (1.50 g) and naringinase (1.50 g) were weighed and dissolved in acetate buffer (pH 4.1, 100 mL) and reaction at 50 °C for 60 h. The enzymolysis process was terminated by extracting the samples with 50 mL of *n*-butanol for three times, and all the extracts were then dried by evaporation at 40 °C. The enzymolysis products were collected for further isolation.

2.5.2. Identification of enzymolysis products by high performance liquid chromatography (HPLC)

HPLC method was used to analyze the component of emzymolysis products for confirming the existence of compound K.

The chromatograph was acquired by an Agilent 1200 Infinity series (Agilent Technologies Co., Ltd., USA) equipped with a binary pump solvent management system and an online degasser. The sample was separated on an Zorbax Eclipse XDB-C18(150 × 4.6 mm i.d., particle size 5 μm)at ambient room temperature (25 °C), and eluted with a gradient of solvent A (acetonitrile) and solvent B (water). The solvent composition varied as follows: 0–10 min, 35% A; 10–12 min, 35%–50% A; 12–22 min, 50% A; 22–24 min, 50%–65% A; 24–34 min, 65% A; 34–50 min, 65%–80% A; 50–51 min, 80%–100% A; 51–52 min, 100%–35% A. After that, the column was reconditioned. All solvents were filtered through a 0.22 μm filter prior to use. The flow rate was kept constant at 1 mL/min, and the sample injection volume was 20 μL. The determine wavelength was 203 nm. Compound K standard solution was used for comparation.

2.5.3. Isolation and analysis of enzymolysis products

The enzymolysis products (65.19 mg) were weighed and dissolved in methanol (5 mL), applied on C-18 column and eluted through step gradients with 150 mL of 70%, 73%, 76%, 79%, 82%, 85%, 90%, 95%, and 100% (*v/v*) of methanol-water with flow rate at 20 mL/min and gathered 10 mL/tube. The eluent was detected using the TLC method. The eluents containing target ingredients were combined and evaporated for obtaining pure enzymolysis products.

2.5.4. Identification of enzymolysis products by NMR

The enzymolysis products were dissolved in MeOH-*d*4. NMR experiments were performed on Bruker DRX400 spectrometer (Bruker Biospin Co., Karlsruhe, Germany) for ^{1}H-NMR and ^{13}C-NMR.

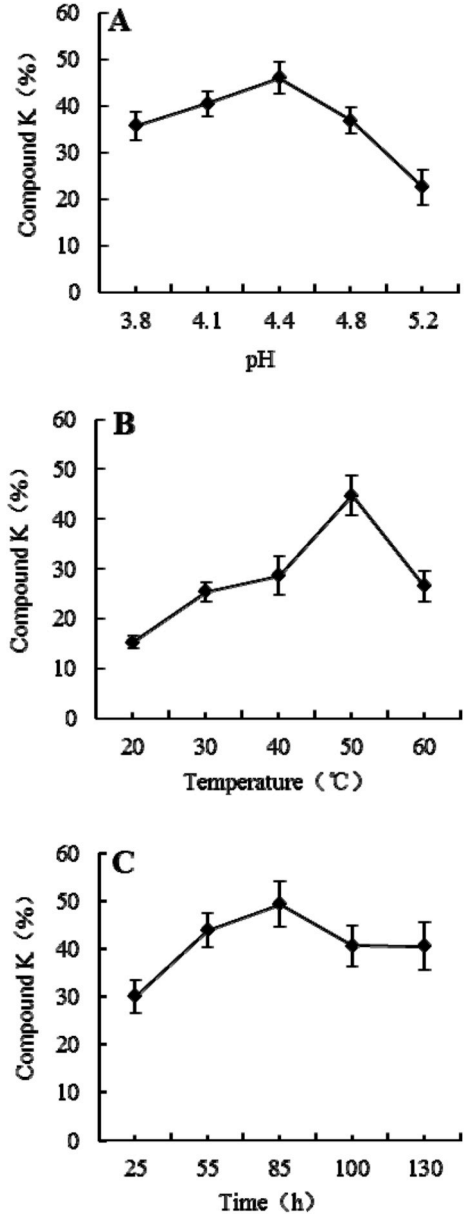

Fig. 4. HPLC analysis of the effect of enzymatic conversion from compound K *via* naringinase at different pH values (3.8, 4.1, 4.4, 4.8 and 5.2) (A), different enzymolysis temperature (20, 30, 40, 50 and 60 °C) (B), and different enzymolysis time (25, 55, 85, 100 and 130 h) (C).

2.6. Optimization of enzymolysis conditions

2.6.1. HPLC method

The HPLC method was the same as 2.5.2 to analyze the single factor and response surface methodology (RSM) experiment for detecting the compound K yield to optimize the enzymolysis parameters.

2.6.2. Preparation of standard solutions

Compound K standard was accurately weighed, and dissolved in methanol to make 2 mg/mL stock solution. The stock solution was then diluted to a series of working solutions with final concentrations of 0.25, 0.5, 1, and 2 mg/mL, respectively.

2.6.3. Optimization with single factor analysis

The emzymolysis parameters were optimized according to the setting of the enzymolysis: pH at 3.8, 4.1, 4.4, 4.8, and 5.2, enzymolysis temperature at 20, 30, 40, 50, and 60 °C, and enzymolysis time at 25, 55, 85, 100, and 130 h. HPLC was used to analyze the optimum parameters of conversion effect. The peak area of compound K was considered as a response value.

Table 1
Reaction variables with coded and actual variable levels for the 3^3 - second order factorial Box-Behnken design and its responses.

Run[a]	Actual and coded levels			Compound K Content (%)[e]
	X_1[b]	X_2[c]	X_3[d]	
1	100	50	4.8	44.88 ± 1.51
2	77.5	40	4.8	35.65 ± 1.10
3	77.5	60	4.8	44.05 ± 0.20
4	55	60	4.3	48.01 ± 0.80
5	55	40	4.3	52.51 ± 2.65
6	77.5	50	4.3	63.86 ± 2.83
7	100	60	4.3	44.83 ± 1.75
8	77.5	50	4.3	62.57 ± 2.90
9	77.5	60	3.8	45.96 ± 0.13
10	55	50	4.8	48.70 ± 1.66
11	77.5	50	4.3	63.38 ± 2.81
12	55	50	3.8	58.52 ± 0.72
13	100	40	4.3	42.68 ± 1.70
14	77.5	50	4.3	60.58 ± 2.25
15	77.5	40	3.8	48.18 ± 1.37
16	100	50	3.8	58.70 ± 1.36
17	77.5	50	4.3	63.88 ± 2.68

[a] In randomized order.
[b] X_1, reaction time (h).
[c] X_2, reaction temperature (°C).
[d] X_3, pH value of buffer solution.
[e] Results are a representative of three separate experiments (expressed as mean value ± S.D.).

2.6.4. Optimization by RSM and verification of statistical method

An overall set of 17 experiments designed with Box-Behnken design, including 5 replicates of the center point, were employed to determine the optimal conditions for enzymolysis pH, temperature, and time. Design Expert Version 8.0.6 Trail (State-Ease, Inc. Minneapolis, MN, USA) was used for data analysis. Experimental data was fitting with the following equation:

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^2 \sum_{i} \sum_{i=j=1}^{n} \alpha_{ij} X_i X_j$$

Where Y is the predicted response; α_0 is the intercept; α_i, α_{ii}, α_{ij} represent the model coefficient for linear, quadratic and interaction terms, respectively. X_i and X_j are independent variables, and n is the number of factors.

Verification of the calculated optimal enzymolysis conditions was performed via conversion with these parameters, and the comparison between the yield of compound K and the predicted value. The fitness of the polynomial model equation was expressed by the coefficient of determination R^2. Models and regression coefficients were considered significant differences when $P < 0.05$ or extremely significant differences when $P < 0.01$.

2.6.5. Statistical analysis

All experiments were performed in triplicate, and the data were expressed as means ± standard deviation (SD) of three parallel measurements.

3. Results and discussion

3.1. HPLC analysis of enzymatic hydrolysate

In Fig. 1A, an absorption peak was found at 28.362 min, demonstrating that the retention time is similar to the compound K standard

Table 2
Analysis of variance for quadric regression model.

Factor	SS	df	Mean Square	*F*-value	*Pr* > *F*	significant
Model	1242.801	9	138.0891	25.2908	0.0002	**
X_1[a]	34.6614	1	34.6614	6.3482	0.0398	*
X_2[b]	1.8346	1	1.8346	0.3360	0.5803	
X_3[c]	181.2897	1	181.2897	33.2030	0.0007	**
X_1X_2	11.0341	1	11.0341	2.0208	0.1981	
X_1 X_3	3.9949	1	3.9949	0.7316	0.4207	
X_2 X_3	28.1501	1	28.1501	5.1556	0.0574	
X_1^2	45.9128	1	45.9128	8.4088	0.0230	*
X_2^2	662.5513	1	662.5513	121.3457	< 0.0001	**
X_3^2	197.4804	1	197.4804	36.1683	0.0005	**
Residual error	38.2202	7	5.4600			
Lack of fit	30.6312	3	10.2104	5.3817	0.0688	
Pure error	7.5889	4	1.8972			
Cor Total	1281.022	16				
$R^2 = 0.9702$	$R^2_{Adj} = 0.9318$					

*$p < 0.05$.
**$p < 0.01$.
[a] X_1, reaction time (h).
[b] X_2, reaction temperature (°C).
[c] X_3, pH value of buffer solution.

(28.323 min) (Fig. 1B). It was initially determined that enzymatic hydrolysate contained components with their structure similar to that of compound K.

3.2. TLC analysis of PPD-type gypenosides isolated by HP-20 macroporous resin

In a TLC spectrum, each lane corresponds to one tube, and each saponin in eluent is indicated by a purple spot. Spots in different height indicate different type of saponin, and the same height indicates that the saponins have the same or similar structure. Moreover, the deeper the purple color, the higher the content of saponins. In this experiment, ginsenoside-Rb_3 was used as a reference for collecting the eluents containing the saponins with similar polarity.

As shown in Fig. 2A, purple spots appeared in the lane 16, and became deeper from the lane 22 to 36, which means that the content of target saponin in eluent in the corresponding tube was higher than others. However, the lane 37 to 43 showed light purple spots, indicating that content of target saponin was lower. Therefore, the eluents of the tube 22 to 36 were collected for further analysis.

3.3. TLC analysis of PPD-type gypenosides purificated by C-18 column

In Fig. 2B, the purple spots appeared from the lane 56 and disappeared in the lane 77. However, spots nearing ginsenoside-Rb_3 and at other height appeared simultaneously from the lane 69 to 77, indicating that the eluent included non-Rb_3 components. Thus, the eluents from the tube 56 to 68 were collected for enzymolysis.

3.4. TLC analysis of enzymatic hydrolysate purificated by C-18 column

As shown in Fig. 2C, the height of spots nearing compound K appeared from the lane 38, but other spots were also observed from the lane 38 to 40. Therefore, the eluents from the tube 41–45 were collected and named as Z_1, and evaporated to dryness for identification of structure by NMR.

3.5. Identification of the enzymolysis end products

The enzymolysis end products were purified, and white powders (22.1 mg) that are freely soluble in methanol were obtained. In Fig. 3, NMR spectrogram shows that the ^{1}H-NMR and ^{13}C-NMR of the Z_1 were consistent with the report by Karikura et al. (1991). The ^{1}H-NMR (MeOH-*d*4) showed 8 CH_3 signals with single peak. The ^{13}C-NMR spectrum showed 36 C-atoms including 2 olefinic C. One glucosyl group was indicated by ^{1}H, ^{13}C-NMR (δH 3.08–3.80, δC 62.4–98.2). From the NMR, and TLC and HPLC with the standards of compound, the structure of the compound Z_1 was identified as compound K.

3.6. The optimal enzymolysis conditions of PPD-type gypenosides by naringinase

3.6.1. Construction of calibration curve of compound K

The relationship between the compound K concentrations (*C*, mg/mL) and the area of peaks (A) was established in following equation with a correlation coefficient (R^2) of 0.9998.

$$A = 6008.7 \times C - 63.185$$

Quantitative determination of compound K in the enzymatic hydrolysate was performed using an external standard method and a calibration curve was established. The identification of compound K in samples was confirmed by comparing its retention time with the standard. All the analyses were repeated twice and the average values were used for the model building. The level of compound K was expressed in mg/(2 mg of PPD-type gypenosides).

3.6.2. Influence of independent variables on enzymolysis effect by single factor test

The effect of pH was mainly on the modification of enzyme conformation, and the native conformation was preferred to reach the maximum enzyme activity. Fig. 4A shows that the conversion rate of PPD-type gypenosides first increased and then decreased with the increasing of enzymolysis pH. The conversion rate increased by about > 35% when pH values increased from 3.8 to 4.8, and the highest value was reached at pH 4.4. The yield of compound K decreased significantly with the pH value from 4.8 to 5.2. Thus, the pH 3.8 to 4.8 were selected for further optimization.

Previous study showed that enzymolysis temperature influenced the reaction process by altering the collision frequency between enzyme and substrate, in addition, enzymolysis temperature also influenced the enzyme activity by changing the enzyme conformation. As shown in Fig. 4B, the influence of enzymolysis temperature on the conversion rate of PPD-type gypenosides was notable. When temperature increased from 20 to 40 °C, the compound K yield also increased gradually. In particularly, the content of compound K reached the maximum at 50 °C. As the temperature continued to rise to 60 °C, the compound K yield

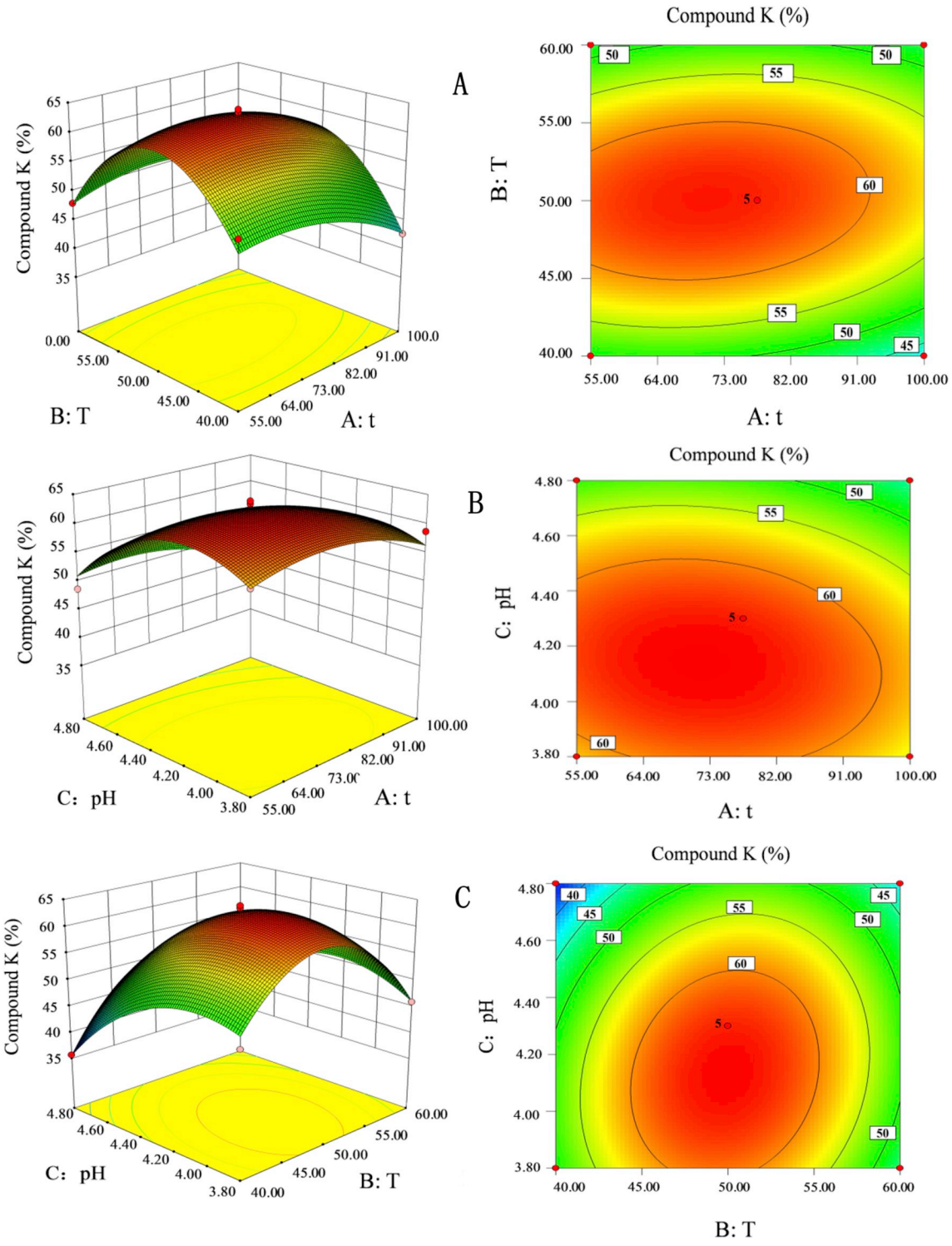

Fig. 5. Three-dimensional curved surface and contour map of the interaction effect of enzymolysis time and temperature (A), enzymolysis pH and time (B), and enzymolysis temperature and pH (C) on compound K yield.

decreased and the enzymolysis effect deteriorated, which might indicate that the naringinase began to denature and inactivate when the temperature further increased. But the yield was still at a relatively high level. Therefore, the temperatures from 40 to 60 °C were selected for further optimization.

Different reaction time would influence the combination of naringinase and PPD-type gypenosides, consequently affected yield of compound K. As shown in Fig. 4C, as the reaction time increased, the yield of compound K first increased and then tended to flatten. From 25 to 55 h, the yield of compound K increased significantly; from 55 to 85 h, the increasing rate was slow. When the reaction time reached 100 h, the yield of compound K gradually decreased. During enzymolysis time from 55 to 85 h, the accumulation of hydrolysate might accelerate the reverse reaction, and resulted in the slower conversion rate of compound K by naringinase. From 100 to 130 h, the enzymolysis reaction reached a platform, the extending enzymolysis time did not

contribute to the increase in the yield. Thus, the enzymolysis time from 55 to 100 h was selected for further optimization, which was based on an increase in degree of enzymolysis hydrolysis.

3.6.3. Analysis and verification of predictive model

Table 1 presents the results of Box-Behnken design experiments regarding the influence of independent variables on compound K yield, and the corresponding values that the compound K yield ranging from 35.65 ± 1.10% to 63.88 ± 2.68%. The experimental data of Box-Behnken design are reflected in the following quadric regression equation:

$$Y=62.86 - 2.08X_1 +0.48X_2 -4.76X_3 +1.66X_1X_2 - X_1X_3 +2.65X_2X_3 -3.3X_1^2 -12.54X_2^2 -6.85X_3^2$$

Where Y is the expected compound K yield, X_1 is reaction time, X_2 is reaction temperature, X_3 is pH.

Table 2 exhibits the evaluation of the quadric regression model equation significance carried out by Fisher's statistical test for the analysis of variance (ANOVA) to verify the feasibility of the equation. The *F* value, 25.2908, suggests that this model was statistically significant, which represents the relationship between the value of response and independent variable. According to Table 2 and the model equation, it can be concluded that the model linear terms X_1 and X_3 could affect the compound K yield significantly ($P < 0.05$). Among these statistically significant variables, pH (X_3) was an extremely significant ($P < 0.01$) factor that influenced the hydrolysis process, followed by enzymolysis time (X_1). Inversely, enzymolysis temperature (X_2) showed less effect on the yield of compound K. The R^2 values and the adjusted determination coefficient (Adj. R^2) were 0.9702 and 0.9318, respectively, suggesting that a high degree of correlation between the observed and predicted values. The *F* value (5.3817) and *P* value of lack-of-fit (0.0688) imply that the lack-of-fit was not significant as compared to the pure error. It indicates that the model equation was adequate for predicting the yield of compound K under any combination of values of the variables.

As shown in Fig. 5, three dimensional surface plots were established to investigate the relationship among different variables and response to obtain the optimal hydrolysis conditions that would maximize the yield of compound K. In Fig. 5A and B, the yield of compound K gradually increased with the reaction time from 55 to 77.5 h, and decreased after 82 h. Therefore, the prolongation of the enzymolysis time did not increase the compound K yield. The experiment results show that at 50 °C and pH 4.3, the reaction reached the maximum within 77.5 h; while excessive pH and temperature of the enzymatic reaction would reduce the yield of compound K. Fig. 5B and C show that the compound K yield increased as the increment of pH from 3.8 to 4.3, but decreased as the pH was higher than 4.3. In addition, there was a similar trend in the effect of enzymolysis time on compound K production (Fig. 5A and C). In the reaction temperature range from 30 to 50 °C, an increment in temperature might contribute to the enhanced enzyme activity. However, if the temperature was further increased above 55 °C, the enzyme might be inactivated.

According to the optimization of the regression model, the optimal enzymolysis conditions of compound K by naringinase might be enzymolysis pH 4.1, enzymolysis temperature 50 °C, and enzymolysis time 71 h, with the yield of compound K at 64.93% in theory. Besides, the verification experiment was performed using this condition, and it indicates that the actual conversion rate was 65.44 ± 4.52%. These results were in good accordance with the predicted values, which indicated that the optimized model was reliable.

4. Conclusions

The results in this study demonstrate that the PPD-type gypenosides could be effectively converted to compound K by naringinase. The optimal conditions of enzymatic hydrolysis is pH value at 4.1, temperature at 50 °C, and time at 71 h, and with a yield of compound K at 65.44 ± 4.52%. This study could provide an alternative and practical method for producing compound K from the biotransformation of gypenosides.

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Appendix A. Supplementary data

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Transparency document

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