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Preparative separation and purification of squalene from the microalga *Thraustochytrium* ATCC 26185 by high-speed counter-current chromatography

Hai-Tao Lu[a], Yue Jiang[a], Feng Chen[b,*]

[a]*Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China*
[b]*Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, China*

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Abstract

High-speed counter-current chromatography (HSCCC) was successfully applied to the preparative separation and purification of squalene from microalgae. Crude squalene was obtained from the microalga *Thraustochytrium* ATCC 26185 by extraction with organic solvents. The crude squalene was further separated using a waterless two-phase solvent system composed of *n*-hexane–methanol (2:1, v/v). The upper phase as the mobile phase was pumped into the column at a flow-rate of 2.0 ml min^{-1} in the tail-to-head elution mode. The fractions purified and collected were analyzed by high-performance liquid chromatography. The method yielded 0.2 mg squalene at 96% purity from 150 mg of the crude squalene (0.14% squalene) with 95% recovery. The separation of squalene by HSCCC was completed in 90 min.
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Keywords: Counter-current chromatography; *Thraustochytrium* ATCC 26185; Squalene; Terpenes

1. Introduction

Squalene is a highly unsaturated aliphatic hydrocarbon and belongs to the triterpene group of oils. Its chemical structure is shown in Fig. 1. Squalene is normally used in its natural form as a moisturizing or emollient agent in pharmaceuticals and cosmetic preparations. More importantly, it is a potential oxidation inhibitor; it can protect cell against free radicals, strengthen the body's immune system and decrease the risk for various cancers [1]. Therefore, the development of a simple and efficient method for the separation and purification of squalene is important.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatographic technique that uses no solid support matrix. Therefore, it eliminates irreversible adsorptive loss of the sample onto the solid support matrix used in the conventional chromatographic column [2]. This method has been recently successfully used for the preparative separation of several natural products [3–8]. However, no report has been published on the use of HSCCC for the separation and purification of squalene from natural sources. The aim of the present study was to develop an HSCCC

*Corresponding author. Tel.: +852-2-299-0309; fax: +852-2-299-0311.
E-mail address: sfchen@hkusua.hku.hk (F. Chen).

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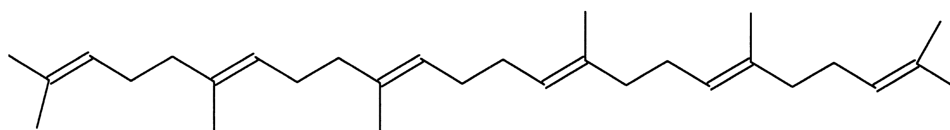

Fig. 1. Structure of squalene.

method for the preparative separation and purification of squalene from the microalga *Thraustochytrium* ATCC 26185 that was considered a potential source of squalene [9].

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (diameter of tube, 2.6 mm; total volume, 332 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a Model Series II HPLC pump (Parma-Tech Research), a Model SPD-10 Avp UV–Vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 20-ml sample loop.

The HPLC system used throughout this study consisted of a Waters 510 pump (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, a Waters temperature control module, a RCM-100/column heater, and a Waters 996 photodiode array detection (DAD) system. The column used was a reversed-phase Symmetry C_{18} column (150×3.9 mm I.D., 5 μm, Waters). Evaluation and quantification were made on a MILLENNIUM chromatography data system (Waters).

2.2. Reagents

Squalene was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, chloroform, acetone, methanol, *n*-hexane, 2-propanol, dichloromethane, ethyl acetate and ethanol were purchased from BDH (Poole, UK).

Freeze-dried microalgal cells (*Thraustochytrium* ATCC 26185) were obtained in our laboratory and stored in the dark at −20 °C.

2.3. Preparation of crude squalene

An 8-g amount of the freeze-dried microalgal cells was weighed and put into a 250-ml flask, to which was added 120 ml of methanol–chloroform (2:1, v/v). The dispersed cells were subjected to shaking extraction on a magnetic mixer at room temperature for 10 h to allow complete cell lysis. The biomass was then removed by filtration using nonabsorbent cotton-wool and the filtrate was passed through activated molecular sieves to remove the residual moisture. The biomass on the cotton-wool was washed twice with a fresh chloroform–methanol solvent system (20 ml each time). All the filtrates were combined and evaporated to dryness under nitrogen gas, and a viscous oily substance was yielded [10]. Then, 40 ml of a mixture of methanol–acetone (7:3, v/v) was added. The mixture was homogenized for 5 min by means of a mechanical shaker (vortex) and stored at −20 °C for 30 h [11]. The supernatant was rapidly filtered through a 0.45-μm membrane, and the residue was washed with 5 ml of cold methanol–acetone (7:3, v/v) solution (−20 °C). All filtrates were combined and evaporated to dryness under nitrogen gas, and 1.4 g of crude squalene was yielded. Finally, the crude squalene was stored in a refrigerator for the subsequent HSCCC separation.

2.4. Measurement of partition coefficient (K)

Approximately 1 mg of squalene was weighed in a 10-ml test tube to which 2 ml of each phase of the

pre-equilibrated two-phase solvent system was added. The test tube was stoppered, and shaken vigorously for 10 min to thoroughly equilibrate squalene with the two phases. The solution was then separated by centrifugation at 4000 *g* for 15 min. Finally, equal volumes of the upper and lower phases were analyzed by HPLC to determine the partition coefficient (K) of squalene. K was expressed as the concentration of squalene in the lower phase divided by that in the upper phase.

2.5. Preparation of two-phase solvent system and sample solution

In the present study, we selected two-phase solvent systems at various ratios. Each solvent mixture was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly and degassed by sonication prior to use. The sample solution was prepared by dissolving the crude squalene in the lower phase of the solvent system used for HSCCC separation.

2.6. HSCCC separation

The upper phase was used as the mobile phase and the lower phase was used as the stationary phase in the tail-to-head elution mode. In each separation, the multilayer-coiled column was first filled entirely with the stationary phase. The mobile phase was then pumped into the tail end of the inlet column at a flow-rate of 2.0 ml min^{-1}, while the apparatus was rotated at 1000 rpm. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 20 ml of the sample solution containing 150 mg of the crude squalene was injected through the injection value. The effluent from the outlet of the column was continuously monitored with UV–Vis detection at 205 nm and the chromatogram was recorded by an L 120 E flat-bed recorder. Peak fractions were collected according to the elution profile. When the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

2.7. Detection

It was impossible to monitor the *n*-hexane effluent continuously because microdroplets of the methanol stationary phase were present in the *n*-hexane mobile phase that produced a random turbidity and a large noise in UV detection. The stationary phase carryover was estimated to be 0.12 ml min^{-1} at 1000 rpm (rotation speed) and at 2 ml min^{-1} *n*-hexane (flow-rate). To stabilize the signal obtained with a UV–Vis detector, it was necessary to add 2-propanol as a postcolumn clarifying agent. A second pump was used to add 2-propanol at a flow-rate of 0.4 ml min^{-1} through a T and a 300 μl mixing reactor made of a knitted PTFE tube (110 cm×0.6 mm I.D.). Such an arrangement allowed a stable and continuous UV detection at 205 nm without applying too high a pressure to the HSCCC apparatus, and minimized the extracolumn band-broadening.

2.8. HPLC analysis and identification of squalene

Analysis of squalene in the crude sample and each peak fraction from HSCCC was performed on a C_{18} column at a column temperature of 30 °C. The mobile phase composed of acetonitrile (100%) was eluted at a flow-rate of 1.5 ml min^{-1} and the effluent was monitored at 205 nm by DAD. The retention time was compared with that of standard squalene. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

Fig. 2 shows HPLC analysis of the squalene crude from the microalgal cells. There are other compounds in the crude extract and the quantity of squalene is relatively small.

In HSCCC, successful separation necessitates the careful search for a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K) for the applied sample [12]. *n*-Hexane was often selected as the nonpolar liquid phase. It forms a biphasic solvent system with many polar and intermediately polar solvents with relatively low mutual solubility. The K values of

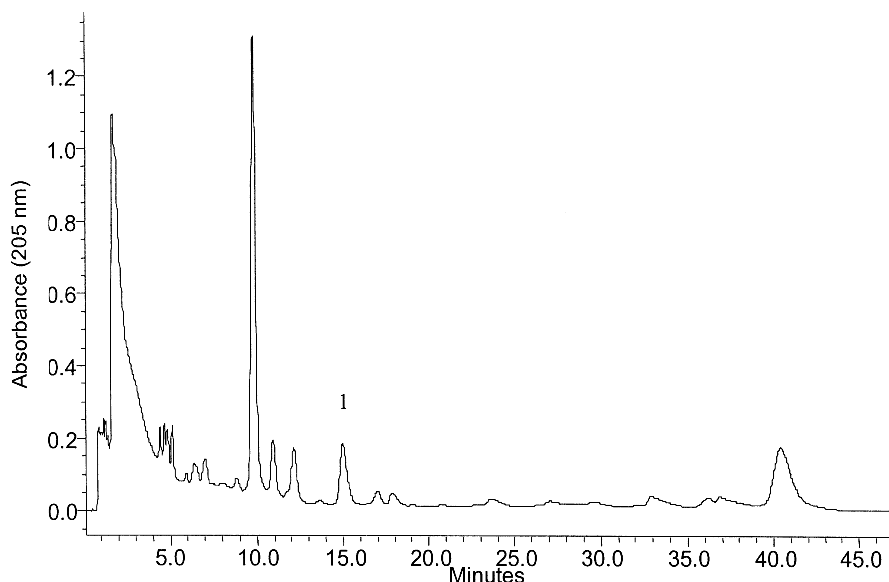

Fig. 2. Chromatogram of crude squalene extracted from the microalga *Thraustochytrium* ATCC 26185 by HPLC analysis, 1 = squalene. Conditions: column: reversed-phase Symmetry C_{18} column (150×3.9 mm I.D., 5 μm); column temperature: 30 °C; mobile phase: 100% acetonitrile; flow-rate: 1.5 ml min^{-1}; detection: 205 nm; injection volume, 20 μl.

squalene in several solvent systems were measured and are given in Table 1.

Preliminary HSCCC was carried out with the two-phase solvent systems composed of *n*-hexane–ethanol–water (4:2.8:1.2, 4:3.7:0.3) and *n*-hexane–methanol–water (4:3.7:0.3) at various volume ratios. Under these conditions, however, the *K* values were very small. The retention time of squalene was too short, and it was difficult to separate squalene from the other compounds. Because squalene is a highly unsaturated aliphatic hydrocarbon, which has strong hydrophobicity, it is freely soluble in *n*-hexane, but only slightly soluble in a strongly polar solvent such as methanol or ethanol and is not soluble in water. As shown in Table 1, the *K* value increased with an increase in the ratio of ethanol and a decrease in the ratio of water. However, when the ratio of ethanol–water was lower than 3.8:0.2, the two-phases combined to form a single phase, which could not be used for HSCCC. Thus, the two-phase solvent

Table 1
K (partition coefficient) values of squalene in several solvent systems

Solvent system	*K*
n-Hexane–ethanol–water (4:2.8:1.2)	0.002
n-Hexane–ethanol–water (4:3.7:0.3)	0.070
n-Hexane–methanol (2:1)	0.136
n-Hexane–methanol–water (4:3.7:0.3)	0.003
n-Hexane–dichloromethane–acetonitrile (4:0.6:3.4)	0.031
n-Hexane–dichloromethane–acetonitrile (4:1.2:2.8)	0.098
n-Hexane–dichloromethane–acetonitrile (4:1.5:2.5)	0.145
n-Hexane–dichloromethane–acetonitrile (4:1.7:2.3)	0.238
n-Hexane–dichloromethane–ethyl acetate–acetonitrile (3.4:0.6:0.6:3.4)	0.025
n-Hexane–dichloromethane–ethyl acetate–acetonitrile (3.1:0.9:0.9:3.1)	0.112

systems composed of *n*-hexane–ethanol–water and *n*-hexane–methanol–water were not suitable for the separation of squalene from the microalga.

Based on physical properties of squalene, waterless two-phase solvent systems were tested. *n*-Hexane had a high solubility in 100% acetonitrile and methanol, which led to a higher solubility of squalene. Dichloromethane and ethyl acetate are often used to adjust the polarity of solvent. We studied the two-phase solvent system composed of *n*-hexane–dichloromethane–acetonitrile at various volume ratios (4:0.6:3.4, 4:1.2:2.8, 4:1.5:2.5 and 4:1.7:2.3). When the ratio of dichloromethane increased, the *K* value also increased. But the densities of the two phases were so similar that the two phases became mutually soluble. We also investigated the two-phase solvent system composed of *n*-hexane–dichloromethane–ethyl acetate–acetonitrile at various volume ratios (3.4:0.6:0.6:3.4 and 3.1:0.9:0.9:3.1). However, it was also difficult to separate squalene from other compounds, because their *K* values in the two-phase solvent systems were also small. Thus, The two-phase solvent systems composed of *n*-hexane–dichloromethane–ethyl acetate–acetonitrile and *n*-hexane–dichloromethane–acetonitrile were also not suitable for the separation of squalene from the microalga.

Finally, we found that the two-phase solvent system composed of *n*-hexane–methanol at a ratio of 2:1 was the best. Although the *K* valve was only 0.136, the peak resolution and the retention time were both satisfactory. Fig. 3 shows preparative HSCCC separation of the crude squalene sample (150 mg) using the selected solvent system composed of *n*-hexane–methanol (2:1). The retention of the stationary phase was 72.6%. HPLC analysis of each fractionated effluent of the preparative HSCCC revealed that squalene corresponding to peak 1 was

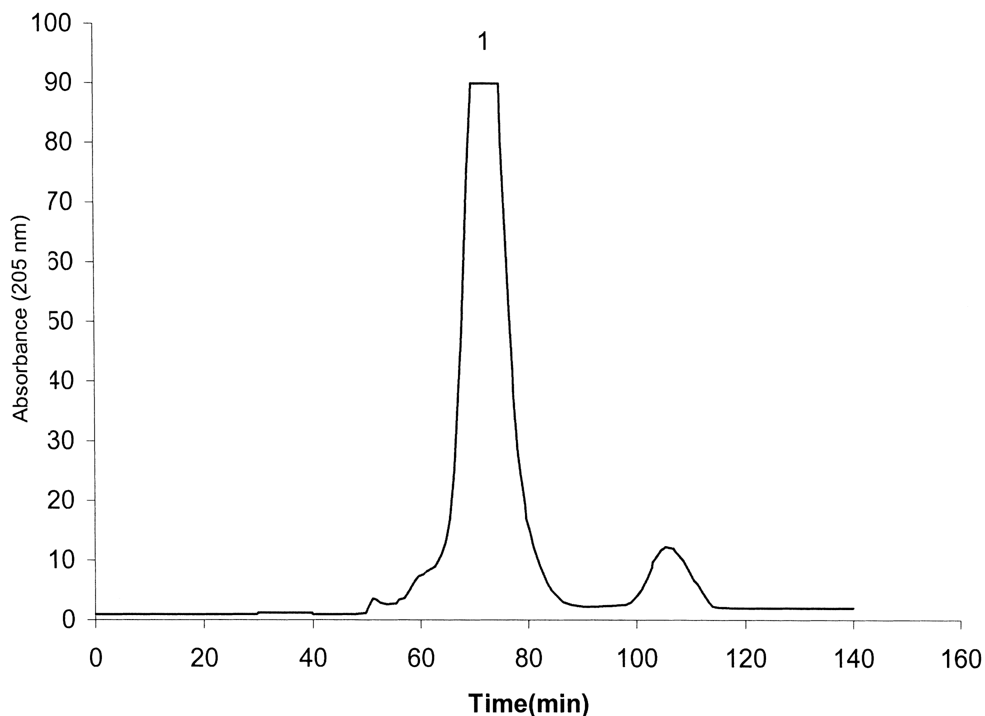

Fig. 3. Chromatogram of crude squalene extracted from the microalga *Thraustochytrium* ATCC 26185 by HSCCC separation, 1 = squalene. Conditions: column: multilayer coil of 2.6 mm I.D. PTFE tube with a total capacity of 332 ml; rotary speed: 1000 rpm; solvent system: *n*-hexane–methanol (2:1, v/v); mobile phase: the upper phase; flow-rate: 2.0 ml min^{-1}; detection: 205 nm; sample size: 150 mg; injection volume: 20 ml; retention of the stationary phase: 72.6%.

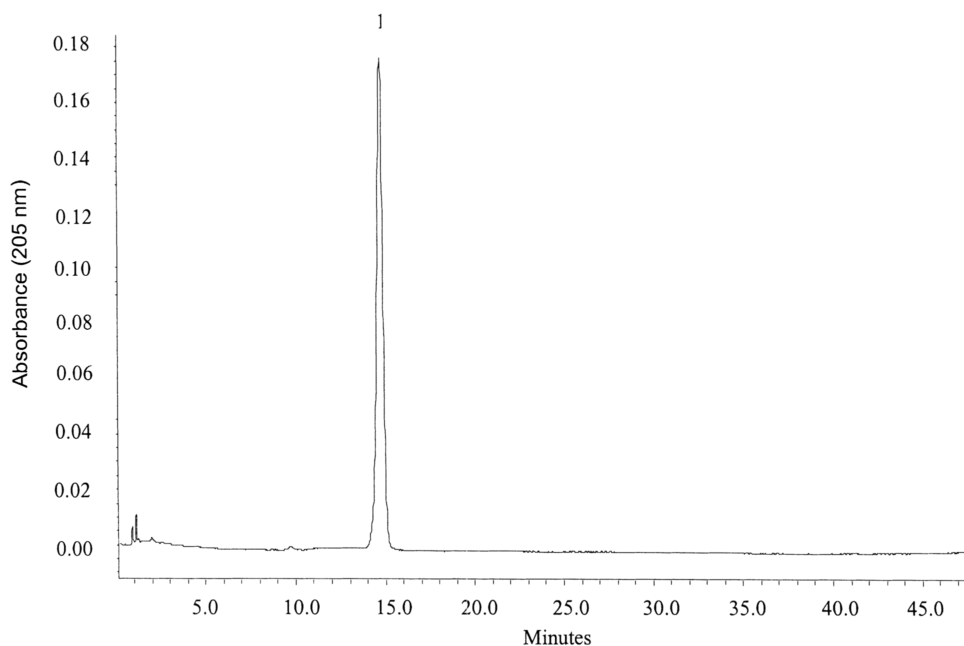

Fig. 4. HPLC chromatogram of squalene purified by the preparative HSCCC, 1 = squalene. Conditions: column: reversed-phase Symmetry C_{18} column (150×3.9 mm I.D., 5 μm); column temperature: 30 °C; mobile phase: 100% acetonitrile; flow-rate: 1.5 ml min^{-1}; detection: 205 nm; injection volume, 20 μl.

over 96% pure. An amount of 0.2 mg squalene (96% pure) was yielded with 95% recovery. The HPLC chromatogram of squalene as purified from the preparative HSCCC is shown in Fig. 4.

In the squalene crude, the content of squalene was approximately 0.14%. The purity of squalene was increased to more than 96% after a one-step separation by HSCCC. This result demonstrated the high resolving power of HSCCC that could be achieved by the careful selection of proper solvent systems.

In order to save solvents and time, the slow eluting compounds after squalene were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was used only once.

4. Conclusion

HSCCC was successfully used to separate and purify squalene (>96%) from the microalga *Thraustochytrium* ATCC 26185. The present study clearly indicates that HSCCC is a powerful tool in biotechnological downstream processes for the separation and purification of bioactive compounds.

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