Orthogonal Design Optimization of Conditions for Regulating Secondary Metabolite Content in *Gentiana macrophylla* (Post-Print)

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Abstract

To optimize conditions for inhibiting content variations of secondary metabolites in Gentiana macrophylla, this study employed a 3-factor, 4-level orthogonal experimental design, comprising 16 treatments, to investigate the effects of lovastatin (MVA pathway inhibitor), fosmidomycin (MEP pathway inhibitor), and sampling time on the contents of four major secoiridoid compounds in Gentiana macrophylla: loganic acid, sweroside, swertiamarin, and gentiopicroside. The results demonstrated that: (1) The content variation of the four secoiridoid compounds was most significantly influenced by sampling time, followed by fosmidomycin concentration, and then lovastatin concentration. (2) Following treatment with the optimal inhibition conditions, the contents of loganic acid, swertiamarin, gentiopicroside, and sweroside decreased by 69%, 36%, 33%, and 4%, respectively. Based on the inhibition conditions optimized through orthogonal methodology, all four compounds could be effectively suppressed. In conclusion, the optimal inhibition conditions for regulating secondary metabolite content variation in Gentiana macrophylla were determined as fosmidomycin 400 $\mathrm{mol \cdot L^{-1}}$, lovastatin 50 $\mathrm{mol \cdot L^{-1}}$, and a sampling time of 6 days. These conditions establish a foundation for further investigating the regulatory mechanisms of MEP and MVA pathways in the metabolic synthesis of secoiridoid compounds.

Full Text

Optimization of Conditions for Regulating Secondary Metabolite Content in *Gentiana macrophylla* Using Orthogonal Experimental Design

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Abstract

To identify optimal conditions for inhibiting changes in secondary metabolite content in *Gentiana macrophylla*, this study employed a three-factor, four-level orthogonal experimental design with 16 treatment groups to investigate the effects of lovastatin (a mevalonate [MVA] pathway inhibitor), fosmidomycin (a methylerythritol phosphate [MEP] pathway inhibitor), and sampling time on the contents of four major secoiridoid glycosides: loganic acid, sweroside, swertiamarin, and gentiopicroside. The results demonstrated that: (1) sampling time exerted the greatest influence on the content variation of the four secoiridoid glycosides, followed by fosmidomycin concentration and then lovastatin concentration; (2) treatment under optimal inhibitory conditions reduced the contents of loganic acid, swertiamarin, gentiopicroside, and sweroside by 69%, 36%, 33%, and 4%, respectively. The orthogonal optimization identified effective inhibitory conditions for all four compounds. In conclusion, the optimal conditions for regulating secondary metabolite content in *G. macrophylla* were determined to be fosmidomycin at 400 $\text{mol}\cdot\text{L}^{-1}$, lovastatin at 50 $\text{mol}\cdot\text{L}^{-1}$, and a sampling time of 6 days. These conditions provide a foundation for further investigation into the regulatory mechanisms of MEP and MVA pathways in secoiridoid glycoside biosynthesis.

Keywords: orthogonal test; MVA pathway; MEP pathway; lovastatin; fosmidomycin; secoiridoid glycosides

Introduction

Gentiana macrophylla, a perennial herb belonging to the family Gentianaceae, is a traditional Chinese medicinal plant with a bitter and pungent taste and neutral properties, acting on the stomach, liver, and gallbladder meridians. The dried roots are used medicinally to dispel wind-dampness, clear damp-heat, relieve arthralgia, and reduce deficiency fever, with clinical applications in rheumatic

arthralgia, muscle cramps, jaundice, and infantile malnutrition fever. The primary bioactive constituents are iridoid compounds, including gentiopicroside, swertiamarin, sweroside, and loganic acid. Secoiridoid glycosides exhibit diverse biological activities such as anti-tumor and anti-inflammatory effects, with gentiopicroside and swertiamarin demonstrating notable anti-inflammatory, analgesic, and hepatoprotective properties.

The biosynthesis of terpenoid compounds primarily occurs through two pathways: the mevalonate (MVA) pathway in the cytoplasm and the methylerythritol phosphate (MEP) pathway in plastids. Based on studies of monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* and transcriptome data mining in *G. macrophylla*, the proposed biosynthetic pathway for gentiopicroside involves IPP synthesis via both MVA and MEP pathways, isomerization to DMAPP, condensation to GPP, and subsequent enzymatic conversion to loganic acid, loganin, secologanin, and finally gentiopicroside, with derivatives including swertiamarin and sweroside, though intermediate steps remain unclear.

The roles of MVA and MEP pathways in metabolite synthesis have attracted considerable attention. Fosmidomycin and lovastatin are specific inhibitors of DXR (MEP pathway) and HMGR (MVA pathway), respectively, and are widely used to investigate secondary metabolite biosynthesis. Studies in *Andrographis paniculata* suspension cells revealed that both pathways contribute significantly to andrographolide production, with DXR and HMGR contributing 76.6% and 77.6%, respectively. Research on *Panax ginseng* hairy roots demonstrated that both inhibitors suppress ginsenoside biosynthesis, suggesting the MVA pathway plays a dominant role. In *Nothapodytes nimmoniana*, fosmidomycin and lovastatin significantly reduced DXR and HMGR transcript levels, indicating the MEP pathway as the primary source for camptothecin precursors. Studies in *Salvia miltiorrhiza* hairy roots showed the MVA pathway is crucial for cell growth, while the MEP pathway primarily drives secondary metabolite accumulation. However, the mechanisms of MVA and MEP pathways in *G. macrophylla* secondary metabolite biosynthesis remain unexplored.

This study employed a three-factor, four-level orthogonal experimental design based on inhibitor mechanisms, treating one-month-old *G. macrophylla* seedlings with varying concentrations of fosmidomycin and lovastatin at different sampling times. By evaluating changes in secondary metabolite contents, we aimed to identify optimal conditions for inhibiting loganic acid, sweroside, swertiamarin, and gentiopicroside accumulation, thereby establishing a foundation for investigating MEP and MVA pathway regulatory mechanisms in secoiridoid glycoside biosynthesis.

[Figure 1: see original paper]

Materials and Methods

1.1 Materials and Main Instruments

Gentiana macrophylla seeds were purchased from Zhengning County, Qingyang, Gansu Province. Seeds were sown in a greenhouse under 16 h light/8 h dark conditions. One-month-old seedlings (with two pairs of leaves, approximately 2 cm height) were used for experiments. Instruments included an LC-20XR HPLC system (Shimadzu, Japan), Welch chromatographic column (4.6 mm × 250 mm, 5 m), Sartorius precision electronic balance, Xiaomei ultrasonic cleaner, and Shanghai Ailang OBS-2100 oil bath.

1.2 Reagents

Loganic acid (batch No. K17S11B124207, purity $ $98%), swertiamarin (batch No. Y25J1G1429, purity $ $98%), gentiopicroside (batch No. Y29A11Q112202, purity $ $98%), sweroside (batch No. P25O10F101344, purity $ $98%), and lovastatin (batch No. Y23D6C7375, purity $ $98%) were purchased from Shanghai Yuanye Biotechnology. Fosmidomycin (batch No. lX040552) was from CHEMEGEn. Methanol, acetonitrile, and phosphoric acid were obtained from Chengdu Kelong Chemical, Shanghai Honeywell, and Tianjin Kemiou Chemical Reagent, respectively.

1.3 Solution Preparation

Lovastatin stock solution (500 mol·L^{-1}): 0.0104 g lovastatin was dissolved in 2 mL ethanol in a 50 mL volumetric flask, mixed with 20 L of 0.6 mol · L^{-1} NaOH, left to stand for 30 min, neutralized with 1 mol · L^{-1} HCl to pH 7, and diluted to 50 mL with purified water. Stored at 4 °C.

Fosmidomycin stock solution (546 mol·L^{-1}): 0.0100 g fosmidomycin was dissolved in purified water in a 50 mL volumetric flask, sonicated, and stored at 4 °C.

Mixed standard solution: 0.95 mg loganic acid, 1.26 mg swertiamarin, 2.52 mg gentiopicroside, and 1.24 mg sweroside were dissolved in methanol in a 1 mL volumetric flask to prepare stock solutions with concentrations of 0.95, 1.26, 2.52, and 1.24 mg · mL^{-1}, respectively. Stored at 4 °C.

1.4 Orthogonal Experimental Design and Sample Treatment

Based on previous studies, a three-factor, four-level orthogonal test L16(4^3) was designed according to . Sixteen groups of 30 seedlings each were treated by spraying. Treated seedlings were maintained at 26 °C under 16 h light daily. At designated times, seedlings were washed, blotted dry, and placed in rotary evaporation flasks. After vacuum drying with methanol for 1.5 h, dried seedlings were weighed and extracted three times with 1 mL methanol via ultrasonication (1.5 h each). The filtered green extract was concentrated under vacuum to obtain

gray-green solids, which were dissolved in 1 mL methanol, filtered through a 0.22 m membrane, and used as sample solution.

1.5 HPLC Conditions and Standard Curve

The mobile phase consisted of 0.1% phosphoric acid water and acetonitrile with gradient elution: 0–10 min, 12% acetonitrile; 10–13 min, 10% acetonitrile; 13–30 min, 12% acetonitrile. Flow rate: 0.8 $mL \cdot min^{-1}$; detection wavelength: 254 nm; injection volume: 10 L; column temperature: 30 °C.

Standard solutions were serially diluted to establish calibration curves. Loganic acid concentrations: 0.95, 0.475, 0.2375, 0.11875, 0.059375, 0.0296875, 0.014844375, 0.007421875, 0.003710938, and 0.001855469 $mg \cdot mL^{-1}$. Swertiamarin: 1.26, 0.63, 0.315, 0.1575, 0.07875, 0.039375, and 0.0196875 $mg \cdot mL^{-1}$. Gentiopicroside: 2.52, 1.26, 0.63, 0.315, 0.1575, 0.07875, 0.039375, and 0.0196875 $mg \cdot mL^{-1}$. Sweroside: 1.24, 0.62, 0.31, 0.155, 0.0775, 0.19375, 0.0096875, 0.00484375, and 0.002421875 $mg \cdot mL^{-1}$.

Linear regression equations and ranges: - Loganic acid: Y = 4,571,241.60X - 11,205.29 (R^2 = 0.999), 0.002–0.475 $g \cdot mL^{-1}$ - Swertiamarin: Y = 6,499,223.38X - 24,589.43 (R^2 = 0.999), 0.003–0.630 $g \cdot mL^{-1}$ - Gentiopicroside: Y = 14,344,350.83X - 442,328.21 (R^2 = 0.999), 0.005–2.520 $g \cdot mL^{-1}$ - Sweroside: Y = 6,047,666.40X - 40,600.10 (R^2 = 0.999), 0.002–1.240 $g \cdot mL^{-1}$

1.6 Statistical Analysis

Data were processed using Excel 2019. Range analysis was performed using Minitab 18 (Minitab Inc.). ANOVA and correlation analysis were conducted using SPSS Statistics 26 (IBM Corp.). Graphs were generated using GraphPad Prism 5 (GraphPad Software). Inhibition rate was calculated as: (treatment group - blank group)/blank group × 100%.

Results

2.1 Analysis of Secondary Metabolite Content

Orthogonal experimental treatments revealed similar trends in content variation for loganic acid, swertiamarin, gentiopicroside, and sweroside across T2–T16 groups (excluding T1 blank control). Compared with T1, loganic acid content decreased under all treatment conditions, with T9, T10, and T14 showing the most significant reductions of 83%, 81%, and 79%, respectively (highly significant difference). Both single and combined inhibitor treatments suppressed loganic acid synthesis, with fosmidomycin demonstrating greater inhibitory efficacy than lovastatin at equivalent concentrations.

In contrast, while most treatments reduced swertiamarin, gentiopicroside, and sweroside contents compared with T1, T4 showed the most pronounced decreases (52%, 50%, and 54%, respectively). Notably, T2, T5, and T12 treat-

ments unexpectedly increased the contents of these three metabolites, suggesting differential responsiveness to inhibitors among metabolites.

[Figure 2: see original paper]

2.2 Multivariate Analysis of Variance

ANOVA revealed that neither fosmidomycin nor lovastatin concentrations significantly affected the accumulation of loganic acid, gentiopicroside, swertiamarin, or sweroside. Only sampling time showed a significant effect on loganic acid content, while no significant effects were observed on the other three metabolites ().

2.3 Correlation Analysis

Correlation analysis demonstrated significant negative correlations between sampling time and metabolite contents, with particularly strong negative correlations for loganic acid, gentiopicroside, and sweroside ($P < 0.01$). Loganic acid content showed significant positive correlations with swertiamarin, gentiopicroside, and sweroside. Swertiamarin exhibited highly significant positive correlations with gentiopicroside and sweroside, while gentiopicroside and sweroside were also highly positively correlated ().

These results indicate that fosmidomycin and lovastatin significantly affect the contents of all four secoiridoid glycosides, validating their utility as effective inhibitors for studying biosynthetic regulation. The negative correlation between sampling time and metabolite content suggests progressive reduction over time, though the maximum effective duration requires further investigation. The positive correlations among the four compounds imply similar regulatory patterns in response to inhibitors.

2.4 Range Analysis

Range analysis identified the order of factor influence on secoiridoid content as: sampling time > fosmidomycin concentration > lovastatin concentration. The optimal inhibitory conditions for each metabolite were: - Loganic acid: fosmidomycin 400 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, 6 days - Swertiamarin: fosmidomycin 0 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, 6 days - Gentiopicroside: fosmidomycin 400 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, 6 days - Sweroside: fosmidomycin 0 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, 6 days

Considering all four metabolites comprehensively, the optimal inhibitory condition was A(4)B(2)C(4): fosmidomycin 400 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, and treatment duration of 6 days ([Figure 3: see original paper]).

[Figure 3: see original paper]

2.5 Verification Experiment

The optimized inhibitory conditions were applied to one-month-old *G. macrophylla* seedlings in triplicate to validate reliability. Results confirmed that all four secoiridoid glycosides decreased compared with the blank control, with loganic acid, swertiamarin, gentiopicroside, and sweroside reduced by 69%, 36%, 33%, and 4%, respectively ().

Discussion and Conclusion

Gentiana macrophylla contains secoiridoid glycosides as its primary medicinal constituents, synthesized via MVA and MEP pathways through GPP formation and subsequent enzymatic reactions to produce loganic acid, gentiopicroside, and related compounds. This orthogonal study identified optimal conditions for inhibiting four key metabolites.

Range analysis revealed that fosmidomycin exhibited stronger inhibitory effects than lovastatin, suggesting the MEP pathway plays a dominant role in secoiridoid biosynthesis in *G. macrophylla*, consistent with transcriptome-based predictions for *Gentiana rigescens*. The more pronounced reduction in loganic acid compared with other metabolites suggests its upstream position in the biosynthetic pathway. The significant positive correlations among the four metabolites indicate a "cascade convergence" regulatory pattern in response to inhibitors. The negative correlation between treatment duration and metabolite content demonstrates time-dependent reduction, though the maximum effective duration warrants further investigation.

The orthogonal optimization established optimal inhibitory conditions of fosmidomycin 400 $\text{mol} \cdot \text{L}^{-1}$, lovastatin 50 $\text{mol} \cdot \text{L}^{-1}$, and 6-day treatment duration, reducing loganic acid, swertiamarin, gentiopicroside, and sweroside contents by 69%, 36%, 33%, and 4%, respectively. These conditions provide an optimal experimental system for investigating MVA and MEP pathway regulatory mechanisms in secoiridoid glycoside biosynthesis in *G. macrophylla*.

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Note: Figure translations are in progress. See original paper for figures.