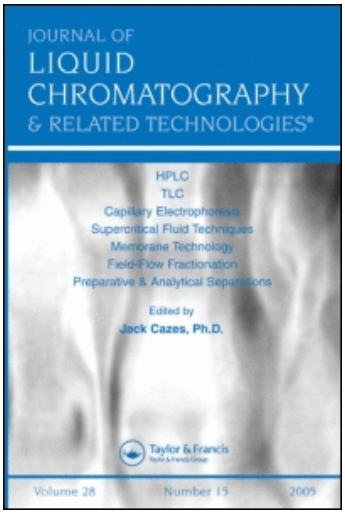

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Abstract: Cordycepin is successfully isolated and purified from *Cordyceps millitaris* in a two-step purification by high-speed countercurrent chromatography. Two solvent systems, ethyl acetate–1-butanol–water (3:2:5, v/v/v) and trichloromethane–methanol–1-butanol–water (2:1:0.25:1, v/v/v/v), were used for the two-step purification. The purity of the prepared cordycepin was 98.1% according to the high-performance liquid chromatographic analysis.

Keywords: Cordycepin, *Cordyceps millitaris*, High-speed countercurrent chromatography, HPLC

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INTRODUCTION

Cordyceps millitaris is one of the traditional Chinese medicinal fungi which have been widely used in traditional Chinese medicine. The studies have shown that cordycepin is one of the most important effective constituents.[1–3] Because of the importance of its biological properties, a large quantity of pure material is urgently needed for further studies.[4,5]

However, the preparative separation and purification of cordycepin from other constituents of the fungus *C. millitaris* by traditional methods are tedious, requiring multiple chromatographic steps resulting in low recovery. High-speed countercurrent chromatography (HSCCC) is a unique liquid–liquid partition technique that uses no solid support matrix. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. HSCCC has been successfully used for the preparative separation of natural products such as traditional Chinese medicinal herbs.[6–9] No report has been seen on the use of HSCCC solely for isolation and purification of cordycepin directly from a crude extract of the fungus *C. millitaris.* We herein report a successful semi-preparative separation and purification of cordycepin from the crude extract of *C. millitaris* using two different solvent systems by HSCCC.

EXPERIMENTAL

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade and purchased from Shanghai Suran Chemical Factory, Shanghai, P. R. China. Methanol used for HPLC analysis was of chromatographic grade and was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, P. R. China.

The cordycepin standard was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. The dried fruiting bodies of *C. millitaris* were supplied by Key Laboratory of Biotechnology for Medicinal Plant of Jiangsu Province, Xuzhou Normal University, P. R. China.

Apparatus

The preparative HSCCC instrument employed in the present study is a Model HHS-400A miltilayer coil planet centrifuge (Shanghai Tonghong Machine Co., Ltd., Shanghai, P. R. China) equipped with a polytetrafluoroethylene multilayer coil of 130 m × 1.6 mm i.d., with a total

capacity of 260 mL. The β value of the preparative column varied from 0.33 at the internal terminal to 0.86 at the external terminal ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 200 to 850 rpm, and 750 rpm was used in the present study.

The system was also equipped with one NS-1007 constant flow pump, a Model 8823B-UV monitor operating at 254 nm, a Yakogawa 3057 recorder and a manual injection valve with a 5 or 10 mL sample loop. The HPLC system used throughout this study consisted of a P3000 pump, a UV3000 detector (Beijing Chuang Xin Tong Heng Science & Technology Co., Ltd. Beijing, P. R. China), and a sample injector (Model: 7725) with a 20 μL loop. Evaluation and quantification were made on a CXTH-3000.

Preparation of Crude Extract[10]

Dried fruiting bodies of *C. millitaris* (20 g) were comminuted and extracted with 400 mL of distilled water by using ultrasonication (40 KHz) for 20 min. The sample extraction procedure was repeated 3 times. The filtrates were combined together and concentrated by vacuum distillation, yielding 8.4 g of crude sample which was stored in a refrigerator for the subsequent HSCCC separation.

Preparation of the Two-Phase Solvent Systems and Sample Solution

A two-step separation strategy was used in the present study on HSCCC with two different types of two-phase solvent systems composed of ethyl acetate–1-butanol–water (3:2:5, v/v/v) and trichloromethane–methanol–1-butanol–water (2:1:0.25:1, v/v/v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. In both solvent systems the upper aqueous phase was used as the stationary phase and the lower phase as the mobile phase.

The sample solution of the first step was prepared by dissolving 500 mg of the crude extract in 10 mL of each phase (1:1, v/v) of the first solvent system. The sample solution of the second step was prepared by dissolving 50 mg of dried peak fraction of the first step separation in 5 mL of each phase (1:1, v/v) of the second solvent system.

The standard working solution of cordycepin (0.1 mg/mL) was prepared in mobile phase of HPLC.

HSCCC Separation Procedure

First, the multilayer coiled column was entirely filled with the upper phase as the stationary phase. Then, the lower mobile phase was pumped into the head end of the column at a suitable flow-rate of 2.0 mL/min while the apparatus was rotated at an optimum speed of 750 rpm. After hydrodynamic equilibrium was reached as indicated by a clear mobile phase eluting from the tail outlet, the sample solution was injected through the injection valve. The effluent from the tail end of the column was continuously monitored by a UV detector at 254 nm, and the peak fractions were collected according to the chromatogram.

HPLC Analyses and Identification of HSCCC Fractions[11]

The crude sample and each purified fraction from the preparative HSCCC separation were analyzed by HPLC with an Alltima C_{18} column (250 mm × 4.6 mm, 5 μm) and column temperature of 25°C. The mobile phase, a mixture of methanol and water (17:83, v/v) was set at a flow-rate of 0.8 mL/min. The effluent was monitored by a UV detector at 260 nm.

RESULTS AND DISCUSSION

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients for the applied material.

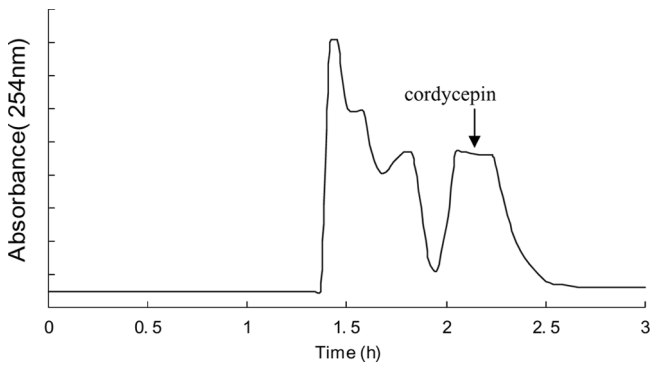

Figure 1. Chromatogram of the crude extract by preparative HSCCC. Conditions: revolution speed: 750 rpm; solvent system: ethyl acetate–1-butanol–water (3:2:5, v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 mL/min; detection: 254 nm; sample size: 500 mg; injection volume: 10 mL; retention of the stationary phase: 25.0%.

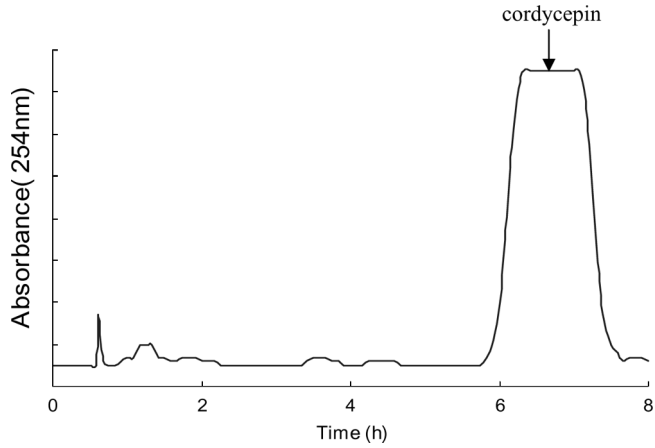

Figure 2. Chromatogram of the second step HSCCC Purification. Conditions: revolution speed: 750 rpm; solvent system: trichloromethane–methanol–1-butanol–water (2:1:0.25:1, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 mL/min; detection: 254 nm; sample size: 50 mg; injection volume: 5 mL; retention of the stationary phase: 73.1%.

After trying several solvent systems, we found that the solvent system composed of ethyl acetate–1-butanol–water (3:2:5, v/v/v) was most suitable for separation of the crude extract of *C. millitaris* by HSCCC. The chromatogram of HSCCC separation is shown in Fig. 1. Each

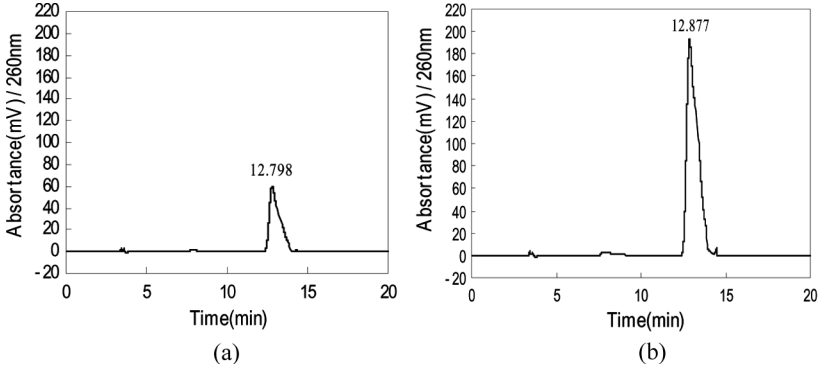

Figure 3. (a) HPLC chromatogram of the standard sample of cordycepin (b) HPLC chromatogram of the standard sample of cordycepin with HSCCC fraction of supposed cordycepin. Conditions: an Alltima C_{18} column (250 mm × 4.6 mm, 5 μm), column temperature: 25°C; mobile phase: methanol and water (17:83, v/v); flow-rate of 0.8 mL/min; detection: 260 nm. The concentrations of standard working solution was 0.1 mg/mL.

fraction was analyzed by HPLC with the C_{18} column and the target fraction was eluted at the retention time of 1.9 h to 2.5 h with a purity of 85.3%. To further improve the purity of cordycepin, the solvent system composed of trichloromethane–methanol–1-butanol–water (2:1:0.25:1, v/v/v/v) was used for the second step HSCCC separation (Fig. 2). The target fraction was eluted at the retention time of 6 h to 7.5 h with a purity of 98.1% by HPLC analysis. The yield of cordycepin after two-step HSCCC separation was 80.1%.

For further confirmation, the HSCCC fraction of supposed cordycepin was mixed with the solution of standard sample of cordycepin where HPLC separation of this mixture showed a single peak as shown in Fig. 3.

CONCLUSION

Using the two-step HSCCC separation method, we were able to purify cordycepin efficiently at a purity of over 98% with a relatively short separation time. The two-step HSCCC separation method with a suitable set of two-phase solvent systems yields high purity cordycepin directly from a crude extract of *C. millitaris*.

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REFERENCES

1. Chang, W.; Lim, S.; Song, H.; Song, B.-W.; Kim, H.-J.; Cha, M.-J.; Sung, J.M.; Kim, T.W.; Hwang, K.C. Cordycepin inhibits vascular smooth muscle cell proliferation. Eur. J. Pharmacol. **2008**, *597* (1–3), 64–69.
2. Yoshikawa, N.; Nakamura, K.; Yamaguchi, Y.; Kagota, S.; Shinozuka, K.; Kunitomo, M. Antitumour activity of cordycepin in mice. Clin. Exp. Pharmacol. Physiol. **2004**, *31*, 51–53.
3. Rottenberg, M.E.; Masocha, W.; Ferella, M.; Petitto-Assis, F.; Goto, H.; Kristensson, K.; McCaffrey, R.; Wiqzell, H. Treatment of A frican trypano somiasis with cordycepin and adenosine deaminase inhibitors in a mouse model. J. Infect. Dis. **2005**, *192* (9), 1658–1665.
4. Chen, S.Z. Study on improved ultillization of deoxynucleoside on *Cordyceps sinensis* with supercritical CO_2 extraction. CN: 1339440, **2002**, *3*, 13.
5. Chen, W.; Wu, W.; Gao, X.-H.; Tang, L.-S.; Shen, M.; Zhang, H.-X. Isolation and preparation of cordycepin by reverse-phase HPLC. Acta Agric. Shanghai **2007**, *23* (1), 59–61.

6. Sun, Q.; Sun, A.; Liu, R. Preparative isolation and purification of linderalactone and lindenenol from *Radix linderae* by HSCCC. J. Liq. Chromatogr. & Rel. Technol. **2006**, *29* (1), 113–121.
7. Yin, H.; Zhang, S.; Luo, X.; Liu, Y. Preparative isolation and purification of two benzoxazinoid glucosides from *Acanthus ilicifolius* L. by high-speed counter-current chromatography. J. Chromatogr. A **2008**, *1205* (1–2), 177–181.
8. Han, X.; Ma, X.; Zhang, T.; Zhang, Y.; Wang, L.; Fan, Y.; Song, T. Isolation of high-purity casticin from *Artemisia annua* L. by high-speed counter-current chromatography. J. Chromatogr A **2007**, *1151* (1–2), 180–182.
9. Wang, X.; Li, F.; Zhang, H.; Geng, Y.; Yuan, J.; Jiang, T. Preparative isolation and purification of polymethoxylated flavones from Tangerine peel using high-speed counter-current chromatography. J. Chromatogr. A **2005**, *1090* (1–2), 188–192.
10. Ling, J.-Y.; Sun, Y.-J.; Lu, P.; Zhang, C.-K. Capillary zone electrophoresis determination of cordycepin in cordyceps spp. Extracted by using ultrasonic. Mycosystema **2002**, *21* (3), 394–399.
11. Zhang, H.-X.; Wu, W.; Chen, W.; Gao, X.-H.; Tang, L.-S. Analysis of cordycepin and adenosine contents in fermentation supernatant of *Cordyceps millitaris* by HPLC. Acta Agric. Shanghai **2005**, *21* (4), 53–56.

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