Optimum Conversion of Major Ginsenoside Rb1 to Minor Ginsenoside Rg3(S) by Pulsed Electric Field-Assisted Acid Hydrolysis Treatment

Chengwen Lu, Yongguang Yin*

Abstract: Ginsenoside Rg3(S) is a primary bioactive component in ginseng, which has pharmacological effects and nutritional activities. In the present study, pulsed electric field (PEF)-assisted acid hydrolysis processing was used to convert major ginsenoside Rb1 to minor ginsenoside Rg3(S). The optimum parameters of PEF assisted acid hydrolysis were analyzed by response surface methodology (RSM). The optimum processing conditions were: electric field intensity, 20 kV cm\(^{-1}\); acid concentration, 0.25 mol/L; pulse number, 10. The conversion rate of ginsenoside Rg3(S) achieved 68.58%, in accordance to the predicted value. The structure of hydrolyzed product was confirmed by \(^{13}\)C-NMR. The results suggested that PEF-assisted acid hydrolysis significantly enhanced conversion rate of ginsenoside Rg3(S).

Keywords: Pulsed electric field; Ginsenoside Rb1; Ginsenoside Rg3(S); Acid hydrolysis.

1 Introduction

Ginseng has been used in a wide range of medicinal therapies and health food for thousands years in Eastern Asia. The major bioactive constituents in ginseng are ginsenosides, which demonstrated various pharmacological and therapeutic activities [1-3]. The minor ginsenoside Rg3(S) is one of the main bioactive components of ginsenosides. It is well known that ginsenoside Rg3(S) possessed an ability to inhibit the lung metastasis of tumor cells, suppressed the tumor cell invasion induced by 1-oleoyl-lysophosphatidic acid (LPA), and provided protection against the cerebral ischemia induced injury in a rat brain [4-6]. The ginsenoside Rg3 prevented cancer either singularly or synergistically, and promoted cisplatin efficacy through inhibiting HO-1 expression in cancer cells [7-8]. Moreover, ginsenoside Rg3 inhibited anti-inflammatory activity in a macrophage cell [9].

However, minor ginsenoside Rg3(S) is only present in red ginseng in small percentages (less than 0.003%) [10-12]. It is difficult to extract minor ginsenoside from ginseng. Up to now, many studies have aimed to produce Rg3(S) by chemical and biological methods. For example, the yield of ginsenoside Rg3(S) from red ginseng was enhanced by citric acid treatment [13]. The protopanaxadiol ginsenosides of ginseng water extraction were hydrolyzed to ginsenoside Rg3(S) under acidic conditions [11]. But the disadvantage of acid hydrolysis processing was high temperature and long hydrolysis time. Moreover, ginsenoside Rg3(S) can be produced by enzymatic and microbial treatment. For example, compared with traditional white ginseng extract, the quantity of ginsenoside Rg3(S) increased 4 times by Celluase-12T treatment [14]. The ginsenoside Rg3(S) was prepared by two glycoside hydrolases processing with high yield [15]. The Microbacterium sp. GS514 had an ability to transform ginsenoside Rb1 to Rg3 [16]. However, the cost of preparation ginsenoside by enzymatic or microbial treatment was high.

Pulsed electric field treatment was used in food sterilization, enzymatic reactions and extraction process [17-24]. Until recently, it was reported that PEF treatment can accelerate the rate of the chemical and enzymatic reactions with high efficiency and less energy consumption [21]. For example, PEF treatment accelerated the alkaline hydrolysis processing and enhanced the extraction ratio of chondroitin sulfate from fish bone [22]. Compared with untreated enzyme, the activity of pectinase increased 21.89 \(\pm\) 1.67\% under the electric field intensity 12 kV/cm [23]. Furthermore, the pulsed electric field combined with
enzyme hydrolysis transformed ginsenoside Rb1 to minor ginsenoside Rd [24].

The ginsenoside Rb1 is especially the most abundant ginsenoside (23%) in ginseng, and its structure can be easily converted to ginsenoside Rg3(S) by hydrolyzing the glucosidic-linkage at the C-20 position (Figure 1). The aim of this study was to optimize the conditions of PEF-assisted acid hydrolysis, which converted ginsenoside Rb1 to ginsenoside Rg3(S). The research data will provide a valuable method for preparing minor ginsenosides and promote the PEF technology application.

2 Materials and Methods

2.1 Materials and Instrumentation

Ginsenoside standards were purchased from Must Biotech Co. Ltd, Chengdu China. HPLC analytical grade solvents were purchased from J.T. Baker, USA. All other chemical reagents were analytical grade and deionized water was used to prepare sample solutions.

The PEF flowing system included a coaxial liquid material chamber, a high-voltage pulse generator, a pump and a fiber-optic temperature-sensing instrument [20]. The PEF processing apparatus was shown in Figure 2. The PEF treatment procedure included a short burst of high voltage to materials between two stainless steel electrodes. The gap of two stainless steel electrodes was 0.1 cm in treatment chamber. The pulse waveform was triangle wave. The PEF frequency was adjustable from 1000 to 3000 Hz, electric field strength ranged from 1 to 50 kV cm\(^{-1}\), and pulse width was 2 μs. The inlet temperature was 20℃ and outlet temperature was 23℃. A digital oscilloscope measured the input voltage and pulse waveform applied to the materials. The PEF system treated materials continuously.

2.2 Preparation of Ginsenoside Rg3(S) with PEF Treatment

Standard ginsenoside Rb1 was dissolved in hydrochloric acid concentration from 0.15 mol/L to 0.35 mol/L. The sample solutions were then pumped into the treatment container and the high voltage pulse was turned on. In order to optimize the condition, the pulse frequency and charge voltage were adjusted. The flow velocity was kept at 2 mL min\(^{-1}\). The reaction mixtures were neutralized with sodium hydroxide, extracted with n-BuOH saturated with H\(_2\)O and measured by HPLC.
2.3 HPLC Analysis of Ginsenoside Rg3(S) Content

The HPLC analysis was performed using Agilent 1100 system with a reverse phase C18 column (4.6×150, 5 μm) and a UV detector. The hydrolyzed products were identified by retention times of the ginsenoside standards. The mobile phase consisted of solvent A (water) and solvent B (acetonitrile) were used for separation. The solvent gradient condition was 20% B (0-20 min), 20% B (20-31 min), 32% B (31-40 min), 43% B (40-70 min), 100% B (70-80 min) at a flow rate of 1mL/min. The column temperature was maintained at 30 °C. The injection volume was 10 μL and the eluent was measured at a wavelength of 203 nm.

2.4 Single Factor Experiments

The PEF parameter: electric field intensity (10, 15, 20, 25, and 30 kV cm⁻¹), pulse number (6, 8, 10, 12 and 14) and acid concentration (0.15, 0.2, 0.25, 0.3 and 0.35 mol/L) were analyzed.

2.5 Experimental Design of RSM

The Box-Behnken design (BBD) was applied to optimize conditions of three variables (Xᵢ, electric field intensity; Xᵢ, pulse number; Xᵢ, acid concentration) [25, 26]. For statistical calculation, the independent variables were coded according to equation (1):

\[ x_i = \frac{X_i - X_0}{\Delta X} \]  

(1)

Where \( x_i \) represented the coded value of the variable \( X_i \), \( X_0 \) was the actual value of \( X_i \) in the centre of the domain, and \( \Delta X \) was the step change value. The three levels of independent variables were depicted in Table 1. The complete design of 17 experimental runs was employed in a random order as shown in Table 2. The polynomial second degreeed model was used by the following equation.

\[ Y = A_0 + \sum_{i=3}^{3} A_iX_i + \sum_{i=3}^{3} A_iX_i^2 + \sum_{i,j=1}^{3} A_{ij}X_iX_j \]  

(2)

where \( Y \) represented the response variable, \( A_0, A_i, A_{ij} \) and \( A_{ij} \) were regression coefficients of variables for intercept, linear, quadratic and interaction coefficients, respectively. \( X_i \) and \( X_j \) were the levels of the independent variables \( i \neq j \).

2.6 Structural Identification

Ginsenoside Rb1 (1 mM) was hydrolyzed by PEF assisted acid hydrolysis treatment. The sample solutions were treated with n-BuOH and evaporated to dryness. The crude residues were dissolved with MeOH and separated on a silica gel column using CHCl₃/MeOH/H₂O (9:3:1). The fraction was evaporated in vacuum and dissolved in pyridine-\( d_5 \). The structure was analyzed via \( ^13 \)C-NMR (a Bruker spectrometer at 500 MHz).

2.7 Statistical Analysis

All experiments were completed in triplicates. The response contour plots of experimental data were analyzed by Design Expert 7.0. The statistical significance was performed by one-way analysis of variance (ANOVA). For each analysis, the significance was considered by confidence level \( P<0.05 \).

Ethical approval: The conducted research is not related to either human or animals use.
3 Results and Discussion

3.1 Effect of Electric Field Intensity on Preparation of Ginsenoside Rg3(S)

In this study, the electric field intensity was an important variable in accelerating the hydrolysis of ginsenoside Rb1. The different electric field intensity was controlled by adjusting electric voltage. The HPLC profile of the reaction mixture was shown in Figure 3. The peak with retention time 45.88 corresponded to ginsenoside Rg3(S). The results demonstrated that the Rg3(S) content was significantly increased by PEF-assisted acid hydrolysis. It can be seen from Figure 4 that the hydrolysis of ginsenoside Rb1 enhanced gradually with the higher electric field intensity. The maximum conversion rate of Rg3(S) achieved 59.38% until the electric field intensity 20 kV cm\(^{-1}\). When the electric field intensity achieved 25 kV cm\(^{-1}\), the conversion rate of Rg3(S) reduced to 51.06%. Therefore, the electric field intensity 20 kV cm\(^{-1}\) was chosen to prepare ginsenoside Rg3(S). The conversion rate of Rg3(S) (59.38%) was higher than the organic synthesis of Rg3(S) (12.8%) [27]. While the electric field intensity exceeded 30 kV cm\(^{-1}\), the electric field electrode caused sparks and wave fluctuation of PEF. This irreversible damage will cause the conversion loss with increased electric field intensity. Therefore, the selection of optimal electric field intensity for PEF processing was shown to be important.

3.2 Effect of Pulse Number on Preparation of Ginsenoside Rg3(S)

Figure 5 showed the conversion rate of Rg3(S) by different pulse number treatment. When the pulse number increased from 6 to 12, the conversion rate of Rg3(S) increased from 47.56% to 63.94%. The results indicated that the maximum conversion rate of Rg3(S) was 63.94% on the condition of pulse number 12. Nevertheless, the conversion rate of Rg3(S) presented decreasing tendency when the pulse number increased from 12 to 14. Under different pulse number variables treatment, the large pulse frequency caused some irreversible changes occurred to molecular structure, leading to decrease the conversion rate of ginsenoside Rg3(S). Therefore, the pulse number 10 was chosen to convert ginsenoside Rb1 to ginsenoside Rg3(S).
hydrolysis processing. The conversion rate of Rg3(S) significantly accelerated during the acid concentration increased from 0.15 mol/L to 0.25 mol/L. The conversion rate of Rg3(S) decreased under the higher acid concentration (0.35 mol/L) processing. These results might be from the interaction between PEF and high acid concentration. The hydrogen ions transferred with electrical pulses by PEF processing. When the PEF-assisted acid hydrolysis reached a balance, there was no significant increase with higher acid concentration. Therefore, the optimal parameter of acid concentration was 0.25 mol/L by PEF-assisted acid hydrolysis processing.

**3.4 Optimization by Response Surface Methodology**

To optimize hydrolysis parameters and understand relationships of the variables, the research data of PEF processing was analyzed by RSM. As shown in Table 2, the conversion rate of ginsenoside Rg3(S) under different experimental conditions was presented. The maximum conversion rate of Rg3(S) achieved 68.58% on the condition of electric field intensity 20 kV/cm, pulse number 10 and acid concentration 0.25 mol/L. Compared with the transformation of ginsenoside Rb1 into Rg3 (41.4%) by microbial strain GS514, the conversion rate of Rg3(S) (68.58%) by PEF-assisted hydrolysis was significantly increased [16] (Cheng et al., 2008). The lowest conversion rate of Rg3(S) was 41.43% on the condition of electric field intensity 15 kV/cm, pulse number 10, and acid concentration 0.15 mol/L. The mathematical model that showed the relationship between the conversion rate of Rg3(S) and hydrolysis parameters was expressed by Eq.3.

\[
Y = +68.04 + 3.12X_1 + 0.42X_2 - 1.32X_3 - 2.24X_4 + 19.98X_1^2 + 14.02X_2^2 + 6.67X_3^2 + 125.1X_4^2
\]

...Eq.3

The value of variation coefficient (1.29) showed a high precision between experimental and predicted values. The coefficient of adjusted determination was 0.9934, indicating that the model was significantly accurate with predicted response. As can be seen in Table 3, the P-value of the model < 0.0001 and the F-value was 267.21.

The response surface was shown in Figure 7 and showed the relationship between conversion rate of Rg3(S) and experimental values of variables. The significant influence variables of the model were electric field intensity, electric field intensity squared, pulse number squared, and acid concentration squared. The interactive effect of electric field intensity and acid concentration, also the
The交互作用的影响脉冲数和酸浓度对诱导作用显著影响。图7c显示了电场强度（$X_1$）保持20 kVcm$^{-1}$，脉冲数（$X_2$）和酸浓度（$X_3$）对响应转化率呈二次影响。当酸浓度为0.15 mol/L时，Rg3(S)的转化率逐渐增加，然后有降低趋势。最大转化率68.58%在理想的加工参数下电场强度20 kVcm$^{-1}$，脉冲数10和酸浓度0.25 mol/L。数据接近预测值68.04%。

### 3.5 结构鉴定

图7：用PEF处理生产Rg3(S)的电场强度，脉冲宽度和酸浓度的等值线图。（a）电场强度和脉冲数（b）电场强度和酸浓度（c）脉冲数和酸浓度。

交互作用的影响脉冲数和酸浓度对诱导作用显著影响。图7c显示了电场强度（$X_1$）保持20 kVcm$^{-1}$，脉冲数（$X_2$）和酸浓度（$X_3$）对响应转化率呈二次影响。当酸浓度为0.15 mol/L时，Rg3(S)的转化率逐渐增加，然后有降低趋势。最大转化率68.58%在理想的加工参数下电场强度20 kVcm$^{-1}$，脉冲数10和酸浓度0.25 mol/L。数据接近预测值68.04%。

### Table 4: $^{13}$C-NMR chemical shifts of ginsenoside Rg3 in pyridine-d$_5$.

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<th>Rg3(S) (ppm)</th>
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<th>2' (ppm)</th>
<th>3' (ppm)</th>
<th>4' (ppm)</th>
<th>5' (ppm)</th>
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carbon positions of ginsenoside Rg3(S) [29]. The sample was confirmed as 3-O-\(\beta\)-D-glucopyranosyl-(1,2)-\(\beta\)-D-glucopyranosyl]-20(S)-propanaxadiol, the similar to standard ginsenoside Rg3(S). The \(^{13}\)C-NMR data confirmed that the metabolite of ginsenoside Rb1 which hydrolyzed under PEF treatment was ginsenoside Rg3(S). Therefore, PEF treatment-assisted acid hydrolysis can convert ginsenoside Rb1 to ginsenoside Rg3(S).

### 4 Conclusion

In the present study, PEF processing was applied to prepare ginsenoside Rg3(S) by hydrolysis of ginsenoside Rb1 under acidic conditions. The results indicated that PEF treatment accelerated the conversion rate of ginsenoside Rb1 to Rg3(S). The metabolite of ginsenoside Rb1 with PEF treatment was identified as ginsenoside Rg3(S) by \(^{13}\)C-NMR. RSM was used to obtain a desirable quadratic polynomial mathematical model for producing ginsenoside Rg3(S) under PEF treatment. The maximum conversion rate of ginsenoside Rg3(S) reached 68.58% under the optimal conditions. These findings suggested that PEF-assisted acid hydrolysis can be used to prepare ginsenoside Rg3(S) with high efficiency and provided data for preparing a bioactive compound by PEF processing.

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**Conflict of interest:** Authors state no conflict of interest.

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