Efficient enzymatic hydrolysis of Protogracillin for clean preparation of Prosapogenin A by response surface methodology optimization

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\textbf{ABSTRACT}

Prosapogenin A (also known as Progenin III and polyphyllin V), a secondary steroidal saponin in 
Dioscorea zingiberensis
tubers, has more superior pharmacological activities than its primary 
form Protogracillin. Conventional preparation methods, such as column chromatography, and 
acidic hydrolysis, are of very low efficiency and limited scale due to tedious procedures and 
large consumption of organic solvent. This study aims to establish a convenient method for 
efficiently obtaining Prosapogenin A by enzymatic hydrolysis of abundant Protogracillin in the 
raw material. In light of the highest hydrolysis performance, β-dextranase was selected from 
four commercial enzymes in this application. After optimization of the conditions by the 
response surface methodology using Box–Behnken design, the enzymatic hydrolysis was carried 
out in 0.20 M HAc-NaAc buffer (pH 4.81) containing β-dextranase/Protogracillin (5.0:1, w/w), and 
the system was constantly kept at 56.7°C water bath for 4 h. Consequently, Protogracillin has 
been almost completely hydrolyzed to be Prosapogenin A and the highest yield was 96.4 ± 
1.4%. The newly proposed approach is efficient and promising for conveniently obtaining 
Prosapogenin A and aimed to provide a laboratory-scale experimental foundation for the 
preparation of Prosapogenin A in industrial applications.

\section{1. Introduction}

\textit{Dioscorea zingiberensis} C.H. Wright (DZW), a tuberous 
herbaceous perennial liana, is a widely grown plant of 
\textit{Dioscoreaceae} family in the China region \cite{1–3}. The 
tubers of this plant, commonly called ‘Yellow Ginger,’ were usually used as raw material for diosgenin pro-
duction in the pharmaceutical industry due to a high 
content of steroidal saponins \cite{4, 5}. Protogracillin (CAS: 
54848-30-5; $M_w$: 1065) was formed by covalent conjunc-
tion between glucose/rhamnose and aglycone skeleton 
by C–O glycosidic bond. This primary glycoside (proto-
gracillin) is a water-soluble furostanol saponin in DZW 
tubers at the amount of 2\%–5\% total steroidal saponins 
(TSS) \cite{6}. Its price is USD1500/kg and can be purchased

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\textsuperscript{\textbullet{}} Supplementary data for this article can be accessed at https://doi.org/10.1080/17518253.2022.2138723.

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from Chengdu Pufei De Biotechnology Co., Ltd. In addition, it can be readily prepared by column chromatography (7, 8). In previous studies, Protogracillin has shown strong anti-thrombotic activity for the treatment of cardiovascular disease (6); however, in vivo bioavailability stays at a very low level for its high polarity (9). Interestingly, one of its secondary glycosides, namely Prosapogenin A (CAS: 19057-67-1; $M_w$: 722), has also demonstrated remarkable and various bioactivities than protogracillin, including antiproliferative (10, 11) and anticancer effects (11). Prosapogenin A with a shorter sugar chain exhibited much stronger antifungal activity (12, 13) than Protogracillin, which could be an ideal lead molecular entity for new drug development (14).

Prosapogenin A was isolated from Dioscorea bulbifera L. tubers in 1999 (15), and some technologies were developed to obtain it by the incorporation of the conventional extraction method and counter-current chromatography (16, 17). Thereafter, there are a few studies that reported recent attempts by prevalent biotransformation (18) or microwave and ultrasound-assisted technologies in the green extraction of bioactive compounds (19–21). However, clean preparation of protosapogenin A is still a challenging task, as the compound is only present in small amounts in raw material of D. zingiberensis. So far, none of those methods was preferred in the industrial application for a few inevitable shortcomings, including time-consuming procedures, large demand for organic solvent or raw materials, and low yield of the desired compound. Therefore, more efforts should be put to establish a novel strategy to efficiently obtain Prosapogenin A.

Hydrolysis of primary glycosides is a universal and popular strategy to prepare bioactive natural compounds of interest. Among them, acid hydrolysis (22, 23) has been employed as the most straightforward measure to completely remove saccharides from the aglycone skeleton. Hence, diosgenin, the main by-product, was tremendously generated during acid hydrolysis of TSS from DZW. In addition, biotransformation of steroidal saponins from Dioscorea tubers (24) and microbial conversion of Parviglucoside was also applied to obtain Prosapogenin A in previous studies (25); however, the crude enzyme has low hydrolytic activity on glycosides, and many unfavorable by-products often lead to tedious separation and purification. High-purity enzymatic hydrolysis is a highly controllable and specific process (26, 27), such as β-glucosidase, cellulase (28), β-glucanase (29), which has been successfully used to specifically hydrolyze glycosidic bonds for the preparation of secondary glycosides (30). Furthermore, the conditions of enzymatic hydrolysis are usually very mild, and it only requires a simple procedure for the construction of the reaction system and results in few by-products (31). Overall, enzymatic hydrolysis has significant strengths because of running cost, environmental friendliness, and daily operation.

In this study, as shown in Figure 4 Scheme 1 we aim to establish a novel reaction system for the efficient conversion of abundant Protogracillin to Prosapogenin A by enzymatic hydrolysis (24, 25). First, the most suitable enzyme, β-dextranase, was screened from four commercial hydrolases. Then, to achieve the highest yield of Prosapogenin A, the hydrolysis conditions were optimized by the response surface methodology (RSM) using Box–Behnken design (BBD).

2. Materials and methods
2.1. Materials and reagents
Protogracillin (purity ≥98% by HPLC-UV) was prepared in our laboratory by column chromatography. DZW was collected in June 2019 from Jianshi County, Hubei Province, China. TSS was extracted by physically separating the DZW, 100.0 g of which was then separated on a silica gel (1.50 kg) column by gradient CH$_2$Cl$_2$-MeOH (12:3–6:3, v/v). Thin Layer Chromatography (TLC) was applied to analyze fractions and similar components (Frac.1–Frac.6) were pooled and combined. 1.0 g of Frac.3 was separated on a preparative C18 column by acetonitrile–water mixture (22%:78%–35%:65%), and then 285.6 mg of protogracillin (purity ≥98% by HPLC-UV) was isolated. The sample was further purified by column chromatography.
was obtained (MS and NMR data shown in Figure S1). Prosapogenin A was prepared by enzymatic hydrolysis of protogracillin and both were identified by MS and NMR, and its purity was ≥98.0% by HPLC-UV. Acetonitrile (ACN, purity ≥99.9%) and methanol (MeOH, purity ≥99.9%) were of HPLC grade and obtained from Omegene LLC (US). CH2Cl2 (purity ≥99.5%) was of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Distilled water (15.0 MΩ cm−1) and ultrapure water (>18.2 MΩ cm−1) were produced by the HealForce water purification system (China). Silica gel (200–300 mesh) for column chromatography and silica gel G for TLC were purchased from Qingdao Ocean Chemical Co., Ltd. (China). C18 packing material was obtained from Silicycle (Canada), β-glucosidase (activity: 100 U/g; 1 U: 1.0 μM of glucose was liberated from salicin per minute at pH 5.0 at 37°C) and β-dextransase (activity: 20,000 U/g; 1 U: 1.0 μM of reducing sugars was liberated from barley β-D-glucan per minute at pH 5.5 at 37°C) were bought from Jinsui Biotech Co., Ltd. (Shanghai, China) and Jiangsu Ruiyang Biotech Co., Ltd. (Wuxi, China). Cellulase (activity: 10,000 U/g; 1 U: 1.0 μM of glucose liberated from cellulose per hour at pH 5.0 at 37°C) was provided by Shanghai Macklin Biochemical Co., Ltd. (China). Naringinase (activity: 100,000 U/g; 1 U: 1.0 μM of α-L-rhamnoside was liberated from p-nitrophenyl α-L-rhamnoside in 5 min at pH 3.7 at 55°C) was supplied by Henan Baikang Chemical Products Co., Ltd. (China). All other chemicals used in the study were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd., China.

2.2. Enzyme screening for the enzymatic hydrolysis

In the first stage, four commercial hydrolases, including β-glucosidase, β-dextranase, cellulase, and naringinase, were compared for the best performance on the conversion of Protogracillin to Prosapogenin A. Before enzymatic hydrolysis, 500 μL of Protogracillin solution (0.600 mg/mL) and 500 μL of enzyme solution (2.400 mg/mL) in 0.20 M HAc-NaAc buffer (pH 4.5) were evenly mixed; therefore, the final concentration of the substrate and the enzyme were diluted to be 0.300 and 1.200 mg/mL, respectively. Then the mixture was incubated in a constant 60°C of the water bath and without shaking or stirring for 12 h. Meanwhile, a control was concurrently performed to prepare a blank solution without enzyme under the same reaction conditions. After the hydrolysis reaction was completed, the whole hydrolysate was lyophilized immediately. Then 0.4 mL of methanol was added into the lyophilized powder to dissolve the resulting product, and the resulting solution was subjected to filtration through a disposable 0.22 μm PTFE membrane syringe before subsequent HPLC-UV analysis of Prosapogenin A. The yield of Prosapogenin A was calculated and set as the index of hydrolysis performance; accordingly, a proper enzyme was selected from those hydrolases for further experiments.

2.3. Optimization of the operating conditions for the enzymatic hydrolysis

Based on the yield of the product, β-dextranase was selected from the four enzymes for having the highest catalytic activity. The effect of four factors, including hydrolysis duration, hydrolysis temperature, β-dextranase/protogracillin ratio, and pH of the buffer, on the yield of Prosapogenin A, was investigated by singly factor experiment. The performance was compared, while Protogracillin was hydrolyzed by β-dextranase in the buffer of various pH at different temperatures for a certain time. Initially, 0.600 mg/mL of Protogracillin (0.5 mL) and 2.400 mg/mL of enzyme (0.5 mL) in 0.20 M HAc-NaAc buffer (pH 4.5) were well mixed, followed by continuous incubation at 60°C for 0.5, 1, 2, 4, 6, 8, 10, and 12 h. Then, the temperature of enzymatic hydrolysis was optimized, while the same hydrolysate system was incubated for 4 h at 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C. Furthermore, enzymatic hydrolysis was performed in the buffer (pH 4.5) at 55°C for 4 h, while the ratio of β-dextranase/protogracillin was 1:1, 2:1, 3:1, 4:1, 5:1, and 6:1. At last, the hydrolysis was conducted in 55°C water bath for 4 h in a buffer solution of different pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, or 6.5). All experiments were done in triplicate, and the average yield of Prosapogenin A and standard deviation (SD) were calculated and the data were presented as Mean ± SD.

2.4. Experimental design by the response surface methodology and calculations

RSM is a commonly-used empirical statistical method for establishing mathematical models, optimizing multifactor tests, and exploring relationships between response variables and explanatory variables (32, 33). Therefore, RSM can be used to predict the effects of various operational factors on the targeted responses, and evaluate the interactions between the varying factors. The current experiment used RSM to conduct data analysis and optimize multiple experimental conditions with the minimum experiments for the maximum response. RSM was performed, and the optimization method solved was achieved using a professional experiment design and data-analysis software of Design-
Expert 8.0.6. based on the results of single-factor exploratory tests. It was observed that there are operating parameters, namely, pH of buffer (X₁), hydrolysis temperature (X₂, °C), and β-dextranase/protogracillin ratio (X₃, mg/mg) have a more important effect on the yield of Prosapogenin A than hydrolysis duration. Therefore, these three factors were selected as input variables for BBD-RSM investigation of the enzymatic hydrolysis Protogracillin process, while the yield of Prosapogenin A is the response (Y). Each variable was divided into three levels (−1, 0, and 1), which allows the establishment of a design of experiment with 17 investigational runs whose five replications were at the central level. All experiments were carried out during 4 h of hydrolysis duration. The ranges of the considered variables are summarized in Table 1.

All experiments were conducted at random to reduce the effects of unknown variations on the observed data caused by external factors. For this, a second-order quadratic model was used to establish the relationship between selected input variables and the yield of Prosapogenin A. The numerical expression of the polynomial statistical model is formalized as follows:

\[ Y = \beta_0 + \sum_{i=1}^{k} (\beta_i X_i) + \sum_{i=1}^{k} (\beta_{ii} X_i^2) + \sum_{i=1}^{k} \sum_{j=1}^{k} (\beta_{ij} X_i X_j) + \varepsilon \]

where \( Y \) represents the yield of Prosapogenin A, and \( X_i \) value was the independent variable affecting the response \( Y \); \( \beta_0 \), \( \beta_i \), \( \beta_{ii} \), and \( \beta_{ij} \) were regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. \( X_i \) and \( X_j \) were the independent coded variables (\( i \neq j, i, j \) range from 1 to \( k \)), \( k \) was the number of tested variables (\( k = 3 \)), and \( \varepsilon \) was a residual term.

The values of the regression parameters, such as the correlation coefficient \( R^2 \), \( p \)-value, and lack-of-fit, were calculated using the analysis of variance (ANOVA) and used for the determination of the relevance and suitability of the predicated model. Based on the 95% confidence level in the developed model, the significance of independent variables on the yield of Prosapogenin A was evaluated.

The concentration of Prosapogenin A in the sample solution was computed by substitution of the peak area of Prosapogenin A into the obtained calibration curve, and the yield of Prosapogenin A (Y%) was then calculated by the below equation:

\[ Y% = \frac{V \times M1 \times C}{m \times M2} \times 100\% \]

where \( V \) represents the volume of hydrolysate in methanol solution; \( M1 \) represents the molecular weight of Protogracillin (CAS: 54848-30-5; \( M_{w} \): 1065); \( C \) represents the concentration of Prosapogenin A in the sample solution; \( m \) represents the weight of Protogracillin added in the enzymatic hydrolysis. \( M2 \) represents the molecular weight of Prosapogenin A (CAS: 19057-67-1; \( M_{w} \): 722).

### 2.5. HPLC conditions and calibration curves

Quantitative analysis of Prosapogenin A was performed on an Agilent 1100 Series HPLC instrument (Agilent Technologies, Santa Clara, CA, U.S.A.) consisting of a G1322A online degasser, a G1312A binary pump, a G1316A ALS autoinjector, a G1316A column thermostat, and a G1315A diode array detector (DAD). Raw chromatogram acquisition and data processing were completed by HP ChemStation Software (Rev. A.07.01 [682]). Twenty microliters of each sample solution was sequentially subjected to chromatographical separation on a Turner C18 HPLC column (4.6 mm I.D. × 250 mm L, 5 μm), and the mixture of ACN (A) and H₂O (B) was constantly pumped at 1.0 mL/min under gradient elution mode. Time program was as follows: 0 min, 25% A: 75% B; 15 min, 33% A: 67% B; 45 min, 60% A: 40% B; 48 min, 60% A: 40% B; 49 min, 25% A: 75% B; 55 min, 25% A: 75% B. Both analytes, Protogracillin and Prosapogenin A, were simultaneously monitored at 203 nm, and the column was kept at 35°C throughout the analysis.

9.70 mg of Prosapogenin A reference was dissolved in MeOH and scaled to 10 mL for subsequent use as a stock solution at 0.970 mg/mL, and a series of standard solutions were prepared by gradual two-fold dilution for six times to be 0.485, 0.243, 0.121 mg/mL, 6.06 × 10⁻² mg/mL, 3.03 × 10⁻² mg/mL, and 1.52 × 10⁻² mg/mL. All the standard solutions were filtered through a disposable 0.22 μm PTFE membrane syringe before HPLC analysis. The peak area (Y) versus the concentration of Prosapogenin A (X) was then plotted for the calibration curve.

### 2.6. Identification of Prosapogenin A

After enzymatic hydrolysis, the resulting reconstituted hydrolysate was subjected to the purification of Prosapogenin A by preparative HPLC using a Hanbon Hedera ODS column (20 mm I.D. × 250 mm L, 5 μm). The chemical structure of Prosapogenin A was identified by ¹H-NMR and ¹³C-NMR (Advance II 400 MHz NMR

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**Table 1.** Experimental parameters and levels for Box–Behnken design.

| Variables                                     | Symbol | −1 | 0   | +1 |
|-----------------------------------------------|--------|----|-----|----|
| The pH of the buffer                          | X₁     | 3.5| 4.5 | 5.5|
| Hydrolysis temperature (°C)                   | X₂     | 50 | 57  | 64 |
| β-dextranase/protogracillin ratio             | X₃     | 2  | 3.5 | 5  |


Spectrometer, Bruker), and the sample solution was also analyzed by UPLC-ESI-QQQ-MS (Shim-pack UFLC SHIMADZU CBM30A system) for MS data acquisition.

3. Results and discussion
3.1. Selection of commercial enzyme
Protogracillin and Prosapogenin A can be separated well and determined by HPLC-UV, and the result is shown in Figure S2. After enzymatic hydrolysis by each of the four commercial hydrolases under the same condition, the yield of Prosapogenin A was calculated. As shown in Figure 1(A), β-dextranase or cellulase has achieved a much higher yield at 96.5 ± 2.0% or 92.1 ± 0.8%, respectively, than β-glucosidase at 62.7 ± 3.5% and naringinase at 0.31 ± 0.07%. In addition, it can be seen from Figure 1(B), a dramatical increase in the yield was noted by either β-dextranase or cellulase in the first 6 h of enzymatic hydrolysis, followed by a plateau till 12 h. Moreover, when the hydrolysis was done from 8 h to 12 h, the hydrolysis capability of cellulase was slightly weaker than that of β-dextranase. While the hydrolysis duration was less than 6 h, the hydrolysis of Protogracillin by β-dextranase was remarkably greater than that of cellulase. Especially, when the hydrolysis duration was 2 h, the yield of Prosapogenin A by β-dextranase was 63.2 ± 3.4%, which was significantly higher than that by cellulase at 30.7 ± 2.3%. Consequently, β-dextranase was selected due to the best catalysis performance.

3.2. Optimization of enzymatic hydrolysis conditions
3.2.1. Hydrolysis duration
The effect of hydrolysis duration (0.5 h–12 h) on the yield of Prosapogenin A was investigated. As shown in Figure 2(A), the yield dramatically increased from 13.7 ± 0.8% to 92.8 ± 5.1% within the initial 4 h, then it enters a plateau with a moderate increase to 96.5 ± 2.0% in the next 8 h. Therefore, to obtain a high yield and reduce the reaction time, 4 h as the optimal hydrolysis duration was selected for subsequent experiments.

3.2.2. Hydrolysis temperature
The effect of hydrolysis temperature on the yield was investigated. As shown in Figure 2(B), the yield of Prosapogenin A increased from 86.3 ± 0.6% to 96.7 ± 0.3% while the temperature ascended from 45°C to 55°C, and the yield only decreased a little even if the temperature was 60°C as β-dextranase is a heat-resistant hydrolase and remains highly active over a wide range of temperatures. However, as the system was further heated up to a higher temperature, the yield of Prosapogenin A began to decline greatly to 12.0 ± 3.8% when the hydrolysis was carried out at 70°C. Accordingly, 55°C was selected for application in further trials.

3.2.3. β-dextranase/Protogracillin ratio
Protogracillin was hydrolyzed at 55°C in 0.20 M HAc-NaAc buffer of pH 4.5 for 4 h, and the effect of β-dextranase/Protogracillin ratio (mg/mg, w/w) on the yield of Prosapogenin A was investigated, and the results are shown in Figure 2(C). When the ratio was set as 1:1, the yield has been 60.4 ± 3.2% only, implying the insufficiency of the hydrolase for the conversion. Then, the yield was rapidly increased with a larger amount of enzyme added into the reaction system, and eventually reached the highest yield at 96.7 ± 0.3% when the ratio was adjusted to be 4:1. Afterward, the ratio of β-dextranase to Protogracillin was further increased to 6:1, nevertheless the yield still maintained at this level in steadily and complete hydrolysis has not been achieved. Therefore, β-dextranase/Protogracillin ratio was 4:1 for the following hydrolysis.

Figure 1. Effect of various hydrolases at 60°C and pH 4.5 (A), and comparison of β-dextranase and cellulase after different hydrolysis durations (B).
3.2.4. pH of buffer

Figure 2(D) illustrates the impact of the pH of the buffer on the hydrolysis performance when the substrate was hydrolyzed in a 55°C water bath for 4 h with a β-dextranase/protogracillin ratio at 4:1. The yield of Prosapogenin A increased gradually to its maximum level after pH value of 0.20 M HAc-NaAc buffer was changed from 3.5 to 4.5. However, when the pH of the buffer was higher, the yield has been markedly decreased to 25.7 ± 0.5% down. Considering the greater yield of Prosapogenin A in more acidic conditions, pH 4.5 was then chosen for this application.

3.4. Optimization of the conditions by RSM-BBD and validation

By combining different levels of variables $X_1$ (pH of buffer), $X_2$ (hydrolysis temperature), and $X_3$ (β-dextranase/protogracillin ratio), an experimental scheme containing seventeen trials was designed and conducted, and then the yield of Prosapogenin A was employed as the index for fitting a model. The experimental data are shown in Table 2. By multiple regression analysis of the experimental data, the relationship between the yield and three tested variables was constructed and expressed to be a second-order polynomial equation. The model was then applied to optimize the hydrolysis conditions. The final equation was as follows:

$$Y = 87.75 + 11.99X_1 - 5.52X_2 + 9.40X_3 + 9.08X_1X_2 - 0.98X_1X_3 - 1.30X_2X_3 - 15.76X_1^2 - 9.48X_2^2 - 1.99X_3^2$$

where $Y$ was the yield of Prosapogenin A (%), $X_1$ was the pH of the buffer, $X_2$ was hydrolysis temperature (°C); $X_3$ was β-dextranase/protogracillin ratio (mg/mg).

The coefficients of the RSM model were verified by analysis of variance (ANOVA) for the quadratic polynomial model in Table 3. These results demonstrate

| No. | $X_1$ pH of buffer | $X_2$ hydrolysis temperature (°C) | $X_3$ β-dextranase/protogracillin ratio | Yield (%) |
|-----|-------------------|-----------------------------------|----------------------------------------|----------|
| 1   | -1                | -1                                | 0                                      | 64.77    |
| 2   | 1                 | -1                                | 0                                      | 78.90    |
| 3   | 1                 | 1                                 | 0                                      | 78.42    |
| 4   | -1                | 1                                 | 0                                      | 27.96    |
| 5   | 1                 | 0                                 | -1                                     | 49.22    |
| 6   | 1                 | 0                                 | -1                                     | 49.22    |
| 7   | -1                | 0                                 | 1                                      | 66.87    |
| 8   | 1                 | 0                                 | 1                                      | 75.11    |
| 9   | 0                 | -1                                | 1                                      | 88.82    |
| 10  | 0                 | 1                                 | -1                                     | 69.86    |
| 11  | 0                 | -1                                | -1                                     | 69.03    |
| 12  | 0                 | 1                                 | 1                                      | 86.14    |
| 13  | 0                 | 1                                 | 1                                      | 80.11    |
| 14  | 0                 | 0                                 | 0                                      | 86.50    |
| 15  | 0                 | 0                                 | 0                                      | 83.90    |
| 16  | 0                 | 0                                 | 0                                      | 89.49    |
| 17  | 0                 | 0                                 | 0                                      | 94.28    |

Table 2. Box–Behnken design matrix and response values of the yield.

Figure 2. Effect of hydrolysis temperature (A), β-dextranase/Protogracillin ratio (B), and the pH of the buffer (C).
that the $P$-value of the regression model was highly significant ($P < .01$), and the lack-of-fit was not significant ($P > .05$), indicating that unknown factors have little effect on the results. The interaction term ($X_1X_2$) and quadratic term coefficients ($X_1^2$, $X_2^2$) were significant ($P < .05$), and the linear coefficient ($X_1$, $X_2$) and the quadratic term coefficients ($X_1^2$, $X_2^2$) were extremely significant ($P < .01$). The determination coefficient ($R^2$) for the quadratic regression model was 0.9133; therefore, 91.33% of the response in yield was due to the pH of the buffer, hydrolysis temperature, and $\beta$-dextranase/protogracillin ratio and their interactions. It showed that the regression equation fits well with the experimental data, and the model can be used to analyze and predict the change of yield under different enzymatic hydrolysis conditions (34). The regression coefficients showed that the most important value among linear terms is the pH value of the buffer (11.99), which is about double that for hydrolysis temperature, however, with the opposite sign (−5.52); and the $\beta$-dextranase/protogracillin ratio is ranked second (9.40). This result illustrates the positive effect of the pH of the buffer and $\beta$-dextranase/protogracillin ratio on the yield of Prosapogenin A, while too high temperature significantly reduces the yield of Prosapogenin A. Among the interaction terms, the highest value was the pH of the buffer and hydrolysis temperature (9.08). This indicates that increasing the pH of the buffer and the hydrolysis temperature within the appropriate range is beneficial to increase the yield of Prosapogenin A. The other two interaction terms showed a negative effect on the yield of Prosapogenin A; however, they were 10-fold lower (−0.98) and 7-fold lower (−1.30) than this of the pH of buffer and hydrolysis temperature but with the opposite sign (35).

According to the experimental data, the three-dimensional response surface was plotted (Figure 3) to reveal the interaction between various factors, and their optimal values achieved the highest yield of Prosapogenin A.

Based on the RSM model, the optimal conditions for the hydrolysis of Protogracillin by $\beta$-dextranase were 4.81 for the pH of the buffer, 56.7°C for hydrolysis temperature, and 5.0:1 for $\beta$-dextranase/Protogracillin ratio. Under these conditions, the maximum yield of Prosapogenin A was 97.14%. To validate the optimum conditions, an experiment for enzyme hydrolysis was performed under the predicted conditions with cautious adjustment (56.7°C, pH 4.81, 1:5 for $\beta$-dextranase/Protogracillin ratio, 4 h) and the experiment was repeated in triplicate and the average yield of Prosapogenin A was 96.4 ± 1.4%, which was highly consistent with the predicted value of the model. Thus, the model development was accurate and reliable for predicting the optimized conditions for the best hydrolysis performance in this study.

### 3.5. Identification of Prosapogenin A

After the preparative HPLC purification, the fraction of Prosapogenin A was collected and the purity was ≥98% by HPLC-UV. Moreover, LC-ESI+-MS, $^1$H-NMR, and $^{13}$C-NMR were applied to confirm the chemical structure of Prosapogenin A (Figure S3).

LC-ESI+-MS: $m/z$ 723 [M + H]$^+$, 577 [M + H-Rha]$^+$, 415 [M + H-Rha-Glc]$^+$; 397 [M + H-Rha-Glc-H$_2$O]$^+$. $^1$H-NMR (400 MHz, Pyridine-$d_5$): $\delta$: 5.74 (1H, s, Rha-H-1‴), 5.33 (1H, d, $J = 4.8$ Hz, H-6), 4.84 (1H, s, Glc-H-1‴), 1.68 (3H, s, Rha-6‴-CH$_3$), 1.15 (3H, s, 21-CH$_3$), 0.90 (3H, s, 19-CH$_3$), 0.88 (3H, s, 18-CH$_3$), 0.72 (3H, s, 27-CH$_3$); 13C-NMR (100 MHz, Pyridine-$d_5$): $\delta$: 142.1 (C-5), 123.0 (C-6), 110.5 (C-22), 103.3 (C-1‴), 101.6 (C-1‴), 82.4 (C-16), 80.9 (C-3), 79.5 (C-2′), 79.2 (C-5′), 79.1 (C-3′), 75.4 (C-4′), 74.1 (C-4″), 73.8 (C-3″), 73.1 (C-2″), 70.7 (C-5″), 68.1 (C-26), 64.2 (C-17), 63.9 (C-6″), 57.9 (C-14), 51.6 (C-9), 43.3 (C-20), 41.7 (C-13), 41.1 (C-12), 40.3 (C-4), 38.8 (C-1), 38.4 (C-10), 33.6 (C-7), 33.5 (C-15), 33.1 (C-23), 32.9 (C-8), 31.9 (C-25), 31.5 (C-2), 30.5 (C-24), 22.4 (C-11), 20.7 (C-19), 19.9 (C-6‴), 18.6 (C-27), 17.6 (C-18), 16.3 (C-21). The above data were consistent with reference (16, 22), accordingly, the compound was identified as Prosapogenin A.

### 3.6. Comparison between the new strategy and conventional methods

The new strategy of this study was validated by comparison with other conventional methods of obtaining Prosapogenin A, and the results are summarized in

| Source | Sum of squares | df | Mean square | F-value | P | Significance |
|--------|---------------|----|-------------|---------|---|--------------|
| Model  | 3984.22       | 9  | 442.69      | 8.19    | .0056 | **           |
| $X_1$  | 1150.67       | 1  | 1150.67     | 21.29   | .0024 | **           |
| $X_2$  | 243.69        | 1  | 243.69      | 4.51    | .0714 |              |
| $X_1^2$| 706.78        | 1  | 706.78      | 13.08   | .0086 |              |
| $X_2^2$| 329.78        | 1  | 329.78      | 6.10    | .0428 |              |
| $X_1X_2$| 3.88          | 1  | 3.88        | 0.072   | .7966 |              |
| $X_1^2X_2$| 6.78         | 1  | 6.78        | 0.13    | .7336 |              |
| $X_1^3$| 1045.45       | 1  | 1045.45     | 19.34   | .0032 | **           |
| $X_2^3$| 378.18        | 1  | 378.18      | 7.00    | .0332 |              |
| $X_1^2$| 16.66         | 1  | 16.66       | 0.31    | .5960 |              |
| Residual| 378.32        | 7  | 54.05       |         |      |              |
| Lack of fit | 306.23    | 3  | 102.08      | 5.66    | .0063 | Not significant |
| Pure error | 72.09   | 4  | 18.02       |         |      |              |
| Cor total| 4362.54     | 16 |             |         |      |              |

*Significant ($P < .05$); **Extremely significant ($P < .01$).
Table 4. Acid hydrolysis of TSS can only obtain aglycone diosgenin but not Prosapogenin A (5). By chromatographic separation, only 45 mg of Prosapogenin A was isolated from 10 kg of dry rhizomes of DZW (yield = 0.00045%) (16) and 3.4 mg from 400 g of dry rhizomes of Dioscorea villosa (yield = 0.00085%) (17). In addition, 82 mg Prosapogenin A was obtained from 15 g TSS by fermentation, and the final yield was about 0.0226% (18). In this study, the abundant primary glycoside Protogracillin was enzymatically hydrolyzed to obtain the rare bioactive secondary glycoside Prosapogenin A, the yield was greatly increased to 0.294%, while the product was of high purity, which did not require subsequent complicated purification. Therefore, our results indicate that enzymatic hydrolysis is more efficient and eco-friendly than conventional methods.

Figure 3. Three-dimensional response surface plots (A, C, and E) and response contour plots (B, D, and F) of the effect of the pH of the buffer ($X_1$), hydrolysis temperature ($X_2$), and β-dextranase/Protogracillin ratio ($X_3$) on the yield of Prosapogenin A.
Table 4. Verification experiments and comparison with other methods.

| Production methods       | Sources         | The yield of Prosapogenin A | References |
|--------------------------|-----------------|-----------------------------|------------|
| Acid hydrolysis          | DZW             | –                           | (5)        |
| Chromatographic separation | DZW         | 0.00045%                    | (16)       |
| Chromatographic separation | Dioscorea villosa | 0.00085%                  | (17)       |
| Fermentation             | DZW             | 0.0226%                     | (18)       |
| The proposed strategy    | DZW             | 0.294%                      | This work  |

4. Conclusion

In this study, a novel method based on enzymatic hydrolysis was developed to efficiently obtain Prosapogenin A from its original glycoside Protogracillin. Compared with conventional methods, the novel technology makes it much easier to obtain the compound conveniently. The conditions of the hydrolysis were optimized by BBD-RSM, and the pH of the buffer was a crucial factor affecting the yield most. The validated yield of Prosapogenin A was very close to the predicted value, indicating that the constructed model was feasible. Consequently, the newly proposed approach was of high performance to produce more bioactive Prosapogenin A from its original, Protogracillin, and it has shown great feasibility and potential to provide a laboratory-scale experimental foundation for the preparation of natural bioactive compounds in industrial application.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the National Natural Science Foundation of China [Grant Number 81303313, 81873196]; Qinglan Project of Jiangsu Province of China (2016).

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