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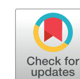
Original Article

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 online 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 $\times$ *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.

2.3. Purification and identification of flammutoxin and a series of degradation products

All purification steps were performed at 4 °C. First, fruiting bodies of *F. velutipes* were cut to pieces and soaked overnight in a buffer containing 5% acetic acid and 0.05 M β-mercaptoethanol at 4 °C. Second, the mixture was homogenized using a blender, and 35% saturated ammonium sulfate was added to the supernatant. Next, the precipitate was collected by centrifugation as mentioned and desalted by dialysis against a buffer (0.05 M Tris–HCl, pH 7.8) for 72 h at 4 °C. The DEAE-650M column (400 × 26 mm) was equilibrated by washing the column with 50 mM Tris–HCl (pH 7.8). Eluting solvents were A: 50 mM Tris–HCl (pH 7.8) and B: 50 mM Tris–HCl (pH 7.8) containing 0.5 M NaCl and eluted with 0–180 mL 0.035 M NaCl, 180–360 mL 0.055 M NaCl, and 180–360 mL 0.055–0.25 M NaCl. One fraction contained 8 mL.

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%), and in-gel trypsin digestion, and MS analysis with combined database search.

2.4. LC-UV-ESI-MS conditions

HPLC analysis of FIP-fve and flammutoxin was performed on a Dionex UltiMate 3000 HPLC system, comprising a DGP 3600 A dual gradient pump, a variable wavelength detector, and a WPS 3000SL autosampler with injection volume set at 10 μL. The UV detector was set to 280 nm for the real-time monitoring of the peak intensity. ESI-MS analysis was performed on an API 3200™ (Applied Biosystems, Foster City, CA, USA) coupled to the HPLC system. Optimized MS parameters were utilized: capillary voltage, 5500 V; turbo heater temperature, 550 °C; nebulizer gas (air, GS1, 50 psi); heater gas (GS2, 50 psi); curtain gas, nitrogen (12 psi); declustering potential (DP), 50 V; dwell time, 150 ms; and pause time, 5 ms. All mass spectra were recorded in the continuous scan mode for positive ions in the scan range of m/z 500–1800. Reconstruction software (Bioanalyst software, Applied Biosystems) was required to generate parent mass.

2.4.1. Columns

Purification was carried out on three columns containing either a C18 or C4 RP media, with pore sizes of 300 Å: Column 1: CTD C18 (5 μm, 150 mm × 2.0 mm I.D.); Column 2: Inertsil C18 (5 μm, 250 mm × 4.6 mm I.D.); and Column 3: ACE C4 (5 μm, 250 mm × 4.6 mm I.D.) The solid-phase extraction (SPE) column for on-line desalting was an Inertsil C18 (5 μm, 50 mm × 4.6 mm I.D.). The column oven temperature was set at 25, 35, and 45 °C, respectively.

2.4.2. Mobile phase and gradients

The mobile phase consisted of the combination of A (0.1% FA in water) and B (0.1% FA in ACN) or the combination of A (0.1% TFA in water) and B (0.1% TFA in ACN). The gradient was linearly varied from 10% B at 0 min and maintained constant at 10% B to 2.5 min, 10%–90% B (v/v) in 20 min, and finally to 10% B at 20.1 min and maintained constant at 10% B to 25 min at a flow rate of 0.8 mL/min for a column with an I.D. of 4.6 mm or a flow rate of 0.2 mL/min for a column with an I.D. of 2.0 mm.

2.4.3. On-line LC-MS desalting system

Using a combined HPLC-MS system, sample desalting is crucial to prevent the suppression of adduct formation during ionization, signal suppression, and ion-source fouling. Hence, two desalting procedures were performed using the crude protein extracts: MWCO centrifugal filtration and on-line desalting. The SPE column was placed before the analytical column by switching the valve to the "inject" position. Analytes were directly eluted with the mobile phase from the SPE column to the analytical column. In a 2D system, ammonium sulfate was flushed out as waste at ca. 2.5 min, and a six-port valve was switched to the position coupling the SPE column with the analytical column (Table 1). The analytes were then transferred into the analytical column.

2.5. Experimental design

This method was developed to determine the major proteins in *F. velutipes*. According to previous research [6,15,17], the soluble proteins of *F. velutipes* were precipitated by addition of ammonium sulfate. Then, cation exchange and size-exclusion chromatography were used to isolate FIP-fve and FTX. The purified proteins could then be subjected to biochemical analysis. RP-HPLC is a very powerful technique for the analysis of proteins because of the excellent resolution and reproducibility of repetitive separations [18]. The goal of this study was to develop an analytical method for the quantification and qualification of biologically active proteins. Therefore, soluble proteins were precipitated by addition of ammonium sulfate and detected by LC-UV-ESI-MS. Mass spectrometry (MS) has emerged as a rapid, sensitive technique for protein identification and characterization. The combination of UV and electrospray-mass spectroscopy (ESI-MS) was employed for the quantitative and qualitative analyses of proteins.

Typically, protein preparation for MS analysis is via precipitation, desalting, purification, and concentration. However, these methods have limitations, such as the use of large sample amounts as well as time-consuming analysis. Instead of dialysis and concentration, centrifugal filter devices were utilized to decrease the analysis time. With the use of precipitation reagents such as ammonium sulfate, a considerable amount of ammonium sulfate precipitate was generated.

Table 1 – Time program of the 2D LC system.

Run time	Left pump (Pump 1)		Run time	Right pump (Pump 2)		
	Flow mL/min	H_2O %		Flow mL/min	A %	B %
0	0.8	100	0	0.8	90	10
	0.8	100	2.5	0.8	90	10
	0.8	100	20.0	0.8	10	90
	0.8	100	20.1	0.8	90	10
25	0.8	100	25	0.8	90	10

A: 0.1% trifluoroacetic acid in water.
B: 0.1% trifluoroacetic acid in acetonitrile.

Hence, the desalting of samples is crucial for mass analysis to prevent the suppression of adduct formation during ionization, signal suppression, and ion-source fouling [19,20]. In the experiment, first, a simplified preparation utilizing MWCO centrifugal filter devices, which provided efficient desalting and sample concentration, and a dual pump system for further desalting, were employed. Next, RP-HPLC, coupled with dual UV-ESI-MS detection, was employed for separation.

2.6. Optimization of on-line desalting HPLC conditions for analyzing proteins in F. velutipes

2.6.1. Desalting

In this study, the soluble proteins of *F. velutipes* were precipitated by addition of ammonium sulfate. Ammonium sulfate is a nonvolatile salt and is incompatible with a mass spectrometric system. For HPLC–MS, desalting is often required before analysis to avoid ion suppression effects and charge-adduct formation. MWCO centrifugal filters were utilized for rapid desalting and sample concentration. Filtration was conducted two times by the addition of distilled water. The majority of salt can be removed by filtration however; a concentrated solution still contains a small amount of salt. A concentrated solution still contains a small amount of salt. To avoid possible clogging on the ESI interface, an on-line desalting SPE was installed before HPLC column to remove the residual salt. Because the salt cannot be retained in the reverse phase column, they will be eluted first and be removed. In a dual pump system, the mobile phase eluting at 2.5 min was considered as waste resulting from desalting of the SPE column. Filter devices as well as the dual pump system was effective for solving salting issues. The desalting system exhibited minimized ion suppression effects in the mass spectrum. Thus, in this study, a highly efficient desalting method is provided.

2.6.2. Evaluation of HPLC conditions

Reversed-phase (RP)–HPLC was employed to separate the proteins of *F. velutipes*. A gradient was designed to determine the protein profile with high resolution and sensitivity while permitting molecules to be rapidly eluted [18]. Typically, the modifiers TFA or FA were added to water and ACN at a final concentration of 0.1% (v/v) and the results were compared. Fig. 1 shows the resolution and selectivity of the proteins from the crude extracts in three columns with TFA and FA. Six chromatograms (A–F) were obtained by LC with UV detection at 280 nm. Chromatograms A & B, C & D, and E & F correspond to separation on the CTD C18, Inertsil C18, and ACE C4 columns, respectively. From the comparison of the resolution in the six modes, TFA exhibited better resolution than FA in three columns. TFA is known to be one of the best "modifiers" for LC and does not interfere with the spectrophotometric detection of the effluents in the UV region. TFA can increase the conductivity and surface tension of water. It is difficult to produce electrospray for solutions with high surface tension and high conductivity, a phenomenon attributed to an

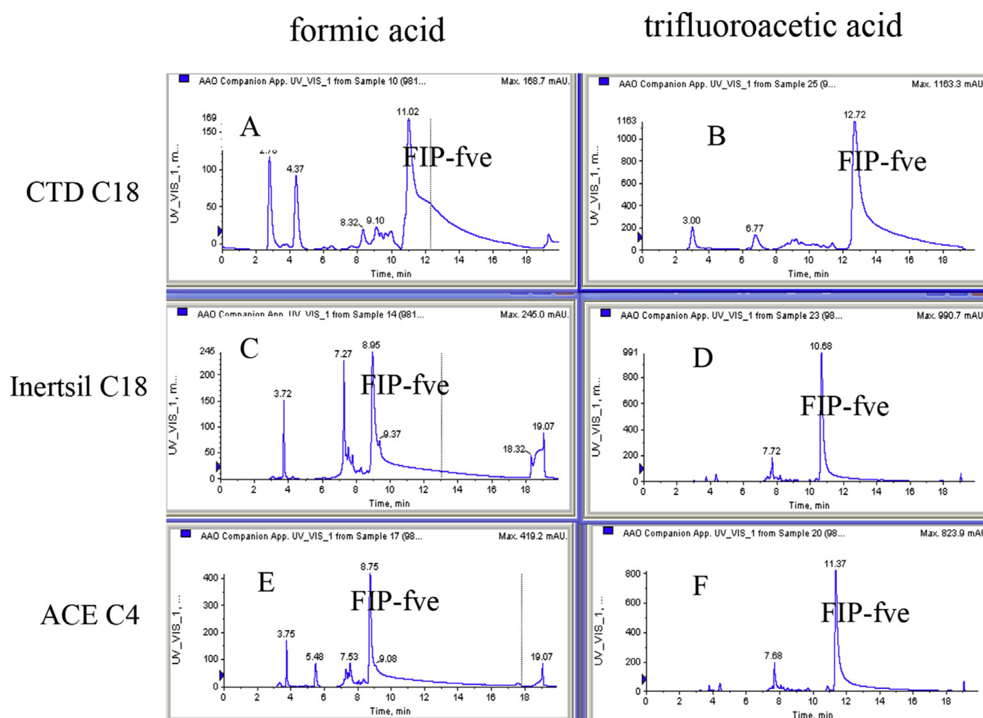

Fig. 1 – UV-280 nm chromatograms of crude protein extract of *Flammulina velutipes* on three commercially available columns with two modifiers (TFA and FA). Inertsil C18 (5 μm, 250 mm × 4.6 mm I.D.); CTD C18 (5 μm, 150 mm × 2.0 mm I.D.); and ACE C4 (5 μm, 250 mm × 4.6 mm I.D.). The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in ACN) or solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN). The proportion of solvent B was linearly increased from 10% to 90% in 15 min, then 10% B at 15.1 min and maintained constant at 10% B to 20 min at a flow rate of 0.8 mL/min for a column with an I.D. of 4.6 mm or at 0.2 mL/min for a column with an I.D. of 2.0 mm.

unstable Taylor cone. For good electrospray, highly charged droplets must be steadily emitted from the tip of a stable Taylor cone, thus, instabilities can cause the frequent breakdown of the spray and reduction in the analyte ion signal [21]. However, LC/MS with a mobile phase containing aqueous 0.1% TFA exhibited adequate sensitivity.

The effects of peak asymmetry were investigated by selecting the CTD C18, Inertsil C18, and ACE C4 columns and the modifiers TFA and FA. The asymmetry value was changed to 13.5, 13.6, 11.1, 12.6, 3.3, and 8.6 for A-F, respectively (Fig. 1). Tailing peaks were observed for the CTD C18 column with both modifiers. The best resolution was observed for Inertsil C18 with TFA.

Two columns were thermostated at 35 °C, resulting in less peak tailing. HPLC-ESI-MS was used to confirm the peak assignments of HPLC-UV analysis.

2.7. *Protein identification*

FIP-fve, FTX and a series of degradation products were collected from the HPLC system for confirmation. The eluted drops were collected, nitrogen purge dried and re-dissolved in 200 μL of distilled water for bottom-up identification approach analysis. The protein was reduced by addition of 10 mM DTT in 25 mM ammonium bicarbonate. Samples were then left at 56 °C for 45 min after which the DTT solution was aspirated, and 55 mM iodoacetamide in 25 mM ammonium bicarbonate was added. Alkylation was performed for 30 min in the dark. For protein digestion, 25 μL of 5 μg/mL trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate containing 2.5 mM $CaCl_2$ (pH 7.8) was added to each sample and incubated overnight at 37 °C. The resulting tryptic fragments were identified from peptide mass fingerprinting.

Reverse phase separation of the peptide mixture was performed in a nano-HPLC system (Agilent Technologies) using a C-18 column (75 μm i.d., 150 mm L, 3 μm particles) at a flow rate of 200 nL/min. The solvent system was as follows: solvent A was 0.1% (w/v) formic acid/H_2O/2% (v/v) acetonitrile, and solvent B was 0.1% (w/v) formic acid/H2O/80% (v/v) acetonitrile. The gradient was isocratic with 2% solvent B, eluted with a linear gradient from 2% solvent B to 60% solvent B over 90 min; the column was then washed using 80% solvent B for 10 min and rebalanced with buffer A for 15 min. The nanoHPLC system was connected on-line to a hybrid LC-MS/MS system (Applied Biosystems API QSTAR XL, MA). On-line MS and tandem MS spectra were obtained using the TOF analyzer with m/z scanning ranges of 400–1200 Da for MS and 75–1500 Da for MS/MS analyses. The MS/MS data were analyzed for identification and quantitation using Mascot Daemon (Matrix Science, London, U.K.; version 2.2.1) and ProteinPilot (Applied Biosystems, USA; version 2.0.1). Search parameters included trypsin as the enzyme with one missed cleavage allowed. Fragment ion mass tolerance and precursor ion tolerance were set to 0.20 Da. Fixed modifications: Carbamidomethyl; Variable modifications: Oxidation.

2.8. *SDS-PAGE*

The SDS-PAGE analyses of samples were performed according to Laemmli [22] in 15% slab gels on a Bio-Rad mini protein II gel apparatus (Hercules, CA, USA). The gels were stained with Coomassie brilliant blue R.

2.9. *In-gel enzymatic protein digestion and identification*

The protein bands of FTX and a series of degradation products were excised from the SDS-PAGE gel. After removing the ethanol solution, gel pieces were incubated in 100 μL distilled water for 10 min at room temperature and then in 40 μL of 50% ACN for 10 min. This step was repeated three times. Subsequently, the supernatants were removed, and the excised gel fragments were equilibrated with 200 μL of 50% ACN containing 25 mM ammonium bicarbonate for removing the Coomassie Blue stain. This step was repeated three times. The enzymatic digestion of proteins is performed in the same manner as described in the previous paragraph.

2.10. *HPSEC-UV-RID system*

The purities of FTX and a series of degradation products were confirmed by HPSEC-RID. The HPSEC High performance size-exclusion chromatography-refractive system included a Metrohm 709 IC pump (Metrohm, Herisau, Switzerland), a column oven (Super Co-150, Enshine, Tainan, Taiwan) equipped with a Rheodyne injector (Cotati, PA. USA) and a 200-μL sample loop, a UV-975 detector (Jasco, Tokyo, Japan) and an OPTILAB DSP interferometric refractometer (P10 cell, 690 nm, Wyatt) with the temperature controlled at 35 °C. The purity was analyzed by using 2 TSK-Gel columns (7.5 × 300 mm), PW_{XL} 4000 and PW_{XL} 3000, connected in series along with the TSK-Gel PWH Guard column, and eluted with 0.3 N $NaNO_3$ at a flow rate of 0.4 mL/min.

3. Results

3.1. *Data of optimized LC-UV-MS conditions*

The soluble protein extracts of *F. velutipes* were analyzed directly by LC-UV-MS. UV and MS spectra were recorded on HPLC-UV-ESI-MS; after chromatography two sharp peaks (16–18 min) were obtained as shown in Fig. 2. Using a 25 min run time, Peak 1 was resolved from the mass chromatogram with a retention time of 17.02 min. The ESI mass spectrum of designated peak 1 exhibited two distributions of multiply charged ions with m/z of 897, 934, 975, 1019, 1068, 1121, 1180, 1246, 1319, 1401, 1495, and 1601, the charge states of which were in the range of +14 to +25, and m/z of 877, 914, 954, 996, 1044, 1096, 1154, 1218, 1290, 1370, 1461, and 1566, the charge states of which were in the range of +14 to +25. The mass spectrum of peak 1 after deconvolution exhibited two major peaks with m/z of 22,413.4 Da and 21,912.5 Da, respectively (Fig. 2). Another peak observed at 17.55 min from the mass chromatogram (Fig. 2). The ESI mass spectrum of the crude extracts exhibited a broad distribution of multiply charged ions with m/z of 1594, 1417, 1276, 1160, 1063, 982, 911, and 851, the charge states of which were in range of +9 to +15. The mass spectrum of peak 2 after deconvolution provided a molecular weight of 12,749.1 Da.

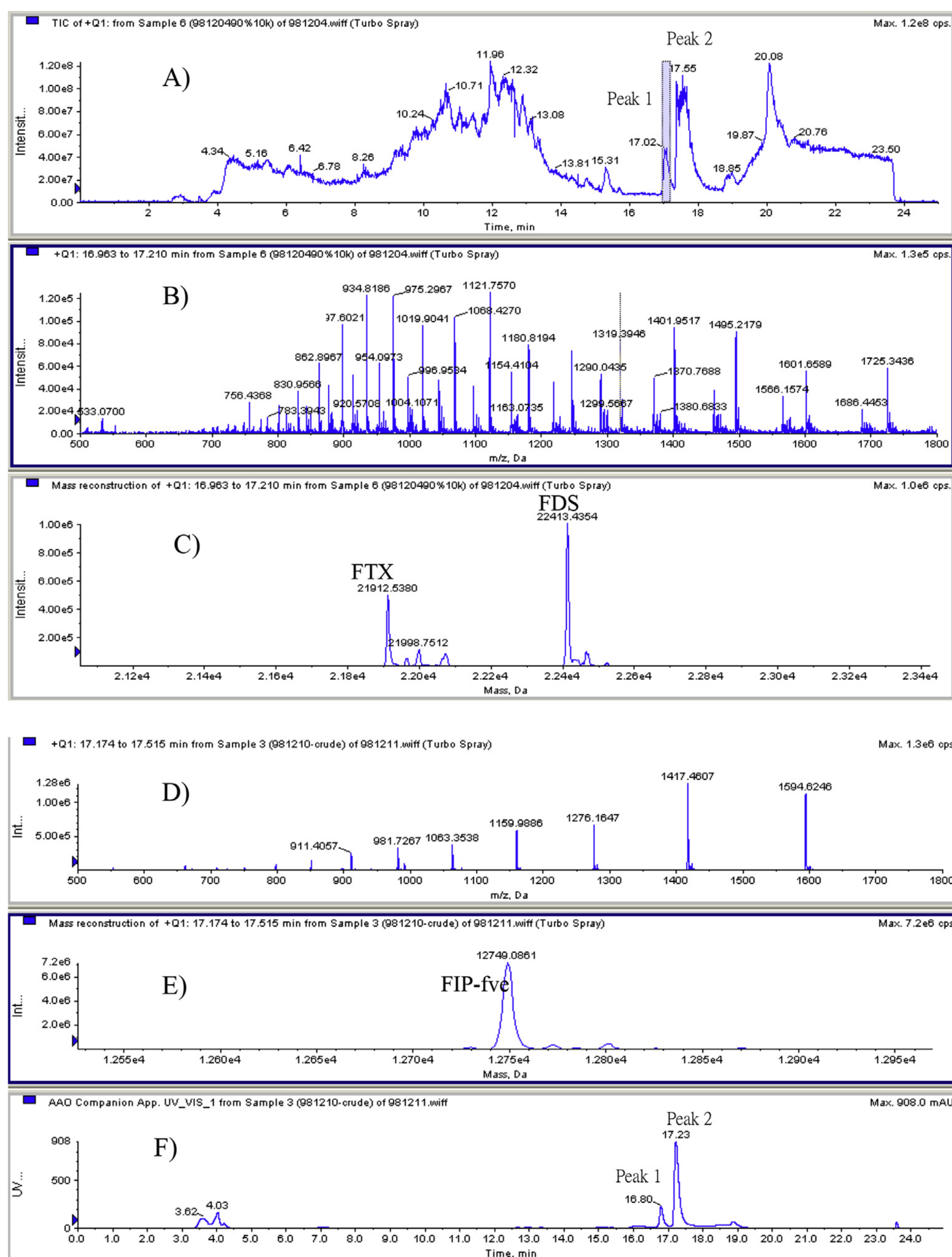

Fig. 2 – HPLC-UV-ESI-MS chromatograms of precipitation of crude protein extract by 95% saturated ammonium sulfate on an Intertsil C18 column. A) TIC: total ion chromatogram of MS, B) MS spectra of the peak of flammutoxin (FTX) and a putative uncharacterized protein, FDS, and C) Reconstructed mass based on the multiply charge states. Bioanalyst software (Applied Biosystems) was employed to generate the reconstructed parent mass. Raw mass spectrum showing the distribution of charge states observed from m/z 500 to 1800.

Fractions corresponding to peak 1 were collected for confirmation. The eluted drops were collected, nitrogen purge dried and re-dissolved in 200 μL of distilled water for bottom-up identification approach analysis. The intact mass of the peptide was obtained from the mass spectrum and screen of Mascot, which was used to determine the candidate proteins. After bottom-up approach analysis, the database search resulted in FTX protein with sequence coverage of 65% and Mascot score of 3385, FDS protein with sequence coverage of 30% and Mascot score of 1808, respectively. Two protein components, FTX and FDS, were resolved from peak 1. The mass spectrum exhibited molecular weights of 21,912.5 and 22,413.4 Da after BioAnalyst™ deconvolution (Fig. 2), which corresponded to those of FTX and FDS, respectively. Further experiments of purification of FTX need to be performed.

Peak 2 fractions at a retention time of 17.1–17.5 min were collected from the HPLC system, followed by further protein identification analysis. The database search resulted in FIP-fve

protein with sequence coverage of 99% and Mascot score of 3736. Lin et al. [6] have isolated FIP-fve from F. *velutipes* and calculated a molecular mass of 12,704 Da from its amino acid composition. Paaventhan et al. [18] have employed ESI-MS for the determination of FIP-fve and reported a mass of 12,745.9 Da, corresponding to the calculated mass of the protein (12,746.3 Da) by considering its N-terminal acetylated serine residue. The results obtained herein were consistent with those reported previously [6,23]. Nevertheless.

3.2. Quantification of FIP-fve and detection of FTX

UV detection was employed to quantify FIP-fve and FTX. FIP-fve and FTX (including FDS) concentration of crude protein extract were 1100 and 840 μg/mL, respectively, and UV detection was performed at 280 nm using bovine serum albumin (BSA) as the protein calibration standard. The calibration curve was linear in the concentration range of 0.29–4.69 mg/mL (r^2 = 0.9999). Eleven milligrams of FIP-fve was obtained from 100 g of fresh fruiting bodies. Since two protein components, FTX and FDS, were resolved from peak 1, the FTX content in fresh fruiting bodies could not be estimated. Further experiments on the purification of FTX need to be performed.

3.3. Identification of FTX and a series of degradation products

The identification of FTX and a series of degradation products was carried out by in-gel enzymatic protein digestion and analysis. The crude extracts exhibited two major protein bands with molecular weights of 13 kDa and 30 kDa, respectively, and one faint band corresponding to a molecular weight of 22 kDa. A similar result has been reported by Ko et al. [6]. SDS-PAGE as well as UV-MS analysis provided corresponding molecular weight measurement for FIP-fve.

Lin et al. (1974) [13] have isolated FTX with a molecular weight of 22 kDa from F. *velutipes*. Bernheimer and Oppenheim (1987) [14] have purified a hemolytic protein of 32 kDa from the same mushroom and assumed that the FTX of Lin et al. would have been proteolytically derived from their 32 kDa protein. Tomita et al. (2004) [24] have cloned and expressed the cDNA encoding an FTX protein, which was a 272 amino acid residue protein with molecular weight of approximately 31 kDa. However, herein, two molecular weights of 31 kDa and 22 kDa were observed from SDS-PAGE and mass spectral analyses of the crude extract with 95% saturated ammonium sulfate. The results obtained herein can prove Bernheimer's hypothesis. Two protein components, FTX and FDS, the molecular weights of 22,413.4 and 21,912.5 Da, were observed from peak 1. The FTX of 22 kDa would have been proteolytically derived from their 30 kDa protein. To illustrate this hypothesis, ammonium sulfate precipitation was used to collect possible degradation products of FTX.

Because the precipitate composition had a relatively high concentration of FIP-fve by addition of 95% saturated ammonium sulfate. This would cause that FTX was difficult to purify from 95% saturated ammonium sulfate fractionation. Ammonium sulfate fractionation and DEAE cellulose column chromatography were used for preparing FTX and its series of degradation products (Figs. 3 and 4). The fractionations were subjected to both LC-UV-MS and SDS-PAGE for molecular weight measurement. FTX and its series of degradation products co-eluted on the LC. Spectral analysis by Bio-Analyst™ deconvolution revealed that the molecular weights of FTX and a series of degradation products are 29,957.2 Da,

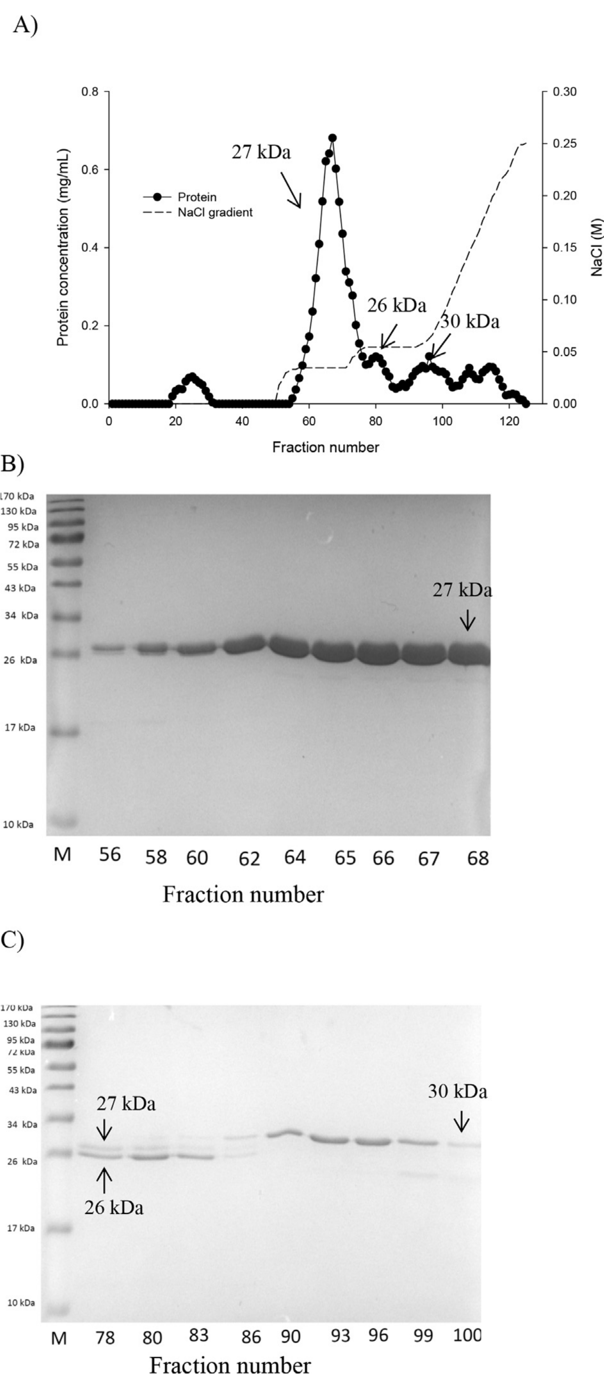

Fig. 3 – Purification of flammutoxin by 35% ammonium sulfate A) Chromatographic profile of flammutoxin fractionation on a DEAE-650M anion-exchange column; B) Lane: M, Molecular weight markers; electrophoretogram of flammutoxin fractionation after anion-exchange chromatography in fractions 56–68 C) fractions 78–100.

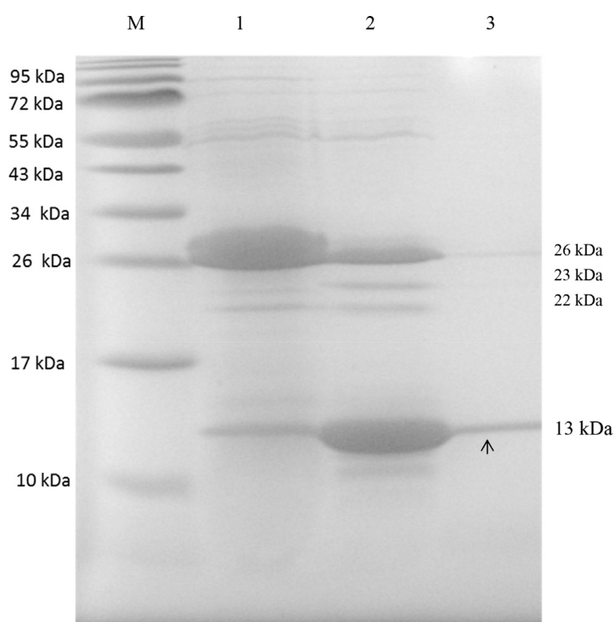

Fig. 4 – SDS-PAGE analysis of precipitation of crude protein extract by different concentrations (0–45%, 45–65% and 65–90%) of saturation ammonium sulfate cut. Lane: M, Molecular weight markers; Lane 1: 0–45% saturation ammonium sulfate cut; Lane 2: 45–65% saturation ammonium sulfate cut; Lane 3: 65–95% saturation ammonium sulfate cut.

27,480.2 Da, and 26,512.5 Da. The in-gel digestion bottom-up experimental results showed FTX protein 30 kDa with a sequence coverage of 40% and a Mascot score of 338, the 27 kDa with a sequence coverage of 52% and Mascot score of 1740 and the 26 kDa with sequence coverage of 37% and Mascot score of 1215, respectively. Their purities were greater than 90%, as confirmed by HPSEC-RID (High performance size-exclusion chromatography-refractive index detection).

The FTX and it degradation products 26–30 kDa proteins was enriched in 0–45% saturation ammonium sulfate fraction, according the results of in-gel trypsin digestion and MS analysis (Lane 1, Fig. 4). Four proteins, FIP-fve (13 kDa) and FTX degradation products (26–27, 22 kDa) and FDS (23 kDa) enriched in 45–65% ammonium sulfate fraction, according the results of in-gel trypsin digestion and MS analysis (Lane 2, Fig. 4). We also confirm this result on LC-UV-ESI, but only degraded FTX (21,912.5 Da) and FDS (22,413.4 Da) were observed. The degraded FTX 22 kDa was not major protein on SDS-PAGE analyses but it exhibited a major FTX mass spectrometric response. We confirmed by using purified proteins that the larger molecules of FTX 30, 27 and 26 kDa co-eluted with FTX 22 kDa and only showed low ionization efficiency on ESI like other large proteins from various sources [25] and could not be observed on LC-UV-MS chromatogram in the crude protein extract (Fig. 2). Therefore, we only found the MS spectra of 22 kDa flammutoxin from MS chromatograms, precipitation of fresh crude protein extract by 95% saturated ammonium sulfate.

4. Discussion

In this paper, an analytical method to simultaneously identify and monitor both FIP-fve and FTX in *F. velutipes* was established using the combination of on-line desalting and HPLC-UV-ESI-MS. Three columns, two modifiers, and three column oven temperatures were investigated for the development of a suitable HPLC method. Generally, TFA causes frequent breakdown of the spray and reduction in the analyte ion signal. However, by the comparison of the ion suppression effects of TFA and FA, no significant difference was observed from TIC chromatograms of FIP-fve. As compared to formic acid, TFA exhibited better resolution. Results revealed that the optimum conditions to obtain high resolution involved the addition of 0.1% TFA as a mobile phase modifier with the Inertsil C18 columns at 35 °C. The procedure was advantageous as only 10 μL of extracts was required for a single analysis and quantitative evaluation of FIP-fve.

The SDS-PAGE results showed that the fresh crude extracts exhibited two major protein bands with molecular weights of 13 kDa and 30 kDa, respectively. While we stored the extracts at 4 °C for several days to evaluate analytical column, optimizing analytical condition for HPLC-UV-ESI-MS analysis as shown in Fig. 1, but we only found FIP-fve from UV chromatograms. However, the fresh crude protein extracts of *F. velutipes* were subjected to analytical column, after chromatography two sharp peaks (16–18 min) were obtained as shown in Fig. 2. According Bernheimer and Oppenheim (1987) [14], 22 kDa of FTX was assumed proteolytically derived from their 32 kDa protein. We also found that FTX was rather unstable. Therefore, To minimize proteolysis of FTX, all purification steps were carried out at 0–4 °C, and stored at −80 °C until used [15,17]. The SDS-PAGE results showed that the fresh crude extracts exhibited FTX bands with molecular weights of 30 kDa, while the mass spectrum showed an average molecular mass of 21,912.5 Da after deconvolution. In addition, we found FTX and FDS were resolved from the same peak from the mass spectrum. Hence, we purified FTX and its degradation products to confirm that the results of HPLC-UV-ESI-MS analysis. FTX and a series of degradation products were isolated from *F. velutipes* using 35% saturated ammonium sulfate on a DEAE cellulose column. FTX and a series of degradation products were isolated with the molecular weights of 29,957.2 Da, 27,480.2 Da, and 26,512.5 Da. However, 22 kDa FTX could not be purified from 35% saturated ammonium sulfate fractionation. Therefore, the precipitate of various ammonium sulfate concentrations (0–45%, 45–65% and 65–90%) were collected to confirm two protein components, the molecular weights of 21,912.5 and 22,413.4 Da, FTX and FDS. The molecular weight of 21,912.5 Da is presumed to be FTX and the molecular weight of 22,413.4 Da is presumed to be FDS by the results of in-gel trypsin digestion and MS analysis of 45–65% ammonium sulfate fraction.

In summary, an HPLC-UV-ESI-MS method was developed, the UV detector was set at 280 nm for the real-time monitoring of the peak of protein, and HPLC-ESI-MS was used to confirm the peak assignments of HPLC-UV analysis for qualitative and quantitative analyses. This method was excellent for the determination of low-molecular-weight proteins in *F. velutipes*.

Instead of dialysis, MWCO centrifugal filter devices and an on-line desalting HPLC-UV-ESI-MS system were utilized. Applications of the analytical method could be extended, including to monitoring the stability of FIP-fve and FTX under different processing conditions, to distinguish products that contain FVP-fve and FTX, and to profile the levels of the bioactive protein in different developmental stages or cultivation techniques. The complete identification and analysis of proteins required digestion followed by the bottom-up identification approach.

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