Purification of Phenylpropanoids from the Scaly Bulbs of Lilium Longiflorum by CPC and Determination of Their DPP-IV Inhibitory Potentials

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ABSTRACT: The scaly bulbs of Lilium longiflorum (Liliaceae) are used as a food ingredient and a traditional medicine in East Asia. A preliminary study revealed that treatment with 100 μg/mL of the ethyl acetate fraction of this plant material inhibited dipeptidyl peptidase IV (DPP-IV) to 58.99%. Phytochemical studies were conducted to identify the active ingredient, and five compounds, namely, 1 (2.9 mg, 75.8% purity at 320 nm), 2 (12.2 mg, 97.9% purity at 320 nm), 3 (3.1 mg, 66.5% purity at 320 nm), 4 (6.8 mg, 96.9% purity at 320 nm), and 5 (6.2 mg, 90.2% purity at 320 nm) were purified from 200 mg of the ethyl acetate fraction of L. longiflorum via centrifugal partition chromatography (CPC) with a two-phase solvent system composed of chloroform/methanol/isopropanol/water (5:2:2:4, v/v/v/v) in an ascending mode. Their structures were identified as 1-O-p-coumaroyl-2-O-β-glucopyranosylglycerol (regaloside D, 1), 3,6′-O-diferuloylsucrose (2), 1-O-p-coumaroyl-2-O-β-glucopyranosyl-3-O-acetylglycerol (regaloside B, 3), 1-O-p-coumaroylglycerol (4), and 4-O-acetyl-3,6′-O-diferuloylsucrose (5), respectively, by 1H and 13C NMR and MS analysis. Compounds 2 and 5 exhibited DPP-IV inhibitory activities with IC50 values of 46.19 and 63.26 μM, respectively. Compounds 1, 3, and 4 did not show activities, indicating that biphenylpropanoids linked via the sugar moiety are more effective than phenylpropanoids with glycerol or glyceryl glucoside. This is the first report of simultaneous separation of five phenylpropanoids from L. longiflorum by CPC and evaluation of their DPP-IV inhibitory activities.

1. INTRODUCTION

Lily (Lilium species) is a well-known horticultural crop with various floral colors and patterns. Its scaly bulbs, leaves, and petals have been used as a food and a traditional medicine for many centuries in East Asia. Especially, the scaly bulbs of Lilium species have been used to treat coughs, sore throats, and hemoptysis because of dry lung or lung heat, and insomnia associated with hallucinations, restlessness, and irritability.1 Previous phytochemical studies investigating Lilium species reported the role of flavonoids,3–5 steroidal saponins,6–7 and phenypropanoids,8–11 as major constituents. These compounds exhibit diverse biological activities including antiviral,3 antitumor,5,6 antidiabetic,5 anti-inflammatory,10 and antioxidant activities.11 Steroidal saponins isolated from the bulbs of Lilium longiflorum showed inhibitory activity against 12-O-tetradecanoylphorbol-13-acetate-stimulated 32P-incorporation into phospholipids of HeLa cells7 and promoted scratch wound closure by stimulating fibroblast activation and migration via selective upregulation of TGF-β type I receptors.5 Phenylpropanoid glycerol glucosides isolated from the bulbs of L. longiflorum attenuated glucose production in a H4IIE rat hepatoma cell line.8

Natural products include secondary metabolites with a wide range of polarities and multiple stereochemistry. Therefore, the separation and purification of these substances is tedious and laborious entailing repeated chromatographic steps. In our previous trial, on the isolation of bioactive principal compounds from the ethyl acetate fraction of L. longiflorum using open column chromatography and preparative high-performance liquid chromatography (HPLC), the substances with the expected purities were not separated because of the interaction with the stationary phase. We observed sample loss and column deterioration with fractions adhering to the solid support.12 Chemical decomposition also occurred while the fractions containing high concentrations of the pure substance...
passed through the solid stationary phase. Therefore, centrifugal partition chromatography (CPC) was used to exclude the interaction between the solid stationary phase and substances to purify the bioactive compounds from *L. longiflorum*. CPC is a solid-free separation technique that uses immiscible two phases of the solvent.13 Because CPC uses only the liquid phase, there is no irreversible sample absorption, sample loss, or sample denaturation.13 CPC can be used to recover the total injected sample and facilitate high sample loading capacity.13 In addition, CPC can be easily converted into a normal or a reverse phase by altering the solvent flow direction and the mobile or stationary phases. Based on the extensive studies investigating CPC development and application,14,15 it is obvious that CPC is an efficient technique for preparative separation and purification of natural products.

Type 2 diabetes mellitus is characterized not only by insulin resistance but also by impaired β-cell function.16,17 Regarding β-cell function, a marked decrease in the incretin effect has been reported in patients with type 2 diabetes.17 Incretin effect refers to the intense stimulation of insulin secretion when sugar was orally administered rather than intravenously injected.17 This effect depends on blood glucose and is reduced in diabetic patients compared with healthy individuals.17 The discovery of incretin hormones, glucose-dependent insulinotropic peptide, and glucagon-like peptide 1 (GLP-1) and their physiological degradation enzyme, dipeptidyl peptidase IV (DPP-IV), has led to the development of GLP-1 agonists and DPP-IV inhibitors, as the new targets for the treatment of type 2 diabetes mellitus.17 DPP-IV inhibitors share the functions of the GLP-1 agonist but can also be administered orally.18 Given the recent studies of DPP-IV inhibitors, synthetic compounds have been used in current pharmacotherapy.19 As well, many active compounds derived from natural sources exhibited DPP-IV inhibitory effects, and represent promising drug candidates.20−23

In the present study, we used CPC to purify major compounds from the ethyl acetate fractions of scaly bulbs of *L. longiflorum* (Figure 1). The DPP-IV inhibitory activities of these compounds were evaluated in vitro.

2. RESULTS AND DISCUSSION

2.1. Preparation of Crude Phenylpropanoids. To identify new DPP-IV inhibitors from herbal medicines and foods, the methanol extract of the scaly bulbs of *L. longiflorum* and its solvent fractions, the *n*-hexane, chloroform, ethyl acetate, and *n*-butanol fractions were tested using a DPP-IV inhibitor screening assay and they (each 100 μg/mL) showed an DPP-IV inhibition of 34.44, 29.07, 57.03, 58.99, and 31.59%, respectively. Because the ethyl acetate fraction exhibited the strongest DPP-IV inhibitory activity, it was subjected to CPC for further separation.

2.2. Selection of the Two-Phase Solvent System. The HPLC profile of the ethyl acetate-soluble fractions of the scaly bulbs of *L. longiflorum* at 320 nm.

![Figure 2. HPLC profile of ethyl acetate-soluble fractions of the scaly bulbs of *L. longiflorum* at 320 nm.](https://dx.doi.org/10.1021/acsomega.9b03649)
unsolved peaks, which prevented the calculation of the K value (data not shown). Thus, we selected chloroform/methanol/isopropanol/water (CHCl3/MeOH/IPA/water), as one of the appropriate solvent systems for phenylpropanoid separation, as reported in the review of strategies for solvent system selection in countercurrent separation.25 Therefore, we separated the compounds, as demonstrated in the HPLC analysis. The HPLC chromatograms of compounds 1–5 were shown below. Compounds 1–5 were obtained by CHCl3/MeOH/IPA/water (3:2:2:3, v/v/v/v), compound 1 eluted rapidly. Because the K values of compounds 2 and 3 obtained by CHCl3/MeOH/IPA/water (3:1:2:4, v/v/v/v) and CHCl3/MeOH/IPA/water (4:3:3:4, v/v/v/v) were very similar and their α values were close to 1, it was predicted that 2 and 3 could not be separated well. Finally, the optimum solvent system comprising CHCl3/MeOH/IPA/water (3:2:2:4, v/v/v/v) was determined to separate the target compounds.

2.3. CPC Separation and HPLC–DAD–ESI/MS Analysis of CPC Peak Fraction. The ethyl acetate fraction (200 mg) of the scaly bulbs of L. longiflorum was dissolved in a 1:1 (v/v) mixture (1 mL each) of the two-phase solvent system (CHCl3/MeOH/IPA/water = 5:2:2:4, v/v/v/v). The ascending mode was applied using the lower organic phase as the stationary phase and the upper aqueous phase as the mobile phase. The stationary phase retention of this system was 68%. The peak fractions (I–V) were separated by CPC, and the separation time was approximately 75 min (Figure 3). Individual peaks were collected and analyzed. Compound 1 (2.9 mg) corresponded to the peak fraction I; compound 2 (6.8 mg) corresponded to the peak fraction II; compound 3 (3.1 mg) to the peak fraction III; compound 2 (12.2 mg) to the peak fraction IV; and compound 5 (6.2 mg) to the peak fraction V. Compounds 1–5 were obtained with purities of 75.8, 97.9, 66.5, 96.9, and 90.2% at 320 nm, based on HPLC–DAD analysis. The HPLC chromatograms of compounds 1–5 are shown in Figure 4.

2.4. Structural Analysis. The chemical structures of the target compounds were identified by 1H and 13C NMR spectroscopy and ESI-MS. The spectroscopic data of each compound were shown below. Compounds 1–5 were identified as 1-O-p-coumaroyl-2-O-β-glucopyranosyl (regaloside D),26 3′,6′-O-diferuloylsucrose,27 1-O-p-coumaroyl-2-O-β-glucopyranosyl-3-O-acetylglycerol (regaloside B),26,28 1-O-p-coumaroylglycerol,8,12 and 4-O-acetyl-3′,6′-O-diferuloylsucrose, respectively, based on comparison with the data reported previously. Compounds 1–5 have been reported from L. longiflorum.6

Table 1. Partition coefficient (K) and separation factor (α) of compounds 1–5 in different solvent systems

| solvent system (CHCl3/MeOH/IPA/water) | K values | α values |
|---------------------------------------|----------|----------|
|                                       | 1 2 3 4 5 | α12 α23 α34 α45 |
| 3:2:2:3                               | 0.07 0.32 0.41 1.06 2.44 | 4.57 1.28 2.58 2.30 |
| 3:1:2:4                               | 0.20 1.02 1.07 2.88 7.14 | 5.10 1.05 2.69 2.48 |
| 4:3:3:4                               | 0.37 1.00 1.04 1.92 3.85 | 2.70 1.04 1.85 2.01 |
| 5:2:2:4                               | 0.10 0.49 0.61 1.58 4.35 | 4.90 1.25 2.59 2.75 |

Figure 3. CPC separation of the ethyl acetate-soluble fractions of the scaly bulbs of L. longiflorum using CHCl3/MeOH/IPA/W (5:2:2:4, v/v/v/v) in an ascending mode. (Fraction I: compound 1; fraction II: compound 2; fraction III: compound 3; fraction IV: compound 4; and fraction V: compound 5). The extrusion was performed after 75 min.
Figure 4. HPLC chromatograms of CPC peak fractions I (a), II (b), III (c), IV (d), and V (e) at 320 nm.
Figure 5. Effects of compounds 2 and 5 on DPP-IV activity. Values are presented as the mean ± SD of three independent experiments.

3. CONCLUSIONS

This is the first report that demonstrated the isolation of five phenylpropanoids including 1-O-p-coumaroyl-2-O-β-D-glucopyranosylglycerol (regaloside D, 1), 3′,6′-O-diferuloylsucrose (2), 1-O-p-coumaroyl-2-O-β-D-glucopyranosyl-3-O-acetylglycerol (regaloside B, 3), 1-O-p-coumaroylglycerol (4), and 4-O-acyethyl-3′,6′-O-diferuloylsucrose (5) from the scaly bulbs of L. longiflorum using CPC. In addition, the DPP-IV inhibitory activity of the extracts and components of the scaly bulbs of L. longiflorum was evaluated in vitro. A two-phase solvent system comprising CHCl₃/MeOH/IPA/water (5:2:2:4, v/v/v/v) in ascending mode was applied for the one-step purification of these compounds with high purity. Compounds 2 and 5 were identified as active components of the scaly bulbs of L. longiflorum, inhibiting the DPP-IV enzyme with IC₅₀ values of 46.19 and 63.26 μM, respectively. Therefore, this study provides a reference for the large-scale isolation of phenyl-
propanoids from the scaly bulbs of *L. longiflorum*. Compounds 2 and 5 represent DPP-IV inhibitors.

4. MATERIALS AND METHODS

4.1. General. CPC was performed using the Gilson CPC 250 system (Gilson Inc., Middleton, WI, USA) equipped with a 250 mL rotor, a 10 mL sample loop, a Shimadzu LC-8A pump (Shimadzu, Kyoto, Japan), and a Shimadzu SPD-10A UV/vis detector. Analytical HPLC—DAD—ESIMS was carried out on an Agilent 1200 series system and an Agilent 6120 quadrupole MS system (Agilent Technologies Co., Santa Clara, CA, USA) equipped with a YMC-Triart C18 column (5 μm, 250 mm × 4.6 mm; YMC Co.) and ChemStation software. The NMR experiments were performed with a JNM-ECA 500 MHz NMR instrument (JEOL Ltd., Tokyo, Japan; tetramethylsilane as an internal standard) and a 600 MHz Varian NMR spectrometer (VNS-600, Palo Alto, CA, USA). All other chemicals and solvents used in this study were of analytical grade.

4.2. Plant Material. The scaly bulbs of the *L. longiflorum* hybrid (cv. White Triumph) were harvested at the experimental farm, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeongeup-si, Jeollabuk-do, Korea) and collected in August 2017. This plant was identified by Sang Hoon Kim, one of the co-authors of this study. The scaly bulbs were dried using an air drying machine at a temperature of 40 °C for 72 h. The voucher specimens (no. RB012) have been deposited at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute.

4.3. Preparation of Crude Sample. The scaly bulbs of *L. longiflorum* were hot-air dried and weighed to 12 kg in dry weight. The dried sample was extracted with methanol (3 × 15 L) overnight at room temperature. The solvent was evaporated in vacuo to yield a methanol extract (447 g), which was suspended in distilled water (1 L) and partitioned with n-hexane (3 × 2 L), chloroform (3 × 2 L), ethyl acetate (5 × 3 L), and n-butanol (5 × 3 L), sequentially. Each solvent fraction was evaporated in vacuo to yield fractions of n-hexane (53.6 g), chloroform (5.8 g), ethyl acetate (18.0 g), and n-butanol (141.6 g).

4.4. Evaluation of Partition Coefficient (K) and Separation Factor (α). The two-phase solvent system was selected according to the partition coefficient (K) of each target compound in the crude sample. The K value was defined as the peak area of the target compound in the stationary phase divided by the peak area of the target compound in the mobile phase. The K value was determined using HPLC as follows: 1 mg of ethyl acetate fraction was added to a 1.5 mL tube, followed by the addition of 500 μL of each phase of the pre-equilibrated two-phase solvent system. The tube was vigorously shaken for 1 min. After the two-phase samples were completely equilibrated, 200 μL of each phase was collected and analyzed by HPLC. The K values of target compounds were obtained using the following equation: 

\[ K = \frac{A_{\text{upper phase}}}{A_{\text{lower phase}}} \]  

where \( A \) represents the area of the two phases and was obtained by dividing the K values of the two compounds \( (\alpha = K_1 / K_2) \). The K values were calculated via a dose–response analysis using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

4.5. CPC Separation. CPC separation was conducted with a biphasic solvent system composed of CHCl₃/MeOH/IPA/water with a volume ratio 5:2:2:4. Each solvent was added to a separation funnel and shaken vigorously. After equilibration, the upper and lower phases were separated and degassed by sonication for 30 min before use. To prepare the injection, the 200 mg crude sample was dissolved in 1 mL of each phase. Depending on the phase density, CPC has two operation modes. In this study, the lower phase including chloroform was heavy, and was used as the stationary phase, selecting the ascending mode. The column was first filled with the stationary phase at a flow rate of 10 mL/min with a rotation speed of 500 rpm, and the mobile phase was pumped into the column at the same flow rate while the instrument was run at 1600 rpm. After reaching a hydrostatic equilibrium, indicating the outflow of the mobile phase from the column, the prepared sample solution was injected. The fractions were collected by a fraction collector for 1 min (or 10 mL) in each test tube and monitored using a UV detector at 320 nm.

4.6. HPLC—DAD—ESI/MS Analysis. The ethyl acetate-soluble fraction and the CPC peak fractions were weighed accurately and dissolved in MeOH at 1.0 mg/mL, followed by filtration through a syringe (0.45 μm). The HPLC analysis was performed using a YMC-Triart C18 column (5 μm, 250 × 4.6 mm; YMC Co., Kyoto, Japan) with binary gradient elution with water (v/v, solvent A) and acetonitrile (v/v, solvent B): 0–40 min, 10–40% B; 40–42 min, 40%–100% B. The injection volume was set to 10 μL and the flow rate was maintained at 0.8 mL/min. Chromatograms were acquired at 320 nm using the DAD detector. The mass spectra were measured between m/z 100 and 1000 in the positive ionization mode (ESI⁺) at a scan rate of 1.06 s/cycles and monitored using a diode array detector. The mass spectrometric conditions were as follows: capillary voltage = 4000 V; drying gas flow = 10 L/min (N₂); nebulizer pressure = 30 psi; and drying gas temperature = 350 °C.

4.7. DPP-IV Inhibitory Activity Assay. DPP-IV activity was analyzed using a DPP-IV inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s protocols. Briefly, the DDP assay buffer (20 mM Tris-HCl, pH 8.0, containing 100 nM NaCl and 1 mM ethylenediaminetetraacetic acid) was used as the assay solution. Human recombinant DPP-IV and its substrate, namely, 5 mM H-Gly-Pro conjugated to aminomethylcoumarin (AMC), were prepared in the same buffer. Diluted assay buffer (30 μL) and diluted enzyme solution (10 μL) were added to a 96-well plate containing 10 μL of the solvent (blank) or solvent-dissolved test compounds. The reaction was initiated by adding 50 μL of a diluted solution of the fluorogenic substrate. Cleavage of the peptide bond by DPP-IV released the free AMC group. Fluorescence at an excitation wavelength of 350 nm and an emission wavelength of 450 nm was monitored using a plate reader (TECAN, Männedorf, Switzerland). The percent inhibition was calculated as follows: ([DPP-IV level of vehicle-treated control — DPP-IV level of test samples]/DPP-IV level of vehicle-treated control) × 100. Subsequently, the 50% inhibitory concentration (IC₅₀) was calculated via a dose–response analysis using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03649.

HPLC chromatograms of the methanol extract and n-hexane, chloroform, ethyl acetate, and n-butanol

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fractions of bulbs of *L. longiflorum* and $^1$H$^3$C, and $^1$H$^{13}$C HMBC NMR spectra and MS data of 1–5 (PDF)

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

CPC, centrifugal partition chromatography; DAD, photodiode array detector; DPP-IV, dipetidyl peptidase IV

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