The Growth Characteristics and Ginsenosides Isolation of Suspension-cultured Crown Gall of *Panax quinquefolium*

西洋参冠瘿组织悬浮培养及其人参皂苷类成分的分离

YU Rong-Min[1*], JIN Qian-Xing[1], SUN Hui[2], YE Wen-Cai[1,2] and ZHAO Yu[3*]

于荣敏[1*],金钱星[1],孙 辉[2],叶文才[1,2],赵 昱[3*]

1. 暨南大学药学院,广州 510632
2. 中国药科大学中药学院,南京 210009
3. 浙江大学药学院,杭州 310031

1. *Department of Phytochemistry and Pharmacognosy, College of Pharmacy, Jinan University, Guangzhou* 510632, *China*
2. *Department of Phytochemistry, China Pharmaceutical University, Nanjing* 210009, *China*
3. *Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou* 310031, *China*

摘 要 对西洋参冠瘿组织悬浮培养生长特征进行了考察,并对其悬浮培养物中的人参皂苷类成分进行了提取、分离和鉴定。研究得到了培养物最大生物量收获时间[18.62 g/L(dry weight)]及其中最高人参皂苷累积时间(620.4 mg/L on the 27th day)。培养基中碳源、磷、氨基氮、硝基氮的利用率分别为 91.8%,100%,81% 和 97%。利用现代分离纯化方法从培养物中分离得到了 4 种人参皂苷类成分,利用理化及谱学技术分别鉴定为假人参皂苷 F_{11}(pseudoginsenoside F_{11},Ⅰ),人参皂苷 Rd(ginsenoside Rd,Ⅱ),人参皂苷 Rb_1(ginsenoside Rb1,Ⅲ)和人参皂苷 Rb_3(ginsenoside Rb_3,Ⅳ)。

关键词 西洋参,冠瘿组织,悬浮培养,人参皂苷,分离

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Abstract The growth characteristics and ginsenosides isolation of the suspension-cultured crown gall of *Panax quinquefolium* were studied. The result showed that the maximum biomass of cultures was 18.6g/L(dry weight) and the content of ginsenosides reached its maximum level of 620.4 mg/L on the 27th day. The utilization rates of sugar, phosphorus, nitrogen in NH_4^+ and nitrogen in NO_3^- were 91.8%, 100%, 81% and 97%, respectively. Four compounds were isolated from the suspension-cultured crown gall and their structures were elucidated as pseudoginsenoside F_{11}(Ⅰ), ginsenoside Rd (Ⅱ), ginsenoside Rb_1 (Ⅲ) and ginsenoside Rb_3(Ⅳ).

Key words crown gall, ginsenosides, isolation, *Panax quinquefolium*, suspension cultures

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* Corresponding author. Yu Rong-Min: Tel: 86-20-85228205; E-mail: rongminyu99@hotmail.com;
ZHAO Yu: Tel: 86-571-87217313; Email: dryuzhao@hotmail.com

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Panax quinquefolium has been widely used for its therapeutic effects[1]. However, the field cultivation of the plant is a time-consuming and labor-intensive process: it takes over six years for its harvest, during which great care is needed since the growth is subject to several conditions, such as soil, climate, pathogens and pests. In addition, the plant has fallen short of supply for a long period due to a great demand on the market.

The major active constituents of *P. quinquefolium* are ginsenosides, a diverse group of steroidal saponins[2]. The use of plant biotechnology, such as cell culture and transgenic techniques, has been considered as a cost-effective approach to production of ginsenosides in large quantities. Crown gall, a kind of irregular tissue caused by the *Agrobacterium tumefaciens* pathogen, is capable of introducing and replicating its own DNA into the plant's nuclear genome. Compared with callus and cell culture, the crown gall cultures grow faster, produces more active constituents and are free of phytohormone appended. Therefore, crown gall cultures may be an effective method of producing useful secondary metabolites in some of valuable medicinal plants. The production of secondary metabolites by crown gall cultures has been studied in many plants[3].

Our previous research has showed that the crown gall cultures of *P. quinquefolium* could produce ginsenosides by using phytohormone-free solid MS (Murashige and Skoog's) medium[4]. In the present paper, the growth characteristics and ginsenosides isolation and structural elucidation of the suspension-cultured crown gall were carried out. Otherwise, the concentrations of residual sugar, phosphorus, nitrogen in NH_4^+ and nitrogen in NO_3^- in the media during the culture period were measured. The structures of four compounds were elucidated by physicochemical and spectral methods as peudoginsenoside F_{11} (Ⅰ), ginsenoside Rd (Ⅱ), ginsenoside Rb_1 (Ⅲ) and ginsenoside Rb_3 (Ⅳ). All of them were firstly isolated from the crown gall cultures of *P. quinquefolium*.

1 MATERIALS AND METHODS

1.1 Cell cultivation

The crown gall tumors were induced by the direct infection of sterile stems of *P. quinquefolium* L. with *A. tumefaciens* C58, and were adapted to culture in phytohormone-free MS liquid medium. The pH value of all media was adjusted to 5.70 before sterilization. The experiments were conducted using 250mL Erlenmeyer flasks containing 50mL of MS medium, inoculated with 4.0g of fresh weight biomass and incubated at 25℃ on an orbital shaker at 110r/min in the darkness. Five flasks were harvested regularly over a period of 30d for the measurement of biomass, sugar, phosphorus, nitrogen and ginsenosides.

1.2 Measurement of major nutrients in medium

The concentration of residual sugar in the medium was determined according to the method of phenol-concentrated sulfuric acid[5]. The residual nitrogen in NH_4^+ and NO_3^- in the medium was measured using phenol-hypochlorite reaction[6] and resorcinol method[7], respectively. Phosphorus of PO_4^{3-} in the medium was determined using ascorbic acid method[8].

1.3 Determination of cells weight

The cell suspensions were filtered and washed several times with sufficient amount of distilled water. The cells were weighed after being dried to a constant weight at a temperature below 50℃.

1.4 Assay of ginsenosides

For sample preparation, 1.0 g of powdered crown gall cultures was extracted with 20mL of methanol, using an ultrasonic bath for 30min after degreasing with 30mL of ether. After filtration and evaporation to dryness, the residue was dissolved in 20mL of water and extracted with 20mL of n-BuOH saturated with water. The n-BuOH extraction was evaporated to dryness, and the residue was dissolved in 10mL of methanol for analysis. The content of total ginsenosides was determined by UV-spectrophotometry[9]. The authentic ginsenosides were obtained from Institute of Drugs and Biological Products Identification of China.

1.5 Extraction and isolation of ginsenosides from the crown gall cultures

The crown gall cultures (470g, dry weight of the biomass) were soaked for 12 h at room temperature and extracted four times with 95% ethanol to afford 60 g of crude extracts. The extracts were dissolved in methanol and filtered. The methanol solution was then subjected to column chromatography on silica gel with gradient elution of chloroform/methanol. Compound I was obtained from the section in the rate of 99% :1% and fractions A and B were 65% :35% and 60% :40% respectively. Further isolation was carried out by using preparative HPLC (GILSON) on a Lichrospher® 100 Rp-18e (5μm) column (Merk) at room temperature. The eluting solvent was 75% methanol, and the

flow rate was 1.0mL/min. The effluent from the outlet of the column was monitored with a UV detector at 203 nm. By this method, compound Ⅱ was obtained from fraction A and compounds Ⅲ and Ⅳ from fraction B.

1.6 Structural elucidation

The structures of four compounds isolated from the crown gall cultures were elucidated on the basis of ^{1}H-NMR and ^{13}C-NMR spectra using Advance DMX500 and Advance 400MHz spectrometer (Bruker) with tetramethylsilane as an internal standard and ESI-MS spectra using a Finigan LCQ Advantage MAX (Thermo) spectrometer.

2 RESULTS AND DISCUSSION

As shown in Figure 1, the suspension cultures showed a lag phase from 0 to 6d, an exponential phase from 7 to 21d and a stationary phase from 22 to 27d after subculture. The biomass reached a peak of 18.62g/L(dry weight) on the 27th day, as almost 6 times as the amount of inoculum. The content of total ginsenosides fluctuated between 2.65 and 3.31 mg per 100mg dry weight. The concentration of total ginsenosides increased slowly at the beginning of cultivation and reached a maximal value of 620.4 mg·L^{-1} on the 27th day.

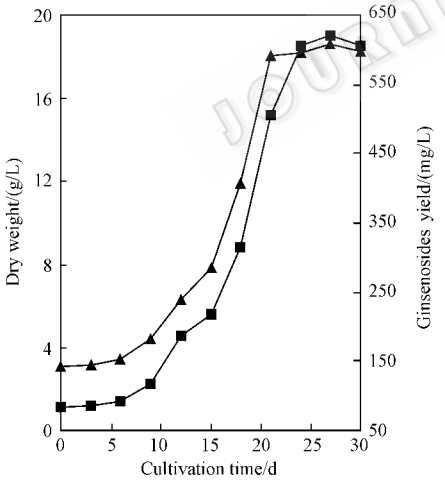

Fig. 1 Time course of growth (▲) and production of total ginsenosides(■) during cultivation of suspended crown gall of *P. quinquefolium*

To understand the growth characteristics of suspension cultures of *P. quinquefolium*, the dynamic changes in the concentrations of residual sugar, phosphorus (PO_4^{3-}), nitrogen (NH_4^+ and NO_3^-) in the media were investigated (Figure 2A and Figure 2B). The results showed that a rapid depletion of sugar in the medium (1.14g·L^{-1} per day) happened during the exponential phase. At the end of the cultivation, there still was 2.16g·L^{-1} (sugar) left in the medium. For each gram dry weight of crown gall, 1.48 g of sugar was consumed, which was about 50% lower than that in callus suspension culture[10]. Phosphorus was taken up rapidly and was depleted from the medium by the 25th day. The results of our experiments showed that the utilization of sugar and phosphorus was 91.8% and 100%, respectively. Similar results were reported in callus suspension cultures of *P. quinquefolium*[11].

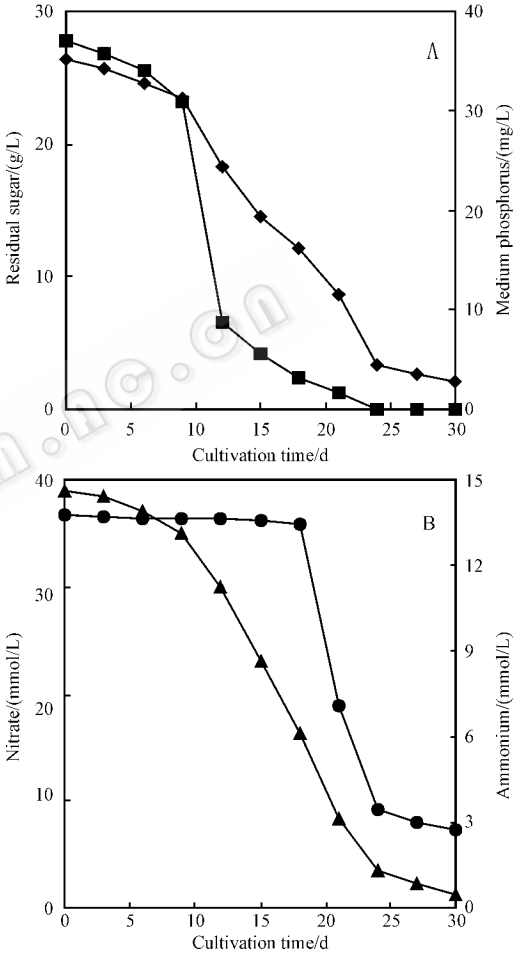

Fig. 2 Changes of phosphorus(A,■), residual sugar(A,◆), NO_3^- (B, ▲) and NH_4^+ (B,●) during cultivation of suspended crown gall of *P. quinquefolium*

As shown in Figure 2B, the depletion of nitrogen in NH_4^+ and NO_3^- was very different. The depletion of nitrate was similar to that of sugar. But the concentration of NH_4^+ changed slightly during initial 18d, and rapidly declined to 3.41mmol/L within 6 d. These results suggest that nitrate and ammonium have different effects on primary and secondary metabolism of the crown gall cultures. In other words, NH_4^+ was unnecessary to the growth of crown gall, but promoted the production of ginsenosides.

These results were similar to those in large-scale cell culture of *P. quinquefolium*[12], but opposite to those in solid culture of the same crown gall (data not shown). Further investigation on the effects of nitrogen source is under way in our laboratory. During the whole cultivation, the utilization of NH_4^+ and NO_3^- was 81% and 97%, respectively.

The information obtained from this experiment is considered to be very important for the large scale production of ginsenosides by plant biotechnology .

The structural identification of compounds was carried out by using ESI-MS, ^{1}H-NMR and ^{13}C-NMR spectra.

Compound I was colorless powder, m.p. 205 ~ 207℃. ESI-MS m/z: 800.3 $[M-1]^-$ ($C_{42}H_{71}O_{14}$, calcd. 800.0), 653.4 $[M-C_6H_{12}O_4]^-$, 491.2 $[M-C_6H_{11}O_4-C_6H_{12}O_5]^-$. ^{13}C-NMR ($C_5D_5N$) δppm: 78.43 (CH, C-3), 74.14 (CH, C-6), 86.59 (C, C-20), 26.84 (CH_3, C-21), 32.64 (CH_2, C-22), 28.66 (CH_2, C-23), 85.50 (CH, C-24), 70.19 (C, C-25), 27.06 (CH_3, C-26), 27.63 (CH_3, C-27), 32.32 (CH_3, C-28), 16.77 (CH_3, C-29), 101.68 (CH, 6-β-*D*-glc-C1), 101.86 (CH, 6-β-*D*-glc-α-*L*-rha-C1), 18.64 (CH_3, 6-β-*D*-glc-α-*L*-rha-C6). Those data were consistent with literature[13]. Therefore, compound I was identified as pseudoginsenoside F11.

Compound Ⅱ was colorless powder, m.p. 206 ~ 209℃. ESI-MS m/z: 946.3 $[M-1]^-$ ($C_{48}H_{81}O_{18}$, calcd. 946.1), 783.3 $[M-C_6H_{12}O_5]^-$, 621.3, 459.4. ^{13}C-NMR (C_5D_5N) δppm: 88.91 (CH, C-3), 18.39 (CH_2, C-6), 83.42 (C, C-20), 22.35 (CH_3, C-21), 36.09 (CH_2, C-22), 23.19 (CH_2, C-23), 125.85 (CH, C-24), 130.81 (C, C-25), 25.73 (CH_3, C-26), 16.57 (CH_3, C-27), 28.06 (CH_3, C-28), 16.25 (CH_3, C-29); 105.09 (CH, 3-β-*D*-glc-C1), 106.03 (CH, 3-β-*D*-glc-β-*D*-glc-C1), 98.25 (CH, 20-β-*D*-glc-C1). Those data were consistent with literature[14]. Therefore, compound Ⅱ was identified as ginsenoside Rd.

Compound Ⅲ was colorless powder, m.p. 197 ~ 198℃. ESI-MS m/z: 1108.4 $[M-1]^-$ ($C_{54}H_{91}O_{23}$, calcd. 1108.3), 945.7 $[M-C_6H_{12}O_5]^-$, 783.3, 621.2, 459.3. ^{13}C- NMR (C_5D_5N) δppm: 88.94 (CH, C-3), 18.32 (CH_2, C-6), 83.40 (C, C-20), 22.37 (CH_3, C-21), 36.16 (CH_2, C-22), 23.18 (CH_2, C-23), 125.87 (CH, C-24), 130.96 (C, C-25), 25.78 (CH_3, C-26), 16.57 (CH_3, C-27), 28.06 (CH_3, C-28), 16.24 (CH_3, C-29); 105.08 (CH, 3-β-*D*-glc-C1), 106.02 (CH, 3-β-*D*-glc-β-*D*-glc-C1), 98.05 (CH, 20-β-*D*-glc-C1), 105.35 (CH, 20-β-*D*-glc-β-*D*-glc-C1). Those data were consistent with literature [14] (1993). Therefore, compound Ⅲ was identified as ginsenoside Rb_1.

Compound Ⅳ was colorless powder, m.p. 197-199℃. ESI-MS m/z: 1078.1 $[M-1]^-$ ($C_{53}H_{89}O_{22}$, calcd. 1078.3), 945.6 $[M-C_5H_8O_4]^-$, 915.8 $[M-C_6H_{12}O_5]^-$, 783.3, 621.2, 459.4. ^{13}C-NMR (C_5D_5N) δppm: 88.97 (CH, C-3), 18.39 (CH_2, C-6), 83.37 (C, C-20), 22.24 (CH_3, C-21), 36.13 (CH_2, C-22), 23.12 (CH_2, C-23), 125.91 (CH, C-24), 130.91 (C, C-25), 25.78 (CH_3, C-26), 16.57 (CH_3, C-27), 28.06 (CH_3, C-28), 16.23 (CH_3, C-29), 105.08 (CH, 3-β-*D*-glc-C1), 105.96 (CH, 3-β-*D*-glc-β-*D*-glc-C1), 98.07 (CH, 20-β-*D*-glc-C1), 105.81 (CH, 20-β-*D*-glc-β-*D*-xyl-C1), 66.87 (CH_2, 20-β-*D*-glc-β-*D*-xyl-C5). Those data were consistent with literature [14] (1993) and therefore compound Ⅳ was identified as ginsenoside Rb_3.

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