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Original Article

Combination of on-line desalting and HPLC-UV-ESI-MS for simultaneous detection and identification of FIP-fve and flammutoxin in *Flammulina velutipes*

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ABSTRACT

A rapid analytical approach, on-line desalting HPLC-UV-ESI-MS method, for the analysis of FIP-fve and flammutoxin (FTX), two important bioactive proteins in the fruiting bodies of *Flammulina velutipes*, was developed. In this study, a highly efficient desalting method is provided using molecular weight cut-off centrifugal filtration and on-line desalting. Sample preparation followed by an on-line desalting HPLC-UV-ESI-MS system was employed for simultaneous desalting and detection and identification of FIP-fve and FTX. Results indicated that using trifluoroacetic acid as a modifier on a C18 reversed-phase column renders effective separation. ESI-MS revealed that the apparent molecular masses of FIP-fve and FTX were 12,749.1 Da and 21,912.5 Da, respectively. Eleven milligrams of FIP-fve was obtained from 100 g of fresh fruiting bodies, and UV detection was performed at 280 nm using bovine serum albumin as the standard protein. The calibration curve was linear in the concentration range of 0.29–4.69 mg/mL (r^2 = 0.9999). FTX and a series of degradation products were isolated from *F. velutipes* using 35% saturated ammonium sulfate on a DEAE cellulose column. The complete identification of FTX and a series of degradation products were carried out by precipitation of various ammonium sulfate concentrations (0–45%, 45–65% and 65–90%), in-gel trypsin digestion, and MS analysis with combined database search. The molecular weights of FTX and a series of degradation products were 29,957.2 Da, 27,480.2 Da, 26,512.5 Da, and 21,912.5 Da.

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1. Introduction

The golden needle mushroom (*F. velutipes*) is a popular edible fungus in Taiwan and Asia. Besides the medicinal and nutritional properties of golden needle mushrooms, their texture and flavor are particularly appreciated. Currently, these mushrooms are well known as a healthy food, owing to the presence of proteins, carbohydrates, vitamins, minerals, and high fiber amount but low fat content [1]. Mushrooms and their components have attracted increasing interest in biomedical sciences and functional food research. Numerous reported studies have primarily focused on two subjects: polysaccharides and proteins. Polysaccharide studies on *F. velutipes* have revealed strong immunomodulatory and antitumor activities, e.g., exhibited by their glucans and heteropolysaccharides [2–5]. The fungal immunomodulatory protein (FIP-fve) is an exceptional compound in *F. velutipes*, which plays a crucial role in the immunomodulatory response [6]. FIP-fve is classified in the family of FIPs, which contains LZ-8, FIP-vvo, and FIP-gts. These FIPs have been isolated and purified from *F. velutipes*, *Ganoderma lucidum (Lingzhi)*, *Volvariella volvacea*, and *Ganoderma tsugae* [6–9]. The immunomodulatory activity of FIP-fve has been demonstrated by its stimulatory activity toward human peripheral blood lymphocytes, inhibition of systemic anaphylaxis reactions, local swelling of mouse foot pads, as well as enhancing the transcription of IL-2, IFN-γ and TNF-α [6]. Previous studies have indicated that FIP-fve is a good candidate for biomedical studies and functional food applications [10,11].

Flammutoxin (FTX), another cellular toxic (bioactive) protein with cardiotoxic and cytolytic activities, has been isolated, characterized and designated by Lin et al. [12]. The FTX protein from the basidiocarps of *F. velutipes* has a molecular weight of 22 kDa and has been reported to cause the lysis of mammalian erythrocytes, the swelling and inhibition of the respiration of Ehrlich ascites tumor cells, electrocardiographical changes in animals with parenterally administered animals, and edema of rat paws [12]. Later, Bernheimer and Oppenheim purified a hemolytic protein of 32 kDa from the same mushroom and referred to it as FTX assuming that the FTX isolated in the study of Lin et al. [13] was derived from their 32 kDa FTX by partial proteolysis [14]. Tomita isolated FTX as a single hemolysin of 31 kDa from *F. velutipes*, determined the N-terminal 28 residues, and studied the molecular basis for the cytolytic action of the protein [15].

Hemolysins have been classically defined as exotoxins capable of causing the lysis of red blood and nucleated cells. Hemolysins are currently believed to be pore-forming toxins. Pore-forming proteins have been proposed to be responsible for gastrointestinal disorders, such as diarrhea and fluid accumulation. Clinical studies have not been reported on intestinal dysfunction caused by the excessive ingestion of *F. velutipes*, because FTX is labile to heat [16].

The aim of this study was to examine the proteins contained in golden needle mushrooms. An analytical method to simultaneously monitor both FIP-fve and FTX is an essential tool for the applications of the *F. velutipes* mushroom as a functional food ingredient or for commercial ready-to-eat pickled products. In this study, the development of an on-line desalting HPLC-UV-ESI-MS method for the quantitative analysis of FIP-fve is reported and the complexity of the detection of FTX is discussed. Further, the findings of this study will be used to establish a general, well-validated, method to determine fungal immunomodulatory protein content in various mushrooms.

2. Materials and methods

2.1. Standards and reagents

HPLC-grade acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Milli-Q grade water (Millipore, Bedford, MA, USA) was used in the mobile phase. An Amicon® Ultra-15 centrifugal filter (with a molecular weight cut-off, MWCO, of 3 kDa) was purchased from Millipore (MWCO, 3 kDa; Merck Millipore, Billerica, MA). All other analytical-grade chemicals were commercially purchased.

2.2. Sample preparation

The golden needle mushroom (*F. velutipes*) is purchased from local markets. First, fresh fruiting bodies of *F. velutipes* (200 g) collected from local markets were washed with water. Second, they were soaked overnight with 200 mL of 5% (v/v) acetic acid and 0.05 M 2-mercaptoethanol [6]. Third, this mixture was homogenized using a blender, and 95% saturated ammonium sulfate was added to the supernatant [6]. After centrifugation at 14,000 × *g* for 30 min at 4 °C, the precipitate was collected, desalted, purified, and concentrated using an Amicon Ultra-15 MWCO centrifugal filter unit. Finally, the extract was filtered using a 0.45 μm nylon filter, followed by immediate transfer into HPLC vials and analysis. The extracts were designated as crude protein extracts of *F. velutipes* and were used to evaluate analytical columns, to optimize analytical conditions and for protein isolation. The concentration of FIP-fve and FTX (including FDS (molecular weight: 20,087 Da, http://www.uniprot.org/uniprot/D2JY92, Last modified: February 9, 2010)) used in this study were 1100 and 840 μg/mL, respectively.

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