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Separation and purification of (−)schisandrin B from schisandrin B stereoisomers

Ka F. Luk[a], Kam M. Ko[b], Ka M. Ng[c,*]

[a] Bioengineering Graduate Program, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong
[b] Department of Biochemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong
[c] Department of Chemical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

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ABSTRACT

Schisandrin B (Sch B), consisting of a mixture of its stereoisomers, namely (−)Sch B and (±)γ-schisandrin, is the most abundant and biologically active dibenzocyclooctadiene lignan present in *Fructus Schisandrae* (FS). The objective of this study is to develop a process for large-scale separation and purification of a single stereoisomer of Sch B, (−)Sch B, which offers the highest desirable bioactivities. To this end, a crystallization-based separation and purification process has been conceptualized. Bench-scale crystallization experiments guided by experimental solid–liquid equilibrium phase diagrams were performed to verify process feasibility. A (−)Sch B product with a purity of 98.5 wt% and a (±)γ-schisandrin-enriched product with a purity of 65.0 wt% were obtained. The (−)Sch B product caused a 32% increase in cellular glutathione level and the (±)γ-schisandrin-enriched product a 26% increase, indicating a potentially more efficacious pharmaceutical preparation.

1. Introduction

Schisandrin B (Sch B) is the most abundant, biologically active dibenzocyclooctadiene lignan found in *Fructus Schisandrae* (FS). There exist four stereoisomers of Sch B, namely (+)γ-schisandrin, (−)γ-schisandrin, kadsuranin, and (−)Sch B. Sch B present in FS is primarily a mixture of (±)γ-schisandrin and (−)Sch B [1], and the weight ratio of (±)γ-schisandrin and (−)Sch B is around 2:8. Sch B, as used in this article, refers to the total amount of (±)γ-schisandrin and (−)Sch B. Recently, among the stereoisomers of Sch B isolated by chiral chromatography, (−)Sch B was found to produce more potent enhancing effect on cellular glutathione and protection against oxidative injury in both cultured cardiomyocytes and hepatocytes [2,3]. Therefore, it is desirable to recover (−)Sch B in a purified form for a more efficacious pharmaceutical preparation.

The conceptual design for large-scale isolation and purification of Sch B from FS has already been developed [4]. The objective of this research is to develop a scalable crystallization-based process, instead of using highly costly chiral chromatography, for the separation and purification of (−)Sch B from Sch B for large scale manufacturing.

Crystallization is a good choice because, with recent developments, it is capable of producing pure solid compounds from a multicomponent mixture. For a particular system, the flowsheet of a crystallization-based separation process can be synthesized based on knowledge of solid–liquid equilibrium (SLE) phase behavior. The key idea is to identify the crystallization compartment/region of the desired component on the SLE phase diagram, and to navigate the feed composition of crystallizers to reach the appropriate region by a sequence of operations, such as cooling, heating, solvent addition or solvent removal [5–7]. Therefore, the SLE phase behavior of the ternary system comprising (±)γ-schisandrin, (−)Sch B and ethanol (the selected solvent) is firstly determined. A crystallization-based process for the recovery of high purity (−)Sch B is then synthesized accordingly. Moreover, one of the challenges in the development of herbal products is to manufacture the products with consistent quality and efficacy [8]. Thus, a biological assay is used to assess the efficacy of the purified Sch B products in this study.

2. Materials and methods

Sch B was purified from a petroleum ether extract of FS which was prepared as described elsewhere [9]. The H9c2 cell line was purchased from the American Type Culture Collection. Cell culture medium and fetal bovine serum (FBS) were obtained from GIBCO BRL Life Technologies. Reduced glutathione (GSH), oxidized glutathione and glutathione reductase were purchased from Sigma. All these chemicals were of analytical grade.

* Corresponding author. Tel.: +852 23587238; fax: +852 23580054.
E-mail address: kekmng@ust.hk (K.M. Ng).

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2.1. Determination of ternary isothermal SLE phase diagram

2.1.1. Preparation of (±)γ-schisandrin and (−)Sch B by preparative HPLC

Pure (±)γ-schisandrin and (−)Sch B were obtained from Sch B using the Agilent HPLC system comprised of the Agilent Preparative Pump and Agilent Photodiode Array Detector. Two Waters Nova-Pak HR C18 6 μm (19 mm × 300 mm) columns were used in series. The mobile phase was acetonitrile and water (60:40, v/v) at a flow rate of 20 mL/min. One (1) mL of 2% (w/v) Sch B solution (in mobile phase) was injected into a running solution of mobile phase through the Alltech sample loop. Fractions were collected according to the HPLC chromatogram which was monitored at the wavelength of 254 nm. The pooled fraction containing (±)γ-schisandrin and that containing (−)Sch B were dried by evaporating acetonitrile in a rotary evaporator, followed by lyophilization to remove the remaining water.

2.1.2. Measurement of compositions of solid and liquid in equilibrium

To measure the SLE phase diagram at 40 °C, predetermined amounts of (±)γ-schisandrin, (−)Sch B and ethanol were loaded into a 5 mL jacketed glass vessel sealed with a glass stopper. The solid–liquid mixture was stirred with a magnetic bar. The temperature of the system was maintained at 40 °C using a PolyScience water circulator. After holding the system at 40 °C overnight, the agitation was stopped to allow the sedimentation of the undissolved solid. A 100 μL air-tight Hamilton syringe was used to sample a small amount of the liquid phase, which was then analyzed by HPLC to determine the liquid phase composition. The solids were collected using a 0.2 μm syringe filter, followed by washing with 1 mL of cold hexane. The solids were then redissolved in 1 mL of methanol and the resulting solution was analyzed by HPLC to determine the solid phase composition. The above procedure was repeated to determine the SLE phase diagram at 10 °C. All HPLC analysis was performed on a Waters Alliance HPLC system comprised of a Waters 2956 Separation Module and a Waters 2996 Photodiode Array Detector. A Nova-Pak C18 4 μm (3.9 mm × 300 mm) column was used. The mobile phase was acetonitrile and water (55:45, v/v) at a flow rate of 1 mL/min. A 20 μL sample was injected and the elution was monitored by UV absorbance at 254 nm.

2.2. Bench-scale crystallization experiments

Based on the SLE phase diagram thus determined, a three-step crystallization process will be synthesized. The feasibility of this separation process was verified by bench-scale experiments as follows.

2.2.1. First crystallization step

Sch B (2 g) and ethanol (8 mL) were loaded into a 20 mL jacketed glass vessel sealed with a glass stopper. The solid–liquid mixture was mixed with a magnetic stir bar and its temperature was maintained by the oil from a Huber oil circulator. The mixture was heated up to 60 °C until complete dissolution. Then, the system was cooled down to 40 °C at a cooling rate of 0.25 °C/min to effect crystallization. After holding the system at 40 °C overnight, the agitation was stopped to allow sedimentation of the solids. A small amount of the liquid phase was collected using a 100 μL air-tight Hamilton syringe and was analyzed by HPLC. The crystals were recovered using a filter flask under vacuum, followed by washing with cold hexane. It was then analyzed by HPLC and the remaining solids were stored for further experiments.

2.2.2. Second crystallization step

The mother liquor obtained from the first crystallization step was poured into a 20 mL jacketed glass vessel. The vessel was heated up to 60 °C in an open system (without being sealed by a glass stopper) to remove some ethanol. The amount of ethanol removal was controlled such that the overall composition in the vessel was inside the (−)Sch B crystallization region at 40 °C. Then, with the vessel sealed by a glass stopper, the system was cooled down to 40 °C at a cooling rate of 0.25 °C/min to effect crystallization and kept overnight with constant stirring. The liquid and solids were separated and analyzed as in the first crystallization step.

2.2.3. Third crystallization step

The mother liquor from the second crystallization step was poured into a 20 mL jacketed glass vessel. A certain amount of ethanol was added into the vessel such that the overall composition in the vessel was inside the (±)γ-schisandrin crystallization region at 10 °C. Then, with the vessel sealed by a glass stopper, the system was cooled down to 10 °C at a cooling rate of 0.25 °C/min to effect crystallization and kept overnight with constant stirring. Again, the liquid and solids were separated and analyzed.

2.2.4. Purification of (−)Sch B by recrystallization

A known amount of the crystals obtained from the first crystallization step was loaded into a 5 mL jacketed glass vessel for the recrystallization experiment. Then, a known amount of ethanol was added such that the overall composition in the vessel was inside the (−)Sch B crystallization region at 40 °C. With the vessel sealed by a glass stopper, the temperature of the system was maintained at 40 °C overnight. The liquid and solids were collected and analyzed as described previously.

The remaining solids were put into a new jacketed glass vessel and a known amount of ethanol was added for another recrystallization step at 40 °C. This purification process by recrystallization was repeated until the purity of (−)Sch B in the solid phase was higher than 98 wt%.

2.3. Quality assurance of purified Sch B products by measuring cellular glutathione response

2.3.1. Cell culture

H9c2 cells, a permanent cell line derived from cardiac myoblasts [10], were used for all experiments. H9c2 cardiomyocytes were cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with 10% (v/v) FBS. The medium contained glucose (4.5 g/L) and glutamine (4.5 mM), supplemented with $NaHCO_3$ (17 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL). Cells were grown under an atmosphere of 5% (v/v) CO_2 in air at 37 °C. The medium was replaced by fresh medium every 2 or 3 days. A stock of cells was grown in a 75 cm^2 culture flask and split before confluence at a sub-cultivation ratio of 1:10. Cells used for experiments were seeded at a density of 3.75×10^4 cells/well on a 12-well culture plate, and cells in each well were allowed to grow to achieve 60–80% confluence within 24 h prior to drug treatment.

2.3.2. Measurement of Sch B-induced glutathione response

H9c2 cardiomyocytes were treated with high purity (−)Sch B product and (±)γ-schisandrin-enriched product (dissolved in dimethyl sulfoxide (DMSO) and mixed with complete medium containing FBS) at a concentration of 6.25 μM (0.2% (v/v) DMSO final concentration) for 16 h. Parallel control (i.e. untreated) cells were given medium containing 0.2% DMSO only. At the end of the incubation period, the control and drug-treated cells were measured for the GSH level as described in Chiu et al. [3].