

# Sipeimine-Producing Endophytic Fungus Isolated from *Fritillaria ussuriensis*

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Ten strains of endophytic fungi were isolated from the bulbs of the traditional Chinese medicinal plant *Fritillaria ussuriensis*. The extract from one of them, Fu7, showed a positive reaction with Dragendorff's reagent and the same  $R_f$  value in thin-layer chromatography (TLC) analysis as authentic sipeimine. A further TLC scan and high-performance liquid chromatography-evaporative light-scattering detection (HPLC-ELSD) showed that one ingredient of the extract of strain Fu7 had a similar absorption curve in the range 200–700 nm and the same retention time as authentic sipeimine. Thus, the fungus produces the bioactive ingredient sipeimine, as does its host plant, and could be used for the production of sipeimine by fermentation.

*Key words:* Endophytic Fungus, *Fritillaria ussuriensis*, Alkaloid

## Introduction

*Fritillaria ussuriensis* Maxim. is a perennial plant of the family Liliaceae. The bulbs of the plant are one of the traditional Chinese herbal medicines (TCM). It has been used as one of the most important antitussive and expectorant drugs in China and other Asian countries for thousands of years (Li *et al.*, 2001; Chen *et al.*, 2006). It is now officially recorded in the National Pharmacopoeia of China (Editorial Board of the Pharmacopoeia of the People's Republic of China, 2005). The main active constituents of the bulbs are steroidal alkaloids, such as sipeimine, peimine, pingpeinine A and B (Xu *et al.*, 1982; Chen *et al.*, 2006).

The natural resource of this medicinal material is now in short supply and becomes vulnerable because of the overcollection of the plant (State Environmental Protection Administration of China and Institute of Botany, 2000). Although this plant has been cultivated at several places in China, the conflict between supply and demand is an ongoing issue, because its growth requirements in the environment are quite stringent and its growth rate is slow. Therefore, it is critical to find an alternative way to produce these alkaloids to satisfy the demand.

In 1993, Stierle and his coworkers reported that an endophytic fungal strain isolated from *Taxomyces andreanae* produces the bioactive compounds taxol and taxane, as does its host plant

(Stierle *et al.*, 1993). Since then, continuing studies have reported that many endophytes – microorganisms that inhabit the tissues of living plants – produce a plethora of substances of potential use in modern medicine, agriculture, and industry (Strobel and Daisy, 2003; Tan and Zou, 2001; Guo, 2001; Liu *et al.*, 2005).

An endophytic fungus isolated from *F. ussuriensis*, that produces the same bioactive compounds (alkaloids) as its host plant, would not only reduce the need to harvest the rare plant but would also preserve our ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valuable product is usually easier and more economically produced, effectively reducing its market price (Strobel and Daisy, 2003).

Therefore, this study was undertaken to ascertain the presence of endophytic fungi in the plant *F. ussuriensis* and to ascertain whether any such fungus produces *Fritillaria* alkaloids. In this way, we hoped to develop a new method of producing these plant-derived pharmaceutical components, to resolve the conflict between natural resource protection and the requirement for TCM plants.

## Material and Methods

### *Plant material*

Fresh bulbs of *F. ussuriensis* were collected from the botanical garden of Northwest University,

Xi'an, China, where the plant was introduced from the Institute of Special Products, Jilin, China.

#### *Authentic drug*

Authentic sipeimine was purchased from the Shaanxi Provincial Institute for Drug Control, Xi'an, China.

#### *Isolation of endophytic fungi*

The endophytic fungal strains were isolated by routine microbiological methods from the fresh bulbs of *F. ussuriensis*. The purified strains were numbered and stored in a refrigerator at 4 °C for later use.

#### *Determination of growth curves*

The mycelial suspension of each strain was inoculated in equal amount into Erlenmeyer flasks containing potato-sucrose liquid medium. The cultures were then incubated at 25 °C on a reciprocal shaker.

Three flasks of each strain culture were harvested every day by filtration, then the mycelia were oven-dried and the average dry weights were used to draw a growth curve for each strain. Both the mycelia and their culture filtrates were preserved for analysis.

#### *Qualitative examination of alkaloids – Dragendorff's reaction*

The alkaloids in dried mycelia and culture filtrates were extracted using ethanol and chloroform. Both the extracts from the mycelia and from the culture filtrates were dried by decompression evaporation, then redissolved in water, and acidified with dilute HCl. Improved Dragendorff's reagent was added to each sample and the changes of the colour and the formation of deposits were observed. An orange colour or deposit constituted a positive result (Shanghai Institute of Materia Medica, 1972).

#### *Strain reselection – thin layer chromatography (TLC)*

The strain that reacted positively with Dragendorff's reagent was cultured in potato-sucrose liquid medium for a suitable time based on its growth curve and the primary qualitative results obtained with Dragendorff's reagent. The product was extracted according to the preselected protocol shown in Fig. 1.

Samples extracted from the selected strain were spotted onto a precoated silica-gel plate together with authentic sipeimine solution. The plate was developed in a preselected solvent system of ethyl acetate/methanol (6:0.2, v/v). The plate was then fixed in a drying oven at 105 °C for about 1 h and the sample components visualized by spraying the plate with improved Dragendorff's reagent.

The relative front ( $R_f$ ) values and the colour of the spots were measured and compared. The target strain was identified according to the results.

#### *Distribution of sipeimine inside and outside the mycelia*

To increase the culture scale of the target strain selected from the TLC reselection, the mycelia and culture filtrate samples were treated separately according to the protocol showed in Fig. 1, but the compounds were not mixed at step ⑦. Both samples were analyzed individually by TLC according to the procedure described above, to determine the distribution of the *Fritillaria* alkaloids inside and outside the mycelia.

#### *Product identification*

##### *TLC scan*

The extracts of the strains selected with strain reselection and the sipeimine standard were applied to a silica-gel plate for TLC analysis and visualized by the colour reaction produced with Dragendorff's reagent. The TLC plate was then scanned with CAMAG TLC Scanner 3 (CAMAG, Muttenz, Switzerland) in the wavelength range 200–700 nm.

##### *High-performance liquid chromatography-evaporative light-scattering detection (HPLC-ELSD)*

Standard solution: 0.05 mg/L sipeimine in methanol. Instrument and operation conditions: HPLC chromatograph, Agilent 1100 system (Agilent Technologies, Santa Clara, California, USA); control system, N2000 chromatography station (Zhejiang University, PRC); chromatographic column, Agilent ZORBAX SB-C<sub>18</sub> (150 mm × 4.6 mm, 5 μm); column temperature, 30 °C; mobile phase, acetonitrile/water (71:29, v/v) containing 0.03 % diethylamine at a flow rate of 0.8 mL/min; sample injection volume, 20 μL; ELSD detector, Alltech 2000 (Alltech, Deerfield, Illinois, USA); gasifying temperature, 85 °C; flow rate of nitrogen gas, 2.1 L/min.

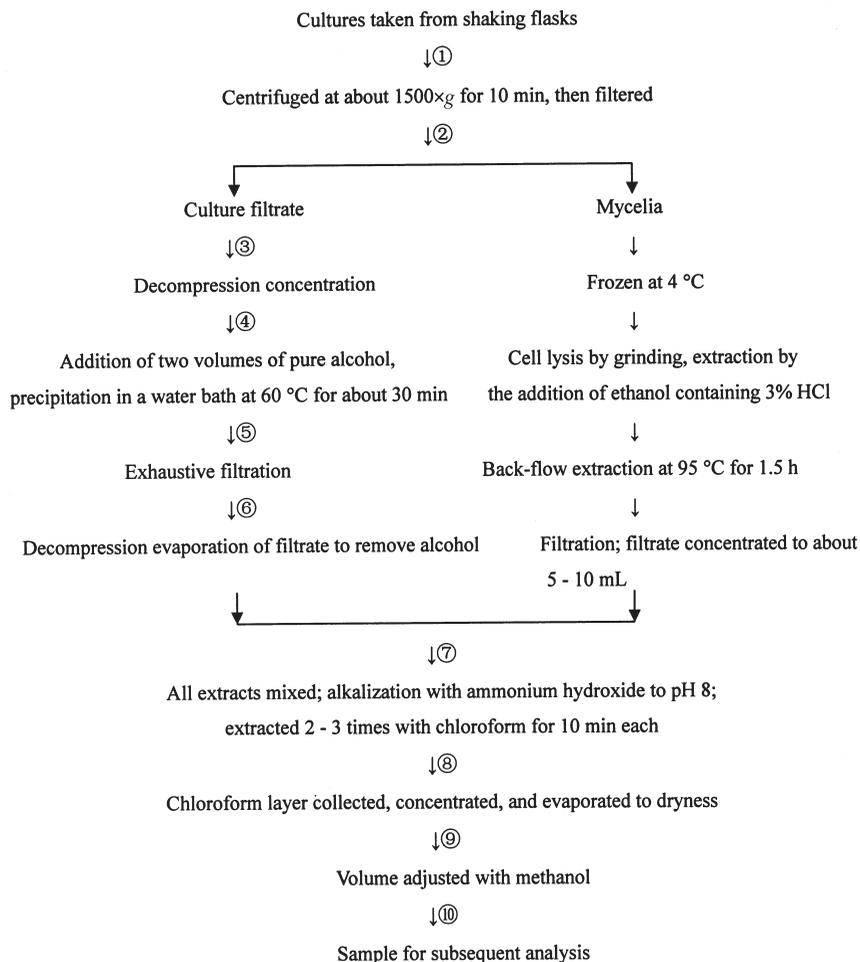


Fig. 1. Protocol for the extraction of cultures.

## Results and Discussion

### *Growth curves of the isolated strains*

Ten endophytic fungal strains (Fu1–Fu10) were isolated from the bulbs of *F. ussuriensis* confirming the proposition that endophytic microorganisms can be found in virtually every plant (Strobel and Daisy, 2003).

The results of the reaction with improved Dragendorff's reagent indicated that both the mycelia and the culture filtrates from strains Fu4, Fu6, Fu7, Fu8, and Fu10 contained alkaloids or similar compound(s). Therefore, they were selected for a further TLC assay. From their growth curves (Figs. 2a and b) it can be seen that these alkaloids were all produced around the stationary phase, which is

consistent with the general opinion that secondary metabolites are often produced after growth has stopped (Griffin, 1994).

### *Reselection of the sipeimine-producing strain*

According to TLC detection, only the sample from strain Fu7 produced a spot with the same  $R_f$  value as authentic sipeimine. So, it was selected for further analysis.

### *Distribution of sipeimine inside and outside the hyphae*

Further TLC analysis showed that the sample from the Fu7 culture filtrate produced four spots,

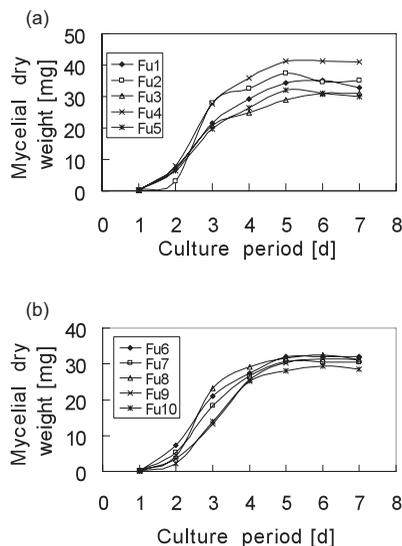


Fig. 2. Growth curves of the strains (a) Fu1–Fu5 and (b) Fu6–Fu10.

and the mycelial extract produced only one spot of very light colour on the silica-gel plate after development. The spot from the mycelial extract and one spot from the culture filtrate had the same  $R_f$  value and colour as authentic sipeimine. Therefore, we inferred that fungus Fu7 produces sipeimine or at least a compound similar to that of its

host plant *F. ussuriensis*. The main product was present in the culture filtrate. Therefore, subsequent assays were mainly performed using the culture filtrate.

#### Product identification

##### TLC scan

From Fig. 3 it can be seen that the Fu7 extract has a similar absorption curve to that of a standard sipeimine solution. Thus, the Fu7 extract should have the same chromophores as sipeimine.

##### HPLC-ELSD analysis

The HPLC-ELSD chromatograms show that both the sample and authentic sipeimine have the same retention times of about 10 min. Therefore, we infer that the sample contains the bioactive compound sipeimine.

Most *Fritillaria* alkaloids are nonchromophoric, which makes the use of direct UV detection without pre- or post-column derivatization impossible. Recently, studies on the use of HPLC coupled to ELSD have increased markedly, and the published results have demonstrated that ELSD is a simple and sensitive direct detection method for the analysis of the major active steroidal alkaloids in *Fritillaria* bulbs (Li *et al.*, 2001; Chen *et al.*, 2006).

Only since the end of the last century the isolation of phytoactive ingredients from endophytic

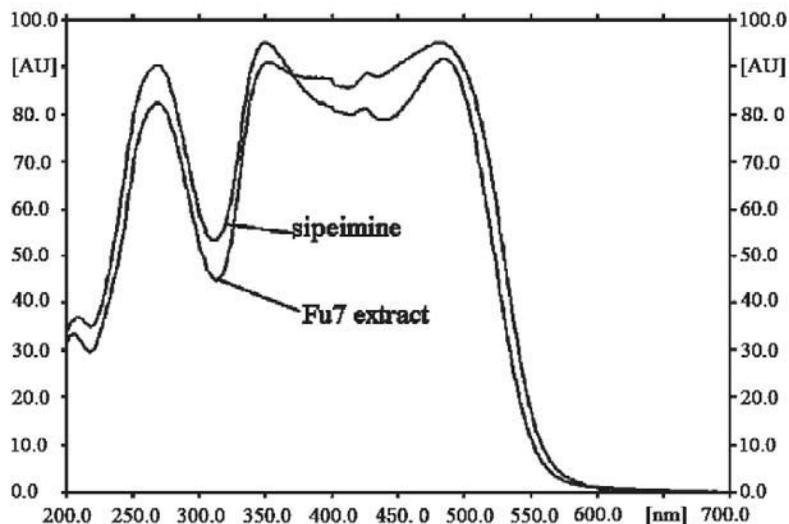


Fig. 3. TLC scan of the Fu7 extract.

fungi has been considered. It has been demonstrated that these endophytes produce a plethora of substances. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of the compounds found through isolation, culture, purification, and characterization of some selected endophytes in the recent past. The prospects of endophytes as new sources of natural products for exploitation in medicine, agriculture, and industry are good (Strobel and Daisy, 2003).

It is likely that the production of certain bioactive compounds by endophytes *in situ* may facilitate the domination of their biological niche within the plant or even provide protection to the plant from harmful invading pathogens. It may also enhance the survival rates of medicinal plants, and stimulate the production of special bioactive compounds (Strobel and Daisy, 2003; Liu *et al.*, 2005). Therefore, it is reasonable to assume that a thorough investigation of the endophytic fungi will significantly increase the yield and improve the quality of medicinal plants.

Our study has shown that the selected strain Fu7 produces the bioactive compound sipeimine as its host plant does, and could be used for sipeimine production by fermentation. However, the yield of sipeimine at present is not high enough for industrial fermentation (data not shown). Therefore, our future aim is to improve the strain and to optimize the culture conditions to enhance the fermentation yield of this strain. In this way, we hope to lay a sound foundation for a new method for producing such active botanical compounds.

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