Preparative Isolation and Purification of Altertoxin I from an *Alternaria* sp. by HSCCC

2008, 67, 863–867

Dejun Hu¹, Miao Liu², Xing Xia³, Daijie Chen⁴, Fengsheng Zhao¹, Mei Ge⁴✉

¹ Department of Pharmacy, Shanghai Jiao Tong University, 200240 Shanghai, China

² School of Life science and Biopharmacy, Shenyang Pharmaceutical University, 110016 Shenyang, China

³ Shanghai Institute of Pharmaceutical Industry, 200040 Shanghai, China

⁴ Shanghai Health Creation Center of Biopharmaceutical R&D, 201203 Shanghai, China; E-Mail: hccbred@gmail.com

Received: 2 January 2008 / Revised: 25 February 2008 / Accepted: 5 March 2008
Online publication: 6 May 2008

Abstract

Altertoxin I (ATX I) is one of the common mycotoxins produced by genus *Alternaria* which is a common food pathogen of fruits and grains. To prepare enough quantity of pure ATX I for further research of mutagenicity and toxicology tests, a novel method using preparative high-speed counter-current chromatography (HSCCC) was developed. The ethyl acetate crude extracts of the acetone washes obtained after fermentation of *Alternaria* sp. was separated using a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (2:5:5:6, v/v). Collected fractions were analyzed by LC and identified by EI-MS and NMR analysis. The technique can isolate mg levels of the target compound per run.

Keywords

High-speed counter-current chromatography (HSCCC)

Mutagenicity

Altertoxin I

Alternaria sp

Introduction

Altertoxin I, also known as ATX I, is a mycotoxin produced by molds of the genus *Alternaria*. *Alternaria* is a common food pathogen responsible for the spoilage of fruits, vegetables, grains and

nuts [1–5]. A variety of toxins produced by *Alternaria* have been associated with mutagenicity of Ames *Salmonella* and adverse health effects, including esophageal cancer in Linxian County, China as well as in areas of Southern Africa [6–10]. Numerous cases of human

toxicity, basically due to the ingestion of food contaminated by species of the *Alternaria* can be found described in the bibliography [11]. *Alternaria* causes serious damage to fruits and produces ATX I, a common mycotoxin with low levels of contamination that have been detected in a diverse variety of food-stuffs which range from wheat to fruit juices [3, 12–16]. Among the major *Alternaria* toxins, ATX I is the most active mutagen in the Ames *Salmonella typhimurium* assay, and exhibits acute toxicity in mice and cytotoxicity to bacterial and mammalian cells [6, 9, 17–20]. ATX I as the major mutagenic species could pose a potent carcinogenic health hazard when combined with a diet high in nitrites and nitrates [6, 9]. Contamination of fruits and grains with ATX I will create a food safety risk. Studies on mutagenicity, toxicology and detection of ATX I are, therefore, a priority for the food/feed industry and regulatory agencies. The study often requires relatively large quantities of pure ATX I.

Isolation of ATX I was done by a multi step protocol based on repeated column chromatography, often followed by a final purification on preparative

thin layer chromatography (TLC) [17, 21, 22]. Currently, there is no practical method for ATX I synthesis, and the commercially available standard is costly. In general, methods currently available for purification of ATX I are time-consuming, require multiple steps, and often generate large amounts of organic solvent wastage.

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatography process developed by Ito [23, 24]. The technique has been widely used in preparative separation of natural products with minimum sample preparation and clean-up procedures [25, 26]. Its theory is well understood and solvents mixtures are known for almost any analyte polarity [27]. The separation and purification of ATX I from extract of the *Alternaria* culture by HSCCC has not been reported so far. The objective of this study was, therefore, to develop a novel method using HSCCC for more efficient purification and recovery of ATX I from the *Alternaria* culture.

Experimental

Apparatus

The HSCCC instrument employed in this study was a TBE-300A high-speed counter-current chromatograph (Tauto Biotechnology, Shanghai, China) with three PTFE (Polytetrafluoroethylene) multi-layer coil separation column connected in series (I.D. of the tubing = 1.8 mm, total volume = 300 mL) and a 25 mL sample loop. The revolution radius was 5 cm, and the β of the multi-layer coil varied from 0.6 at internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distant from the coil to the holder shaft, and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1,000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument, Beijing, China) was used to control the separa-

tion temperature. The effluent was continuously monitored with a UV-Monitor 8823B (Beijing Binta Instrument Technology Co., Beijing, China) at 254 nm. The data were collected with a model N2010 chromatography workstation (Zhejiang University, Hangzhou, China). The fractions were collected in intervals of 4 min with a DC-1000 Model auto-fractional collector (Eyela, Tokyo, Japan).

Agilent 1100 LC system was used, which included a G1311A QuatPump, a G1315B UV-vis photodiode array detector, a G1313A Auto-sampler, a G1332A degasser and Agilent LC workstation.

Reagents, Cultural Media and *Alternaria* sp. Isolate

All organic solvents used for preparation of the crude extract and HSCCC separation were of analytical grade (Shanghai Chemical Reagent Corporation, Shanghai, China). Acetonitrile used for LC was of chromatographic grade (Merck), and distilled water. DMSO was used as the solvent for NMR determination. Potato dextrose agar (PDA), peptone and starch were purchased from Beijing Comwin Pharm-Culture Corporation (Beijing, China). ATX I and *Alternaria* sp. CPCC 480171 were obtained from Shanghai Health Creation Center of Biopharmaceutical R&D (Shanghai, China). The fungus was stored on PDA slant at 4 °C, and grown on PDA plates at 27 °C for 5 days before.

Fermentation of *Alternaria* sp. and Extract Preparation

Alternaria sp., CPCC 480171 was cultured in 500 mL flasks containing potato-dextrose liquid medium (potato 200 g, dextrose 20 g, H₂O 1,000 mL, total volume 200 mL) for 4 days at 25 °C, 120 rpm. 800 mL of the resultant culture was used to inoculate a 30-L GUJS-30C fermenter (Zhenjiang Oriental Biotech Instrument Co, China) containing 18 L

of SDP medium (peptone 2%, dextrose 1% and starch 2%, pH 6.5) plus 0.1% corn oil. After the sterilization cycle at 121 °C for 45 min, the medium was cooled to 27 °C and inoculated. The fermenter was incubated at 27 °C and maintained at 0.5 bar overpressure with an agitator speed of 300 rpm and an air flow rate of 10 L air min⁻¹.

After 5 days the cultured broth was harvested and centrifuged. The biomass of the fungal strain was collected and extracted with acetone (3 L × 3) to yield a crude extract that was a dark reddish solid. The dried extract was dissolved in 1.5 L water and then re-extracted with ethyl acetate for three times. 24.7 g ethyl acetate extract was obtained after evaporated to dryness under vacuum at 40 °C with a rotary evaporator (N-1000, Eyela, Japan). The ethyl acetate crude extract was stored at 4 °C before HSCCC separation.

Selection of Two-Phase Solvent System

The two-phase solvent system was selected according to the partition coefficient (K) value of the target compound. The K values of ATX I were determined by LC as follow: 1 mg of the crude extract was added to a test tube, to which 2 mL of each phase of a pre-equilibrated two-phase solvent system was added. The test tube was then capped and shaken vigorously for 1 min to thoroughly equilibrate the sample between the two-phases. The upper and lower phases were evaporated to dryness and dissolved with methanol to 2 mL separately. Then they were analyzed by LC. The partition coefficient (K) was expressed as the ratio of the peak area obtained from the upper phase to that of the lower phase.

Preparation of Two-Phase Solvent System and Sample Solution

Two-phase solvent systems of *n*-hexane-ethyl acetate-methanol-water with

different volume ratio were prepared and the lower phase was used as the mobile phase and upper phase was used as the stationary phase. Each solvent mixture was thoroughly equilibrated in a separation funnel at room temperature after shaking violently. Then the upper phase and lower phase were separated and degassed in a supersonic bath for 30 min before use. The sample solutions were prepared by dissolving the crude extract in a mixture of upper phase and lower phase in a ratio of 1:1(v/v) of the selected system.

HSCCC Separation Procedure

The HSCCC system was operated in head to tail mode with the upper phase as stationary phase. The multilayer-coiled column was first entirely filled with the upper phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 2.0 mL min⁻¹, while the apparatus was run at a revolution speed of 800 rpm. A crude extract (600 mg) dissolved in 20 mL mixture of the upper and lower phase was loaded via the injection valve after the system reached hydrodynamic equilibrium. The whole separation experiment was conducted at room temperature (25 °C). The effluent was continuously monitored by a UV detector at 254 nm and collected with a fraction collector set at 4 min for each tube. Fractions that contained only ATX I as identified by LC were combined and evaporated to dryness under vacuum at 40 °C.

LC Analysis and Identification of Target Compound

The LC analysis was performed with a reversed-phase Phenomenex Luna C₁₈ column (250 mm × 4.6 mm I.D., 5 µm) at room temperature (25 °C). The binary mobile phase consisted of methanol and water in the gradient mode as follows: 0–20 min, 30–70% methanol; 20–25 min, 70–100% methanol. The

Table 1. The partition coefficient (*K*) values of Alvertoxin I in different biphasic solvent systems used in HSCCC

Solvent system ^a	<i>K</i>
<i>n</i> -Hexane–ethyl acetate–methanol–water (1:3:3:3, v/v)	0.39
<i>n</i> -Hexane–ethyl acetate–methanol–water (1:2:2:3, v/v)	2.72
<i>n</i> -Hexane–ethyl acetate–methanol–water (2:5:5:6, v/v)	0.91
<i>n</i> -Hexane–ethyl acetate–ethanol–water (2:5:5:6, v/v)	1.27
<i>n</i> -Hexane–ethyl acetate–ethanol–water (3:5:6:6, v/v)	0.32
<i>n</i> -Hexane–ethyl acetate–ethanol–water (4:5:5:6, v/v)	0.62
Dichloromethane–methanol–water (2:3:3, v/v)	0.11
Dichloromethane–methanol–water (3:4:3, v/v)	0.28

^a Solvent ratios were by volume

effluent was monitored at 254 nm and flow rate was at 1.0 mL min⁻¹ constantly.

Identification of the HSCCC peak fraction that contained only ATX I was carried out by electrospray ionization mass spectrometry (ESI-MS), ¹H-NMR, ¹³C-NMR spectra and ¹H-¹³C-COSY.

Results and Discussion

Optimization of HSCCC Conditions

The first and critical step in a HSCCC experiment is the selection of a good solvent system, which can provide an ideal partition coefficient (*K*) for the target compound. A suitable *K* value for HSCCC should be preferably in the range of 0.5–1.0. A much smaller *K* value elutes the solute closer to the solvent front with lower resolution while a much larger *K* value tends to give better resolution but broader, more dilute peaks due to a longer elution time [25].

In order to achieve efficient resolution of target compound (ATX I), various two phase solvent systems with different ratios were tested. The measured *K* values for ATX I in these different solvent systems are summarized in Table 1. Among them, two solvent systems, (a) *n*-hexane–ethyl acetate–ethanol–water (4:5:5:6, v/v), and (b) *n*-hexane–ethyl acetate–methanol–water (2:5:5:6, v/v) gave good *K* values at 0.62 and 0.91, respectively.

For further evaluation of the selected two solvent systems, the ethyl acetate extracts of *Alternaria* sp. were analyzed by LC and the chromatogram is given in Fig. 1a. Table 2 lists the distributions of the 7 peaks (Fig. 1a) in the two selected solvent systems. In system (a), the *K* values of peaks 5, 6 and 7 are so similar that it may result in poor separation. In system (b), the *K* value of peak 6 is significantly different from the other peaks. At room temperature the settling time and the volume ratio (volume of the upper phase divided by that of the lower phase) of the solvent system (b) were 21 s and 0.38, respectively. The settling time correlates to the retention of the stationary phase and a good volume ratio could avoid excessive solvent waste [24]. The solvent system (b), providing a reasonable volume ratio between the upper and lower phases with a reasonable range of the settling times in 25 s was, therefore, used for all later HSCCC runs.

The HSCCC chromatogram is shown in Fig. 2. The peaks A–E in Fig. 2 were individually collected and analyzed by LC. Peak A in the HSCCC chromatogram consisted of peak 1 and peak 2 in the LC chromatogram (Fig. 1a); Peak B consisted of two peaks (peaks 3 and 4) in the LC chromatogram (Fig. 1a); peak C, peak D and peak E were corresponding to peaks 5, 6 and 7 (Fig. 1a), respectively. The peak D eluted from 230 to 260 min contained the target compound ATX I (Fig. 1b) with a purity over 95%. The influence of revolution speed, flow rate of the mobile phase was also investigated. The results indicated that when

Fig. 1. The results of LC analysis of the crude sample of **a** *Alternaria* sp. and **b** the purified peak **D** (ATX I) by HSCCC, column: Phenomenex Luna C₁₈ column (250 mm × 4.6 mm I.D., 5 μm) at room temperature, elution: methanol and pure water in gradient mode as follow: 0–20 min, 30–70% methanol, 20–25 min, 70–100% methanol, flow rate: 1.0 mL min⁻¹; detection at 254 nm (peak **A** in the LC chromatogram is peak **1** and peak **2**; Peak **B** in the LC chromatogram is peak **3** and peak **4**; Peaks **C**, **D** and **E** are peaks **5**, **6** and **7**, respectively)

Table 2. Partition coefficient of the seven peaks in the two solvent systems

	Peaks						
	1	2	3	4	5	6	7
Solvent system (a)	0.03	0.02	0.28	0.25	0.54	0.62	0.77
Solvent system (b)	0.02	0.02	0.22	0.23	0.58	0.91	1.21

Solvent systems (a) *n*-hexane–ethyl acetate–ethanol–water (4:5:5:6, v/v), and (b) *n*-hexane–ethyl acetate–methanol–water (2:5:5:6, v/v)

the flow-rate was 2.0 mL min⁻¹, the revolution speed was 800 rpm, and separation temperature was 25 °C,

retention percentage of the stationary phase was 53% and good separation results were achieved.

The Yield and Structural Identification

From 18 L fermentation culture, a total of 45 mg ATX I with purity of over 95% were obtained from our HSCCC separation method. The recovery rate of ATX I was 86.8% based on the original ATX I concentration in the ethyl acetate crude extract analyzed by LC.

The ESI-MS, ¹H-NMR and ¹³C-NMR data were used for the structure identification of ATX I, which was in accordance with the literature [28]. ESI-MS: *m/z* 353.1 [M + H]⁺. ¹H-NMR (400 MHz, DMSO, TMS) δppm: 8.03 (1 H, d, *J* = 8.8 Hz, H-12), 7.98 (1 H, d, *J* = 8.8 Hz, H-1), 7.03 (1 H, d, *J* = 9.2 Hz, H-2), 6.94 (1 H, d, *J* = 8.8 Hz, H-11), 4.55 (1 H, m, H-7), 2.85–3.37 (5H, m), 2.30 (1 H, m, H-8), 2.50 (1 H, m, H-5), 12.70 (1 H, 10-OH), 12.29 (1 H, 3-OH), 5.22 (1 H, 7-OH), 5.33 (1 H, 6a-OH). ¹³C-NMR (400 MHz, DMSO, TMS) δppm: 206.1 (C-4), 204.2 (C-9), 161.1 (C-10), 160.5 (C-3), 140.8 (C-9b), 138.4 (C-3b), 132.9 (C-12), 132.5 (C-1), 124.9 (C-12a), 123.6 (C-12b), 117.9 (C-9a), 116.6 (C-2), 115.6 (C-11), 113.9 (C-3a), 68.1 (C-6a), 64.7 (C-7), 51.5 (C-6b), 47.5 (C-8), 34.9 (C-6), 33.6 (C-5). Two dimension-NMR also supported the structural assignments. The structure of peak **D**, therefore, was positively identified as ATX I (Fig. 3).

Conclusion

The HSCCC method has been developed successfully for the purification of ATX I at a semi-preparative scale in this paper. It is a significantly simplified purification procedure to a higher recovery rate as compared with the conventional methods related to column chromatographic techniques, which may cause irreversible adsorption of target compounds to the column. The minimum use of organic solvents with a gentle and fast separation process makes the HSCCC a more environmentally friendly approach. A total amount of 45 mg of ATX I was obtained from the crude sample. The purity of the ATX I was over 95% after only one-step

Fig. 2. HSCCC chromatogram of the crude extract of *Alternaria* sp. in the solvent system of *n*-hexane–ethyl acetate–methanol–water (2:5:5:6, v/v), stationary phase: upper phase, mobile phase: lower phase, flow rate: 2.0 mL min⁻¹, revolution speed: 800 rpm, temperature: 25 °C, sample size: 600 mg crude extract dissolved in a 20 mL mixture of upper and lower phase (1:1, v/v) of solvent system, detection at 254 nm (the fraction of peak **D** were collected from 230 to 260 min and purified to over 95% estimated by LC analyses)

Fig. 3. The structure of Alvertoxin I

separation. The ESI–MS and NMR analyses were employed to positively identify the isolated ATX I. The present study indicates that HSCCC is a very powerful technique for the separation and purification of a relatively large amount of highly pure ATX I in a short period.

Acknowledgments

Financial support from the National Infrastructure of Natural Resources for Science and Technology (Grant no: 2005DKA21203) is gratefully acknowledged.

Open Access

This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. Bottalico A, Logrieco A (1998) In: Sinha HK, Bhatnagar D (eds) *Mycotoxins in agriculture and food safety*. Marcel Dekker, New York, pp 65–108

2. Solfrizzo M, De Girolamo A, Vitti C, Tylkowska K, Grabarkiewicz-Szczesna J, Szopinska D, Dorna H (2005) *Food Addit Contam* 22:302–308
3. Drusch S, Aumann J (2005) *Food Nutr Res* 50:33–78
4. Solfrizzo M, De Girolamo A, Vitti C, Visconti A, Van Den Bulk R (2004) *J AOAC Int* 87:101–106
5. Nielsen KF (2003) *Fungal Genet Biol* 39:103–117
6. Schrader TJ, Cherry W, Soper K, Langlois I, Vijay HM (2001) *Teratog Carcinog Mutagen* 21:261–274
7. Woody MA, Chu FS (1992) In: Chelkowski J, Visconti A (eds) *Alternaria: biology, plant diseases and metabolites*, Elsevier, New York, pp 409–434
8. Panigrahi S (1997) In: Miller JD, Trenholm, HL (eds) *Handbook of plant and fungal toxicants*. CRC Press, Boca Raton, pp 319–337
9. Schrader TJ, Cherry W, Soper K, Langlois I (2006) *Mutat Res* 606:61–71
10. Liu GT, Qian YZ, Zhang P, Dong ZM, Shi ZY, Zhen YZ, Miao J, Xu YM (1991) In: O'Neill IK, Chen J, Bartsch J (eds) *Relevance to human cancer of N-nitroso compounds, tobacco and mycotoxins*, Lyon: International Agency for Research on Cancer, pp 258–262
11. Ikins WG (1991) In: Fung DY, Matthews RF (eds) *Instrumental methods of quality assurance in foods*, Marcel Dekker, New York, pp 117–154
12. Li FQ, Yoshizawa T (2000) *J Agric Food Chem* 48:2920–2924
13. Hasan HAH (1996) *Acta Microbiol Immunol Hung* 43:125–133
14. Li FQ, Toyazaki N, Yoshizawa T (2001) *J Food Prot* 64:567–571
15. Scott PM, Weber D, Kanhere SR (1997) *J Chromatogr A* 765:255–263
16. Nawaz S, Scudamore KA, Rainbird SC (1997) *Food Addit Contam* 14:249–262
17. Stack ME, Prival MJ (1986) *Appl Environ Microbiol* 52:718–722
18. Osborne LC, Jones VI, Peeler JT, Larkin EP (1988) *Toxicol In Vitro* 2:97–102
19. Boutin BK, Peeler JT, Twedt RM (1989) *J Toxicol Environ Health* 26:75–81
20. Lehmann L, Wagner J, Metzler M (2006) *Food Chem Toxicol* 44:398–408
21. Stinson EE, Osman SF, Pfeffer PE (1982) *J Org Chem* 47:4110–4113
22. Chu F (1981) *J Am Oil Chem Soc* 58:A1006–A1008
23. Ito Y (1986) *CRC Crit Rev Anal Chem* 17:65–143
24. Oka H, Harada KI, Ito Y (1998) *J Chromatogr A* 812:35–52
25. Ito Y. (2005) *J Chromatogr A* 1065:145–168
26. He J, Yang R, Zhou T, Tsao R, Young JC, Zhu H, Li XZ, Boland GJ (2007) *J Chromatogr A* 1151:187–192
27. Friesen JB, Pauli GF (2007) *J Chromatogr A* 1151:51–59
28. Okuno T, Natsume I, Sawai K, Sawamura K (1983) *Tetrahedron Lett* 24:5653–5656