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Application of High-speed Countercurrent Chromatography for the Purification of High-purity Illudin S from *Omphalotus japonicus*

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Illudin S from mushroom, such as *Omphalotus japonicus* and *illudens*, is a natural sesquiterpene analog with strong antitumor and antiviral activities. These illudins compounds are highly effective against various drug-resistant cancers that show extreme cytotoxicity an *in vitro* assay. However, it is difficult to obtain a sufficient amount of highly pure illudin S from a natural product by simple, efficient and low-cost purification techniques. Here, we offer to apply the high-speed countercurrent chromatography for the preparative purification of illudin S from mushroom extract. For a two-solvent system, the optimal condition of hexane/ethyl acetate/methanol/water (1/5/1/5, v/v/v/v) was optimized to obtain pure illudin S from a crude extract. This purified component was evaluated by liquid chromatography (high-purity >99%) and tandem mass spectrometry. The yield amounts of illudin S (1.3 mg/about 10 g *Omphalotus japonicus*) at one running are determined by liquid chromatographic calibration. It is concluded that by requiring a natural material and cost-effectiveness, our method represents a significant improvement over complicated techniques for the purification of illudin S from natural materials.

Keywords High-speed countercurrent chromatography, illudin S, *Omphalotus japonicus*, liquid chromatography

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Introduction

Illudin compounds are a family of natural sesquiterpene structures that are a source of food poisoning for humans, originally isolated from the mushrooms *Omphalotus japonicus* or *illudens*. In an earlier time, Nakanishi *et al.* isolated lampterol, which was destined to called illudin S (Fig. 1), from *Omphalotus japonicus*.[1] On the other hand, further study found that illudin S has the same structure of lampterol. Subsequently, illudin S has received attention concerning antitumor activity.[2] Illudin S displays potent *in vitro* and *in vivo* anticancer activity against even multi-drug resistant tumors, and is metabolically activated to an unstable intermediate that binds to DNA.[3-5] Based on the antitumor effect of illudin S, irofulven (derivatized illudin S) was developed and investigated as a novel anticancer dug.[6,7] Moreover, recent papers concerning illudin S have revaluated various pharmacological effects such as antiviral in an HSV-I/CV-1 assay, glutathione reductase inhibition and a metabolism pattern with enzymes.[8-11] Therefore, the native illudin S regent has been needed to desired for pharmacological experiments, such as anticancer effects, various biological activity and the appraisement of food poisoning, on *in vitro* and *in vivo* assays. However, the useful standard of illudin S involves high-priced regents that are non-commercially available for the versatile assay of pharmacology. Thus, it has been demanded for developments in separation techniques of illudin S relevant to biology and biomedical research including fundamental applications.

In the extraction procedures of illudin compounds, ethyl acetate extract was dried over anhydrous sodium sulfate, filtered, evaporated to dryness (vacuum, 50°C), and redissolved in acetonitrile.[12] A synthetic chemistry paper showed that these illudinoids were prepared using a solid-phase extractive purification.[13] Kasahara and Itou reported that a methanol extract was purified by a multi step procedure with silica-gel chromatography.[14] However, these reports show that these purified and prepared illudin S are not clear purity and involve effective recovery. In addition, there is little chromatographic purification of illudin S from natural materials or extract because of an unavailable technique for applications of high-purity illudin S. Thus, a useful and compliant purification system performs necessary processes that are required for various

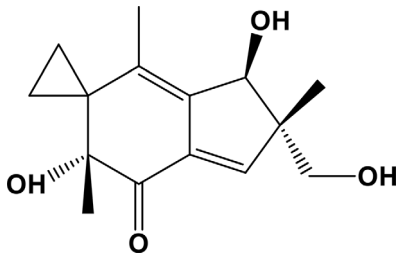

Fig. 1 Chemical structure of illudin S. CAS, 1149-99-1; molecular weight, 264.32; molecular formula, $C_{15}H_{20}O_4$.

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assays of biology and biomedical research. In this application study, high-speed countercurrent chromatography (HSCCC) was employed for the application of illudin S. The HSCCC technique was applied for the useful and effective separation of various compounds.[15–17] In actuality, we reported that HSCCC provided a useful and effective purification system for natural ingredients from complex materials.[18–20] Thus, in this study, the first application was shown to develop a useful, efficient and simple purification of high-purity illudin S from *Omphalotus japonicus* extract by the HSCCC technique.

Experimental

Reagents and chemicals

An illudin S solution (1000 μg/mL in methanol) was obtained from Hayashi Chemical Co., Inc (Osaka, Japan). HPLC-grade water, methanol, *n*-hexane, ethyl acetate, *n*-butanol and formic acid (FA, for HPLC 99%) were obtained from Wako Chemical Co., Inc. (Osaka, Japan). Purified water used for the mobile phase and sample preparation was obtained from a PURELAB Flex 5 system (ELGA, London, UK). A stock solution of Illudin S (100 μg/mL) was adjusted using methanol. The mushroom *Omphalotus japonicus* was sampled in 2017, and an extract was diluted by water/methanol (50/50, v/v) and stored at –20°C.

LC separation of illudin S

The LC system comprised an LC-20AD pump, an SIL-20AC autosampler, a CBM-20A controller, an SPD-M20A detector and a CTO-10AS column oven (Shimadzu Co., Kyoto, Japan). Reversed-phase (RP) analysis was performed using a TSK-GEL ODS-100Z column (2.0 × 150 mm, 3.0 μm, Tosoh Co. Inc., Tokyo, Japan) at 40°C. An injection volume of 10 μL was used, and the total run time of the analysis was 20 min. The mobile phase, comprising solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol), was delivered at a flow rate of 1.0 mL/min. The gradient elution settings were as follows: solvent A/B = 98/2 (0 – 5 min), 2/98 (15 min), and 2/98 (15 – 20 min). The elution of the components was monitored by ultraviolet/visible absorbance from 190 to 800 nm (for the monitoring wavelength, 254 nm).

MS evaluation of illudin S

A Waters Xevo TQD triple quadrupole mass spectrometer was operated with an electrospray ionization (ESI) source in the positive mode. The ionization source conditions were as follows: capillary voltage of 2.0 kV, extractor voltage of 3 V, RF lens voltage of 2.5 V, source temperature of 150°C and desolvation temperature of 400°C. The cone and desolvation gas flows were 50 and 800 L/h, respectively, which were obtained using a nitrogen source (N_2 Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). MS and the product ion scan ranges were adjusted from *m/z* 100 to 400 and 50 to 300. The cone voltage and collision energy were selected to be 20 V and 10 eV, respectively.

Extraction of Omphalotus japonicus

Omphalotus japonicus samples (10 g) were cut into small pieces and transferred to 50 mL propylene centrifuge tubes. To these 25 mL of methanol was added to the centrifuge tube and homogenized using a Geno/Grinder automated tissue homogenizer (1500 rpm for 10 min, SPEXSample Prep Co., Metuchen, NJ). The mixture was centrifuged at 10000 rpm for 10 min (HITACHI High-speed Micro Centrifuge CF16RN, Hitachi, Tokyo, Japan). The pellet was then homogenized and centrifuged three times with 25 mL of an extraction solution. The obtained supernatants were concentrated by a rotary evaporator.

HSCCC purification of illudin S from Omphalotus japonicus extract

For evaluating the two-phase solvent system, the crude *Omphalotus japonicus* extract was diluted by water/methanol (50/50, v/v), and the solution (100 μL) in the test tube was evaporated to dryness at 50°C. These residues were added to the two mutually equilibrated solvent phases (250 μL each) in a test tube, and mixed to equilibrate. After settling, equal volumes of the upper and lower phases (50 μL each) were transferred into separate test tubes and diluted by water/methanol (50/50, v/v). Then, the samples were assayed by the above-mentioned LC assay. The peak area at 12.6 min of illudin S at 254 nm in each phase was assessed for the determining the *K* value. The *K* value was calculated as "*K* = (peak response of illudin S in the upper phase solvent)/(peak response of illudin S in the lower phase solvent)".

HSCCC was performed using an Easy-Prep CCC (multi-layer coil planet centrifuge, Kutsuwa Co., Ltd., Hiroshima, Japan) with a 7.6-cm orbital radius that produces a synchronous type-J planetary motion with a maximum speed of 1500 rpm. This centrifuge was equipped with three column holders and three multilayer coiled columns. Each multilayer coiled column on the holder consists of nine coiled layers of 1.6 mm i.d. polytetrafluoroethylene tubing with a capacity of approximately 115 mL. All three columns and other tube are connected in series to provide a total capacity of real 349.3 mL. The fraction system (PU 714M LC pump, UV702 detector, SC 762 system controller and PLC 761 fraction collector) was from GL Sciences Co. (Tokyo, Japan). The volatile phase of the two-phase system composed of hexane/ethyl acetate/methanol/water (1/5/1/5) at room temperature was thoroughly equilibrated. The separation was performed in reversed phase (head-to-tail mode), with the column initially filled with the less-polar upper stationary phase. Then, *Omphalotus japonicus* (about 10 g) extract was dissolved in 1.5 mL of each phase, and was then filtered by 0.45 μm size for LC. Finally, these supernatants were then loaded into the column. The column was rotated at 1000 rpm while the mobile phase was being pumped into the head of the column at a flow rate of 2.0 mL/min. The retention of the stationary phase was observed to be 63%. Next, fraction A was recovered, evaporated to dryness, assayed using the LC assay.

NMR evaluation of purified illudin S by HSCCC

Samples were studied as solutions in 0.8 mL of $CDCl_3$ at room temperature. ^{1}H NMR spectra were recorded on a JEOL JMN-400 spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. Data are reported as follows: chemical shift in ppm (δ), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, bs = broad singlet, m = multiplet), coupling constant (Hz).

Results and Discussion

LC assay of illudin S in Omphalotus japonicus

The mushroom *Omphalotus japonicus* usually contains several polarity-soluble components, which are mixed together proportionally to detect many peaks in the LC chromatogram. In this study, a precious standard solution of illudin S was used to evaluate reversed-phase chromatographic separation based on

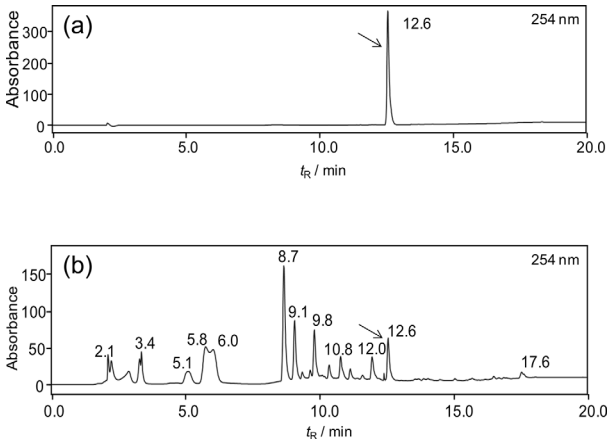

Fig. 2 LC chromatograms of illudin S standard and *Omphalotus japonicus* extract. (a) LC chromatogram and structure of illudin S (concentration level; 100 µg/mL). (b) LC chromatogram of *Omphalotus japonicus* extract.

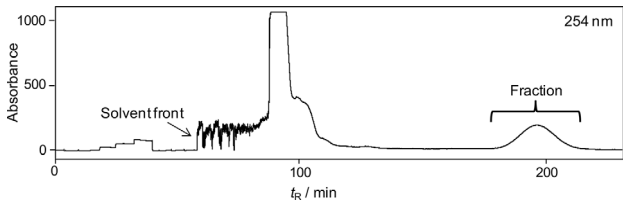

Fig. 3 HSCCC chromatogram of *Omphalotus japonicus* extract. The fraction is from 180 to 210 min.

a C_{18}-based column, mobile phase solvents, the retention time, the peak shape and detection. The optimal chromatograms of illudin S and *Omphalotus japonicus* extract are shown in Fig. 2. Figure 2(a) shows a chromatogram of an illudin S standard solution with a retention time of 12.6 min. Figure 2(b) shows a chromatogram of the extract solution from *Omphalotus japonicus* with many components and illudin S (12.6 min). On the other hand, Fig. 4(a) shows a chromatogram of the purified fraction (illudin S) from HSCCC. Based on these results, we decided to monitor these components by the reversed-phase mode using a simple mobile phase (0.1% FA in water/methanol) and TSK-GEL 100Z, and to measure the limit of detection (S/N = 3, LOD = 0.1 µg/mL) and the limit of quantification (S/N = 10, LOQ = 0.5 µg/mL) values, and calibration curves by 254 nm. Seven-point calibrations (range from 0.5 to 100 µg/mL, r^2 >0.999) were performed for illudin S using the developed LC assay. In addition, we investigated the extraction solutions (methanol, acetonitrile and ethyl acetate) of illudin S from *Omphalotus japonicus* using the developed LC assay. This result showed the same responses of illudin S (retention time, 12.6 min) in these extraction solutions. Thus, we selected the economical and low-cost methanol for this study.

HSCCC purification of illudin S in Omphalotus japonicus

For the purification methods of the effective components from plant samples, various techniques were reported, such as supercritical fluid extraction, hybrid molecularly imprinted polymers and modified filter paper.[21-23] On the other hand, the HSCCC technique is a very simple, useful and effective

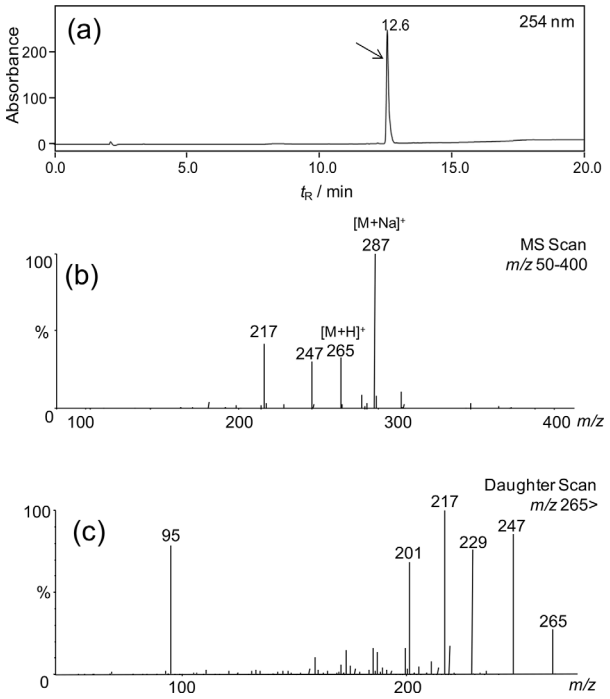

Fig. 4 LC chromatogram and MS spectra of fraction from HSCCC purification. (a) LC chromatogram of fraction from HSCCC purification. (b) MS spectrum of fraction from HSCCC purification on ESI-positive scan mode. (c) MS/MS spectrum of fraction from HSCCC purification on product ion scan of *m/z* 265.

Table 1 Partition coefficients (K) values (n = 3) for illudin S purified using HSCCC

Two-phase solvent system	Partition coefficients (K) value
Hexane/ethyl acetate/methanol/ water (v/v/v/v)	
3/5/3/5	0.10 ± 0.03
2/5/2/5	0.10 ± 0.17
1/5/1/5	0.73 ± 0.03
0/5/0/5	1.57 ± 0.13
Ethyl acetate/butanol/water (v/v/v)	
4/1/5	2.33 ± 0.31
3/2/5	2.84 ± 0.32
2/3/5	3.43 ± 0.22
1/4/5	3.64 ± 0.13
0/5/5	3.66 ± 0.01

purification for natural plants. Thus, we used HSCCC for the purification of illudin S in *Omphalotus japonicus*. Based on a referenced report,[24] the optimal K value was evaluated using the two-phase solvent system and the LC assay. For achieving successful HSCCC separation to obtain pure standards, the two-phase solvent system should satisfy the following requirements: (1) the settling time of the two-phase solvent system with samples should be less than 30 s, (2) the K value of the targeted compound should be close to 1.0, (3) the separation factor should be greater than 1.5, (4) the two-phase solvents should be nearly equal volumes for each phase, and (5) the two-phase solvents should be a volatile solvent system. We used *n*-hexane/ethyl acetate/methanol/water and ethyl acetate/*n*-butanol/water for suitable conditions of (1), (4) and (5). The results of the K

value are given in Table 1. Based on these results, we selected the hexane/ethyl acetate/methanol/water (1/5/1/5) two-phase solvent system which provided a useful *K* value (0.73) regarding to (2) for HSCCC purification. Using this two-phase solvent system, the retention of the stationary phase was 63% in the HSCCC column. The total separation time was about 4 h, and the total elution volume was 480 mL for the HSCCC system. This HSCCC chromatogram of fraction (illudin S) is shown in Fig. 3.

Evaluation of fraction from HSCCC purification

A fraction was high-purity >99% by LC assay, and identified to illudin S by the retention time (Fig. 4(a)). In addition, the yield amounts of illudin S 1.3 mg/about 10 g *Omphalotus japonicus*) at one running are determined by the above-described calibration curve. Based on the ESI-MS spectra and a previous report,[12] this fraction was identified as being illudin S which showed *m/z* 265 $[M+H]^+$ and *m/z* 287 $[M+Na]^+$ in MS scan, and *m/z* 247 $[M–H_2O+H]^+$, *m/z* 229 $[M–2H_2O+H]^+$, and *m/z* 217 $[M–H_2O–CH_2O+H]^+$, respectively. In this result, we can identify illudin S based on the MS spectra (Figs. 4(b) and 4(c)). In addition, the ^{1}H NMR data showed that ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.38 – 0.41 (m, 1H), 0.80 – 0.84 (m, 1H), 0.91 – 0.96 (m, 1H), 1.07 – 1.12 (m, 1H), 1.18 (s, 3H), 1.35 (s, 3H), 1.66 (s, 3H), 3.43 – 3.54 (m, 2H), 4.70 (s, 1H), 6.43(s, 1H). For the stereochemistry of illudin S, a previous study showed the enable data and Fig. 1.[25,26]

Conclusions

In this study, we described the development of a very easy, useful and preparative purification of high-purity illudin S from *Omphalotus japonicus* by HSCCC. We evaluated this fraction of the subsequent LC and MS for the identification of illudin S. It is suggested that this main fraction of illudin S from *Omphalotus japonicus* is high-purity >99% by LC assay. Thus, HSCCC purification can be described for the useful chromatographic preparation and isolation of illudin S.

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