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Simultaneous determination of free ergosterol and ergosteryl esters in *Cordyceps sinensis* by HPLC

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Abstract

The caterpillar-shaped Chinese medicinal mushroom *Cordyceps sinensis* consists of the fruiting body (CsA) and the host caterpillar (CsB). Ergosterol is a principal sterol in fungi and can indicate the level of mycelia of *C. sinensis*. Ergosterol is present in two forms, as free ergosterol and esterified ergosterol, which have different physiological functions. The relative abundances of free to esterified ergosterol are different among the various species. In the present study, a gradient reversed-phase high-performance liquid chromatography (HPLC) method was developed for the simultaneous determination of free and esterified ergosterol in CsA and CsB of *C. sinensis*. The results showed that CsA and CsB had similar ergosterol compositions, but the level of ergosteryl esters in CsB was much higher than that in CsA, indicating that CsA and CsB might be in different growth phase or have different physiological functions for the growth and multiplication of *C. sinensis*.

Keywords: *Cordyceps sinensis*; Fruiting body; Host caterpillar; Ergosterol; Ergosteryl ester; HPLC

1. Introduction

The caterpillar-shaped Chinese medicinal mushroom *Cordyceps sinensis* (Berk.) Sacc., which consists of the fruiting body (CsA) and the host caterpillar (CsB), has long been used in China as a food or herbal medicine to treat numerous diseases (Bok, Lermer, Chilton, Klingeman, & Towers, 1999). Pharmacological studies have shown that *C. sinensis* possesses wide biological activities including anti-oxidation, immuno-potentiation, antitumorigenesis, antiinflammation, and stimulation of testosterone biosynthesis (Bok et al., 1999; Buenz, Bauer, Osmundsonc, & Motley, 2005; Chen, Huang, & Huang, 2005; Li, Li, Dong, & Tsim, 2001; Lo, Tu, Lin, & Lin, 2004; Siu et al., 2004; Weng, Chou, Lin, Tsai, & Kuo, 2002; Yu, Wang, Huang, & Duh, 2006).

Ergosterol is a principal sterol in fungi (Czub & Baginski, 2006), and is present in two forms, as free ergosterol and esterified ergosterol, and the relative abundances of free to esterified ergosterol are different among the various species (de Sio et al., 2000). Ergosterol can indicate the level of mycelia in *C. sinensis* and is a useful chemical marker for evaluating the quality of *C. sinensis*, at least which represents part of biological functions of *C. sinensis* (Li, Yang, & Tsim, 2006). Ergosterol and its peroxidation products may contribute to potential health benefits and significant pharmacological activities (Bok et al., 1999). Recent studies showed that ergosterol could be metabolized in vivo to generate newer bioactive products which had been found to be able to inhibit the proliferation of skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines (Slominski et al., 2005).

A number of methods have been reported for the determination of ergosterol, but most studies to date have focused on the determination of free egosterol, which is easily separated by an isocratic elution procedure (Abramson &

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Smith, 2003; Headley, Peru, Verma, & Robarts, 2002; Jasinghe & Perera, 2005; Sun, Gao, Ling, & Lou, 2005; Varga, Bartók, & Mesterházy, 2006). Because these methods were unable to separate and quantify free ergosterol and ergosteryl esters simultaneously, the samples or extracts were first subjected to alkaline saponification for the hydrolysis of ergosteryl esters before chromatographic analysis (Abramson & Smith, 2003; Headley et al., 2002; Jasinghe & Perera, 2005; Larsen, Axelsen, & Ravn, 2004; Lau, Lee, Chan, & Fang, 2006; Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002; Sun et al., 2005; Varga et al., 2006; Yuan, Wang, Liu, Kuang, & Huang, 2006). Free ergosterol is more important for cell integrity and contributes to a variety of cellular functions. The ergosteryl ester sequestered in cytosolic lipid particles is an inert storage form of sterol and may serve as intermediates for the supply of ergosterol. Because free ergosterol and ergosteryl ester are released from the fungal cell membrane and the cytosolic lipid particles, respectively (Shobayashi et al., 2005), it may be of interest to develop a method to quantify free ergosterol and ergosteryl esters simultaneously, which may be diagnostic of fungal species (de Sio et al., 2000). Although, ergosterol has been isolated from *C. sinensis*, cultured *C. militaris* (Li et al., 2004), and *C. cicadae* (Weng et al., 2002), little is known of the difference of both free and esterified ergosterol between CsA and CsB. In the present study, a gradient reversed-phase high-performance liquid chromatography (HPLC) method is developed for the simultaneous determination of free ergosterol and ergosteryl esters in *C. sinensis*.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany). HPLC-grade dichloromethane was obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Ergosterol was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Water was purified using a Millipore Simplicity system (Billerica, MA, USA).

2.2. Sample preparation

Natural *C. sinensis*, obtained from Tibet, China, was divided into CsA and CsB, which were ground into powder and dried at 50 °C, respectively, and then extracted with methanol/dichloromethane (75:25, v/v) at room temperature. The extracts were centrifuged at 10,000 rpm for 5 min. The extraction procedure was repeated three times. The total extracts were filtered through a 0.45 μm filter and sampled for HPLC analysis.

2.3. Saponification of ergosteryl esters

Ergosteryl esters were saponified by adding 1 ml of freshly prepared methanolic NaOH at the concentration of 0.4 M to 9 ml of CsB extract for hydrolyzing ergosteryl esters at ambient temperature. The concentration of NaOH in the reaction mixture was 0.04 M (Yuan et al., 2006). The reaction mixture was sampled to HPLC for monitoring the hydrolysis of ergosteryl esters during the saponification process.

2.4. HPLC method

HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector from Waters Corporation (Milford, MA, USA). The sample extracts were separated and analyzed by using a Waters Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm) at room temperature. The mobile phase consisted of solvent A (methanol/water, 80:20, v/v) and solvent B (methanol/dichloromethane, 75:25, v/v). A gradient procedure was used as follows: starting at sample injection, 0% of B for 5 min; a linear gradient from 0% to 100% of B for 19 min; 100% of B for 20 min. The flow rate was 1.0 ml min^{-1}. Chromatographic peaks were identified by comparing the retention times and spectra against the known standard. The detecting wavelength was set between 220 and 400 nm, and the chromatographic peaks were measured at a wavelength of 280 nm to facilitate the detection of ergosterol and ergosteryl esters. Aliquots of 20 μl were directly injected into the HPLC for the determination. All injections were repeated three times.

2.5. Method validation

The stock standard of ergosterol was prepared at 300 μg ml^{-1}, and additional calibration levels were prepared by a serial dilution with ethanol. The standard calibration curve was constructed using these ergosterol standard solutions. The linear regression analysis was carried out by plotting the peak areas against the concentrations of ergosterol. The linearity was demonstrated by a correlation coefficient (r^2) greater than 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on a signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. The intraday and interday precisions were determined on the same day and three different days, respectively. Precision was calculated as a relative standard deviation (%RSD) for the repeated measurements. For recovery studies on added ergosterol, known volumes of ergosterol standard solutions were added to 0.1 g of CsB at three levels. The spiked samples were extracted with methanol/dichloromethane (75:25, v/v) following the described procedure. Background levels were subtracted in all recovery determinations.

3. Results and discussion

This study described an HPLC method for the separation of fat-soluble active ingredients in *C. sinensis*. The reversed-phase column was chosen for the separation of

free ergosterol and ergosteryl esters. In comparison with free ergosterol, ergosteryl esters were strongly retained on the reversed-phase column. In the previous separation condition, ergosteryl esters could not be eluted from the chosen column by methanol (Yuan et al., 2006). In the present study, a shorter reversed-phase column was chosen. While eluted by methanol, the retention times of ergosterol and two ergosteryl esters were approximately 5, 60 and 80 min, respectively. The gradient elution could provide narrower peaks compared with isocratic elution. Therefore, a mixed solvent of methanol, water and dichloromethane as the mobile phase and the gradient elution procedure were used to separate free ergosterol and ergosteryl esters in the extracts of *C. sinensis*. Adding dichloromethane in the mobile phase could minimize the peak breadth and reduce the retention times. Under the present dichloromethane gradient conditions, ergosterol was eluted to obtain a narrower peak and the elution times of ergosteryl esters were reasonable for the simultaneous determination of free ergosterol and ergosteryl esters (Fig. 1). The elution peaks were identified by using a photodiode array detector and taking the spectrum of each peak during elution. The identification of ergosterol was achieved by comparing its retention time and spectrum against the known standard. Since, the esterification of ergosterol did not change its UV absorptive character, the spectra of ergosteryl esters were similar to the spectrum of ergosterol (Fig. 2). The spectra of peaks 1 and 4 were also similar to the spectrum of ergosterol, and they are identified as ergosterol analogues 1 and 2, respectively. The external standard method was used for the determination of ergosterol. The contents of ergosteryl esters were also measured by comparing the peak area with the ergosterol standard. For the further identification of ergosteryl esters, the extract of CsB was saponified by adding a newly prepared sodium hydroxide solution for the hydrolysis of ergosteryl esters to produce free ergosterol (Yuan et al., 2006). The increase in the ergosterol content and the decrease in the contents of peaks 6 and 7 during the saponification process indicated that peaks 6 and 7 were the esters of ergosterol.

Under the HPLC conditions tested, the calibration curve ($A = 35747C + 16755$, $r^2 = 0.9999$) was established by plotting the peak area (A) against the ergosterol concentration (C) in the range of 5–300 μg ml^{-1}. The LOD and the LOQ for free ergosterol with a 20 μl injection were 0.01 and 0.03 μg ml^{-1}, respectively, corresponding to 0.2 and 0.6 ng injected on the column. The LOD and the LOQ for ergosteryl esters with a 20 μl injection were 0.02 and 0.07 μg ml^{-1}, respectively, corresponding to 0.4 and

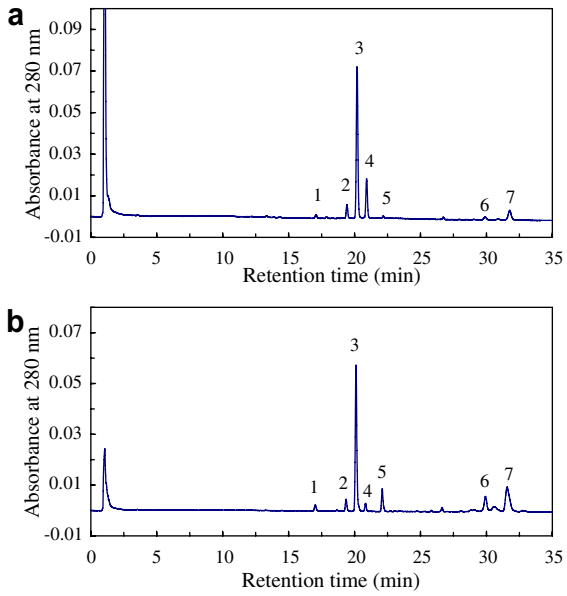

Fig. 1. Chromatograms of the dichloromethane/methanol (25:75, v/v) extracts from CsA (a) and CsB (b) of *C. sinensis* by this developed HPLC method. Peak identification: (1) ergosterol analogue 1; (2) unknown; (3) ergosterol; (4) ergosterol analogue 2; (5) unknown; (6) ergosteryl ester 1 and (7) ergosteryl ester 2.

Table 1
Precision of the determination of free ergosterol and ergosteryl esters in intraday and interday analysis

Ergosterol (mg g^{-1})	Intraday analysis ($n = 4$)		Interday analysis ($n = 12$)	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Free ergosterol	1.601 ± 0.015	0.94	1.594 ± 0.017	1.07
Ergosteryl ester 1	0.255 ± 0.005	1.96	0.251 ± 0.005	1.99
Ergosteryl ester 2	0.600 ± 0.008	1.33	0.611 ± 0.010	1.64

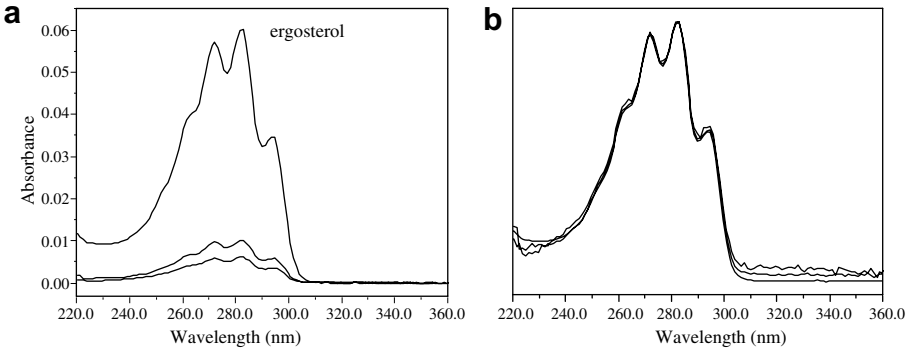

Fig. 2. (a) UV spectra of free ergosterol and ergosterol esters 1 and 2, and (b) their similarity.

Table 2
Contents of free, esterified and total ergosterols in CsA and CsB of *C. sinensis* ($n = 3$)

Sample		Free ergosterol (mg g^{-1})	Ergosteryl ester 1 (mg g^{-1})	Ergosteryl ester 2 (mg g^{-1})	Total ergosterol (mg g^{-1})	Esterified ergosterol (%)
1	CsA	1.843 ± 0.019	0.062 ± 0.003	0.205 ± 0.006	2.110	12.7
	CsB	1.601 ± 0.015	0.255 ± 0.005	0.600 ± 0.008	2.456	34.8
2	CsA	1.853 ± 0.021	0.057 ± 0.003	0.020 ± 0.001	1.930	4.0
	CsB	2.215 ± 0.035	0.257 ± 0.006	0.283 ± 0.008	2.755	19.6
3	CsA	1.706 ± 0.020	0.053 ± 0.003	0.026 ± 0.002	1.785	4.4
	CsB	1.809 ± 0.025	0.268 ± 0.008	0.294 ± 0.009	2.371	23.7

1.4 ng injected on the column. Four replicate determinations were performed on the same day and twelve replicate determinations were made on three different days. The RSD values for free ergosterol and ergosterly esters were satisfactory (Table 1). For recovery, an ergosterol standard was spiked into 0.1 g of the sample at the levels of 1.0, 1.5 and 2.0 mg g^{-1}. The spiked samples were extracted and assayed, and the recoveries of ergosterol were found to be between 99.1 and 101.4%.

As an application, the contents of ergosterol, ergosteryl esters 1 and 2 in CsA and CsB of *C. sinensis* were determined (Fig. 1 and Table 2). The results show that CsA and CsB have similar ergosterol compositions (free ergosterol, ergosteryl esters, and ergosterol analogues). Unlike other medicinal mushrooms which grow on live or decaying portions of trees, *C. sinensis* grows on the host caterpillar which eventually is totally invaded by the mycelia of *C. sinensis* (CsB), and grows out from the mummified shell of the dead host to form CsA for the dispersal of spores (Li, Su, Dong, & Tsim, 2002). Therefore, the two parts (CsA and CsB) show similar fungal structure and ergosterol compositions.

The results also show that the relative levels of ergosterol, especially the ergosteryl ester contents, in CsA and CsB of *C. sinensis* have significant differences. As can be seen from Table 2, although the variation in ergosterol, ergosteryl esters 1 and 2 contents between individual CsA and CsB of *C. sinensis* is significant, the contents of esterified ergosterol in CsB are much higher than CsA, indicating that CsA and CsB may be in different growth phase or have different physiological functions for the growth and multiplication of *C. sinensis*, and thus may have different bioactivities, as is recorded in the traditional Chinese medicinal book (Lo et al., 2004).

In conclusion, this developed HPLC method allows the simultaneous determination of free ergosterol and ergosteryl esters in *C. sinensis* and other fungi. Although, ergosterol and ergosteryl esters only are a portion of the bioactive constituents in *C. sinensis*, the contents of ergosterol and ergosteryl esters may be suitable markers for evaluating the quality of *C. sinensis* and other fungi.

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