

Protein identification from two-dimensional gel electrophoresis by connecting ZipTip with a home-assembled nanoflow system

Chia-Hsien Feng · Chi-Hsien Chou · Lea-Yea Chuang ·
Chi-Yu Lu

Received: 10 February 2010 / Accepted: 18 June 2010 / Published online: 4 July 2010
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Abstract Mass spectrometry (MS) is a powerful technique for protein identification in proteomic research. Two-dimensional gel electrophoresis (2-DE) combined with MS is a significant method for protein separation and identification. For protein identification, peptide sequencing is usually carried out by an effective but expensive nanoflow liquid chromatographic system combined to tandem mass spectrometry (MS/MS). However, protein identification based on such method is time-consuming, and contamination may occur as a result of column overloading. In this study, we establish an alternative nanoscale system for protein identification using MS/MS. The system consists of several devices that can be purchased from commercial sources and can be connected to an electrospray ionization quadrupole-time of flight (ESI-Q-TOF) MS in order to analyze proteins from 2D gels. This inexpensive strategy provides an attractive alternative method for rapid identification of proteins using a nanospray source. In addition, the device is disposable

so that contamination is avoided. It is shown that peptide sequencing based on this device using ESI-Q-TOF MS is accomplished within 10 min.

Keywords Peptide sequencing · Nanoflow · Nanoscale · Home-assembled · Nanospray

Introduction

Mass spectrometry (MS) is a powerful technique for the identification of trace levels of desired analytes in biological matrix [1–3]. Tandem mass spectrometry (MS/MS) plays an important role in analyzing complex protein samples in proteomic study because of its capabilities for rapid and sensitive protein identification and quantitation [4–6]. Two-dimensional gel electrophoresis (2-DE) combined with MS is the core platform for proteomic research. The major merit of 2-DE is its abilities to separate thousands of proteins according to their molecular weight (MW) and pI [7–10]. After digestion, 2-DE allows these isolating proteins to be identified by MALDI-MS or LC-MS/MS [11]. Today, MALDI-MS and LC-MS/MS have become important tools for protein identification by peptide mass fingerprinting (PMF) and peptide sequencing. However the latter is more suitable for protein identification, because peptide sequencing is based on both peptide molecular mass and product ion information, not based on peptide molecular masses alone [12, 13].

In the determination of trace amounts of desired analytes in biological samples, sample separation and purification are very important. For MS, these issues are especially critical because the salts and undesired components may reduce the ionization efficiency and sensitivity. This is the so-called ion suppression effect. To overcome this problem, mini sample separation and purification tools have been

C.-H. Feng
Department of Fragrance and Cosmetic Science,
College of Pharmacy, Kaohsiung Medical University,
Kaohsiung 807, Taiwan

C.-H. Chou
Center for Resources, Research and Development/Graduate
Institute of Medicine, College of Medicine,
Kaohsiung Medical University,
Kaohsiung 807, Taiwan

L.-Y. Chuang · C.-Y. Lu (✉)
Department of Biochemistry, College of Medicine/Center
of Excellence for Environmental Medicine,
Kaohsiung Medical University,
Kaohsiung 807, Taiwan
e-mail: cylu@kmu.edu.tw

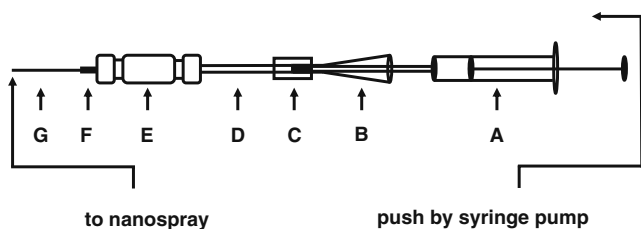


Fig. 1 Schematic diagram of the nanoscale separation system that combines ZipTip, syringe and syringe pump. Each part from A to G represents: A, 50 μL syringe; B, ZipTip; C, PTFE tubing; D, capillary PEEK tubing; E, zero-dead-volume union; F, nanotight tubing sleeve and G, fused-silica capillary. Syringe pump is used to load the mobile phase and push the eluent into nanospray source

developed, such as ZipTip [14]. ZipTip is widely used for sample concentration and desalting for protein and peptide analysis. It contains a small amount of stationary phase immobilized in the sharper point of the tip body. After equilibrating, binding and washing steps, target compounds are desorbed from the stationary phase. The elution solution can be deposited onto the target plate of the MALDI-MS or injected onto the LC-MS/MS system directly. The design of ZipTip is disposable, therefore, sample cross-contamination would be avoided.

In this study, we used several commercial parts to connect ZipTip with ESI-MS/MS. According to this setup, tryptic digestion peptides would be eluted from ZipTip to the nanospray source by syringe pump with a flow rate of 600 nL min^{-1} immediately. This on-line disposable ZipTip strategy shortened the analytical time of peptide separation and protein identification. Application of this method to identify proteins from 2D gels was successful.

Experimental

Materials and chemicals

Sequence-grade modified trypsin was obtained from Promega (Madison, WI, USA, <http://www.promega.com>). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, <http://www.sigmaldrich.com>). Acetonitrile (ACN), formic acid (FA), trifluoroacetic acid (TFA) and other analytical grade reagents were supplied by E. Merck

(Darmstadt, Germany, <http://germany.merck.de>). The water used was Milli-Q grade (Millipore, Bedford, MA, USA, <http://www.millipore.com>). All chemicals and reagents for electrophoresis were purchased from Amersham Biosciences (Uppsala, Sweden, <http://www.amershambiosciences.com>). ZipTips with C18 resin were purchased from Millipore. Gastight 50 μL syringe (needle gauge 22 s) was purchased from Hamilton (Reno, Nevada, USA, <http://www.hamiltoncompany.com>). Zero-dead-volume union and PTFE tubing (1.6 mm OD and 750 μm ID) were from Vici (Schenkon, Switzerland, <http://www.vici.com>). Capillary PEEK tubing (800 μm OD and 65 μm ID) and nanotight tubing sleeve (1.6 mm OD and 395 μm ID) were from Upchurch Scientific (Oak Harbor, USA, <http://webstore.idex-hs.com>). Fused-silica capillaries of 25 μm inner diameter and 375 μm outer diameter were obtained from Polymicro Technologies (Phoenix, AZ, USA, <http://www.polymicro.com>).

Instrumentation

All setup used for 2-DE were purchased from Amersham Biosciences. Tandem mass spectra were acquired by Waters-Micromass electrospray ionization quadrupole-time of flight (ESI-Q-TOF) Global mass spectrometer with a syringe pump. (Manchester, UK, <http://www.waters.com>). The Cap LC system is comprised of three pumps (pumps A, B and C), an autosampler, an inline degasser, a sample cooler, a syringe pump and a switch valve (Manchester, UK). A trapping column (C18 PepMap, 300 μm ID, 5 mm, LC Packings, Sunnyvale, USA, <http://www.lcpackings.nl>) was used for peptide enrichment and desalting. The separation was performed on a Micro-tech Scientific Inc. (Vista, CA, USA, <http://www.cvcmicrotech.com>) 10 cm RP C18 nano-LC column (150 μm inner diameter; 375 μm outer diameter; 3 μm particle size).

Cell culture

The Mesangial 13 cell lines (glomerular mesangial cells from an SV40 transgenic mouse) were purchased from Food Industry Research and Development Institute, Bioresource Collection and Research Center (Hsinchu,

Table 1 On-line ZipTip step-by-step procedures

Step	Description
1	Insert the syringe needle into the ZipTip
2	ZipTip wet, equilibrate, bind peptides and desalt by drawing and pulling the plunger of the syringe by hand
3	Remove the syringe needle from the ZipTip
4	Load the syringe with the elute peptide solution and insert the syringe needle into the ZipTip again
5	Pull the syringe by syringe pump and elute the peptide into the nanospray source

Table 2 Solution for on-line ZipTip procedures with nanoESI-MS/MS

Step	Description	Solution	Volume (μL)
1	Wet	ACN: 0.1% TFA=50:50 ($v v^{-1}$)	10
2	Equilibrate	0.1% TFA	10
3	Bind peptides	load tryptic peptides	10
4	Desalt	0.1% TFA	10
5	Elute peptide	ACN: 0.1% TFA=50:50 ($v v^{-1}$)	10

Taiwan). The Mesangial 13 cell lines were cultured in a 3:1 mixture of DMEM and Ham's F12 medium, supplemented with 5% fetal bovine serum, 14 mM Hepes, 2 mM glutamine, and $100 \mu\text{g mL}^{-1}$ penicillin-streptomycin at 37 C under 5% CO_2 .

2-DE

IEF was performed using an Ettan IPGphor II system. Ready to use 13 cm Immobiline DryStrips (pH 3–10 NL) were rehydrated overnight for 12 h at room temperature in 250 μL rehydration buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 20 mM DTT, 0.5% IPG buffer and a trace of bromophenol blue. Mesangial 13 cell lysate ($\sim 100 \mu\text{g}$) was mixed with 100 μL rehydration buffer containing 100 mM DeStreak reagent instead of 20 mM DTT. After cup-loading by the manufacturer's protocol, protein focusing was conducted for a total of 20,000 Vh. The IPG strips were equilibrated for 15 min in 10 mL equilibration buffer (50 mM Tris-base, pH=8.8, 6 M urea, 30% $v v^{-1}$ glycerol, 0.2% $w v^{-1}$ SDS and 1% $w v^{-1}$ DTT), and followed by 10 mL of the same solution containing 2.5% $w v^{-1}$ iodoacetamide instead of DTT for 15 min. The 10% SDS polyacrylamide gels (13 cm) were employed for the second dimensional separation. The second dimension was performed by 10% SDS polyacrylamide gels (13 cm) using the Hoefer

SE 600 vertical chambers. The second dimension separation was performed sequentially with a constant voltage of 70 V for 0.5 h, and 120 V for 12 h. The separated gels were fixed, washed and visualized by silver staining, and then the spots were analyzed using Image Master 2D Platinum system.

In-gel digestion

An in-gel digestion protocol was adapted as described by Wang [15]. Briefly, silver stained protein spots were excised, destained, washed and dehydrated in ACN for 20 min. The solution was then aspirated and gel pieces were then brought to complete dryness. Gel pieces were rehydrated with a freshly prepared solution of sequence-grade modified trypsin ($20 \text{ ng } \mu\text{L}^{-1}$ in a 25 mM ammonium bicarbonate buffer) at 37 °C for 16 h. The tryptic peptides were extracted with 50% ACN containing 5% FA by sonication in a water bath for 15 min. The extracted solutions were evaporated to dryness in a SpeedVac and then redissolved in 10 μL 0.1% TFA.

Sample loading, desalting and separation by on-line ZipTip

As shown in Fig. 1, one open end of ZipTip (Fig. 1 part B) was connected to a capillary PEEK tubing (Fig. 1 part D) by a PTFE tubing (Fig. 1 part C) and the other end was

Fig. 2 Illustration of ZipTip operative procedures by this home-assembled device. The detailed descriptions are listed in Table 1

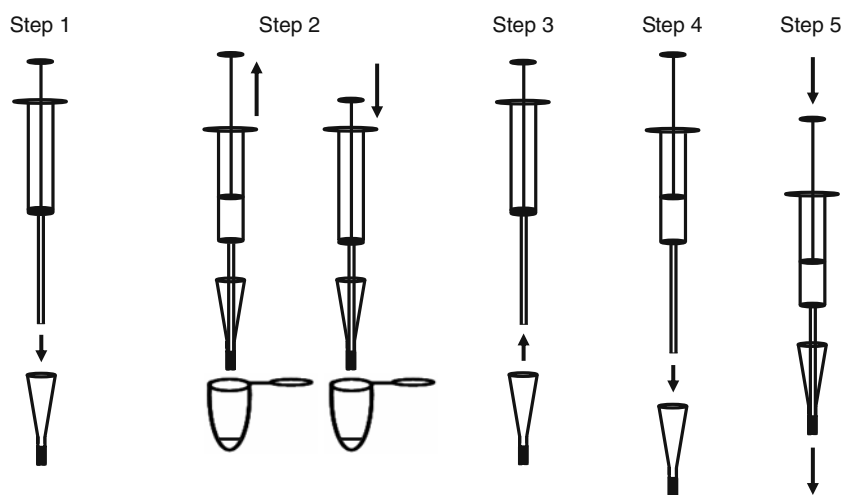


Table 3 Tryptic peptides (from BSA) identified by using standard nanoLC-MS-MS system and home-made ZipTip system

Method Coverage (%)	Standard nanoLC-MS-MS 33	Home-made ZipTip system 23
Identified sequence	DDSPDLPK	YLYEIAR
	LCVLHEK	QTALVELLK
	DLGEEHFK	SHCIAEVEK
	QTALVELLK	KQTALVELLK
	SHCIAEVEK	LVNELTEFAK
	CCTESLVNR	HLVDEPQNLK
	KQTALVELLK	SLHTLFGDELCK
	LVNELTEFAK	YICDNQDTISSK
	HPEYAVSVLLR	EYEATLEECCA
	HLVDEPQNLK	LKPDNTLCDEFK
	SLHTLFGDELCK	KVPQVSTPTLVEVSR
	YICDNQDTISSK	RPCFSALTPDETYVPK
	TCVADESHAGCEK	HPYFYAPELLEYANK
	LGEYGFQNALIVR	
	ETYGDMADCCEK	
	DDPHACYSTVFDK	
	LKPDNTLCDEFK	
	KVPQVSTPTLVEVSR	
	HPYFYAPELLEYANK	

connected to a syringe (Fig. 1 part A). Then the other end of the capillary PEEK tubing was connected to a zero-dead-volume union (Fig. 1 part E). Finally, the other side of the zero-dead-volume union was connected to a length of nanotight tubing sleeve (Fig. 1 part F) and fused-silica capillary (Fig. 1 part G). On-line ZipTip step-by-step procedures are summarized in Table 1. In the 50 μL syringe, the different mobile phase was established by loading different solutions one-by-one in specified order. The loading order is shown in Table 2, which is standard for ZipTip procedures [14]. After wetting and equilibrating, peptides were retained on ZipTip and then desalting solution (1% TFA) was aspirated through ZipTip. Finally, tryptic peptides were eluted from ZipTip and directly into the nanospray source with a flow rate of 600 $\text{nL}\cdot\text{min}^{-1}$ (Fig. 2).

Protein identification by nanoESI-Q-TOF

The elutant from ZipTip was directed to the nanospray source by a 20 μm i.d. and 90 μm o.d. fused-silica capillary. A voltage of 3.2 kV was applied to the nanosource capillary. The MS was operated in positive ion mode with a cone voltage of 80 V and a source temperature of 80 $^{\circ}\text{C}$. TOF analyzer was set in the V-mode. MS/MS spectra were acquired in a data-dependent acquisition mode in which the three most abundant multiple-charged (+2 and +3) ions were selected for CID. MS/MS spectra were collected for each of these top three ions. The precursor ion was excluded for 7 s. During the auto survey of MS and MS/MS scans, collision energies were set at 10 and

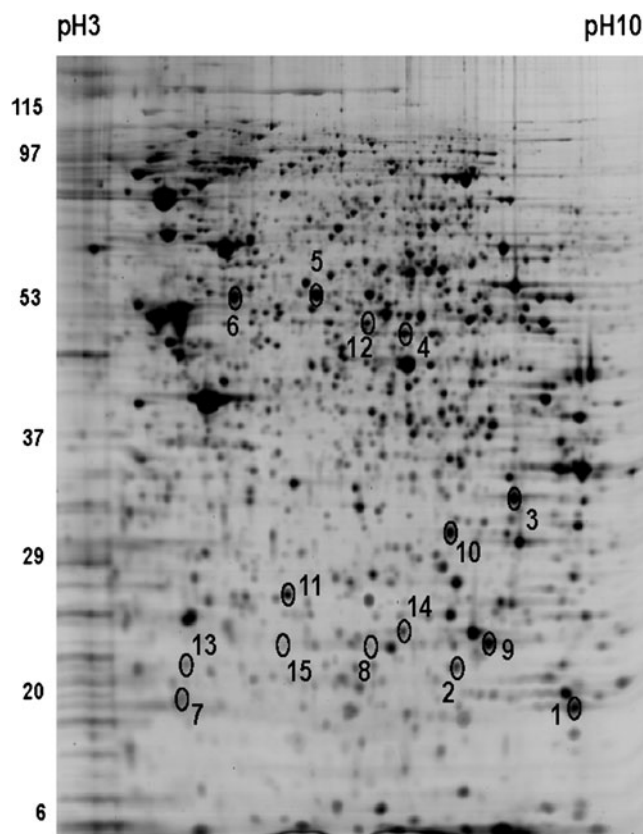


Fig. 3 2-DE image of mesangial 13 cell after silver staining: strip pH range 3–10 and molecular weight range 6–115 kDa. The circles indicate the gel spots for protein identification using the nanoscale system coupled with nanoESI-Q-TOF. The results of protein identification are listed in Table 4

30 V using argon as the collision gas. Individual fragment spectra obtained for each of the precursors were processed by using MassLynx 4.0 software (Manchester, UK) to obtain centroid MS/MS data and the corresponding peak lists in the format of pkl files. These peak list files were then submitted to MASCOT (<http://www.matrixscience.com>) search engine to get corresponding protein identity. The MASCOT program was set with the following parameters: database, NCBI nr; taxonomy, Mus.; enzyme, trypsin; peptide mass tolerance, 0.3 Da; MS/MS ion mass tolerance, 0.3 Da; peptide charge, 2+ and 3+; data format, pkl; instrument, ESI-QUAD-TOF. Variable modifications such as oxidation of methionine and carbamidomethylation of cysteine were selected, and up to one missed cleavage was allowed.

Results and discussion

In current proteomic analysis, the separation prior to mass spectrometry is usually carried out by a nanoflow LC system for peptide sequencing. However, nanoscale LC device is very expensive and not easy to handle. ZipTip is a useful tool for minor peptides desalination and enrichment after protein digestion from 2-DE. The advantages associated with ZipTip are that they are disposable, easy to handle, and sample contamination is avoidable. Nevertheless, it is unable to connect on-line with mass spectrometry. In standard

ZipTip procedures, peptides would be desorbed from the stationary phase after the desalting step. Followed by the transfer of the peptide solution into a clean vial or the deposition onto a target plate for MS analysis via ESI or MALDI MS, respectively.

In this study, we combine commercial ZipTip, syringe and syringe pump to create an on-line nanoscale protein identification system. ZipTip is widely used for sample concentration and desalting for protein and peptide analysis in proteomic research. The C18 sorbents supply the stationary phase to trap the desired peptides, then the target peptides would be eluted into the nanospray source immediately. ZipTip was used as the nanoscale column and the syringe pump was applied to push the mobile phase with a flow rate of 600 nLmin⁻¹. Isocratic chromatography was used and the eluent was ACN: 0.1% TFA=50:50 (v v⁻¹). Sample separation was done within 10 min, and this nanoscale setup was fast and inexpensive for protein identification. In order to test the efficiency of this system, a comparison of the performance with standard nanoLC-MS-MS (gradient chromatography with a flow rate of 200 nLmin⁻¹) was studied. We used trypsin-digested BSA (200 fmol) as the tested protein. The results are shown in Table 3. According to the consequences, this isocratic nanoLC system was workable and its performance was acceptable for protein identification.

This home-made nanoLC system was applied to identify proteins from silver-stained 2-DE. To examine the feasibility

Table 4 Protein lists of identified 2-DE spots by using on-line ZipTip with nanoESI-MS/MS

Spot number ^a (Accession No.)	Protein name	MW ^c	pI ^c	Queries matched	Coverage (%)	Score ^d
1 (Q9WVA4)	Transgelin-2	23,582	6.59	12	41	323
2 (P49722)	Proteasome subunit alpha type 2	25,909	8.39	9	36	299
3 (NP_034829)	Lactate dehydrogenase A	36,475	7.62	12	44	497
4 (NP_033786)	Aldehyde dehydrogenase 2	56,502	7.53	11	29	459
5 (NP_031978)	Protein disulfide isomerase associated 3	56,643	5.88	18	27	473
6 (CAA38762)	Heat shock protein 65	60,903	5.91	18	26	665
7 ^b (AAC35744)	Type II peroxiredoxin 1	21,778	5.20	4	22	256
(AAB06983)	Phosphatidylethanolamine binding protein	20,847	5.19	3	18	125
8 (P14602)	Heat shock protein beta-1	23,000	6.12	11	52	317
9 (NP_033441)	Triosephosphate isomerase 1	26,696	6.90	9	38	330
10 (EDL35843)	Esterase D	33,149	8.27	7	29	164
11 (AAC53295)	Proteasome activator PA28 alpha subunit	28,685	5.73	7	30	243
12 (CAA83428)	CCT (chaperonin containing TCP-1) beta subunit	57,411	5.97	3	5	139
13 (Q9CPU0)	Lactoylglutathione lyase	20,796	5.24	4	31	108
14 (NP_036098)	Proteasome (prosome, macropain) subunit, alpha type 6	27,355	6.34	5	21	269
15 (P14602)	Heat shock protein beta-1	23,000	6.12	9	43	229

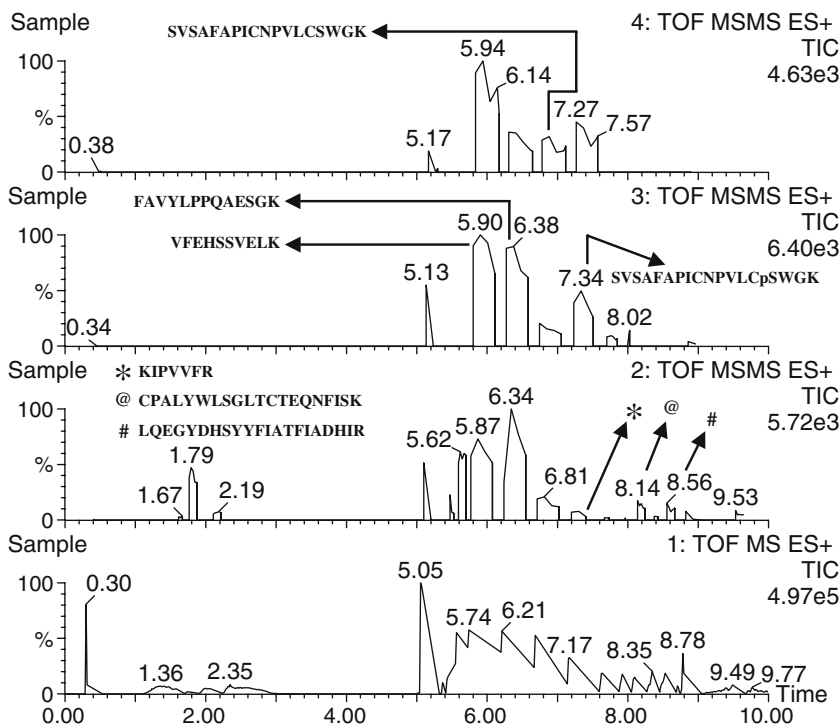
^a The spot number is as indicated in Fig. 3

^b More than one protein was identified in some spots

^c The MW and pI were calculated values based on the identified proteins

^d The confidence scores are based on search results from MS/MS data analysis using the Mascot search engine

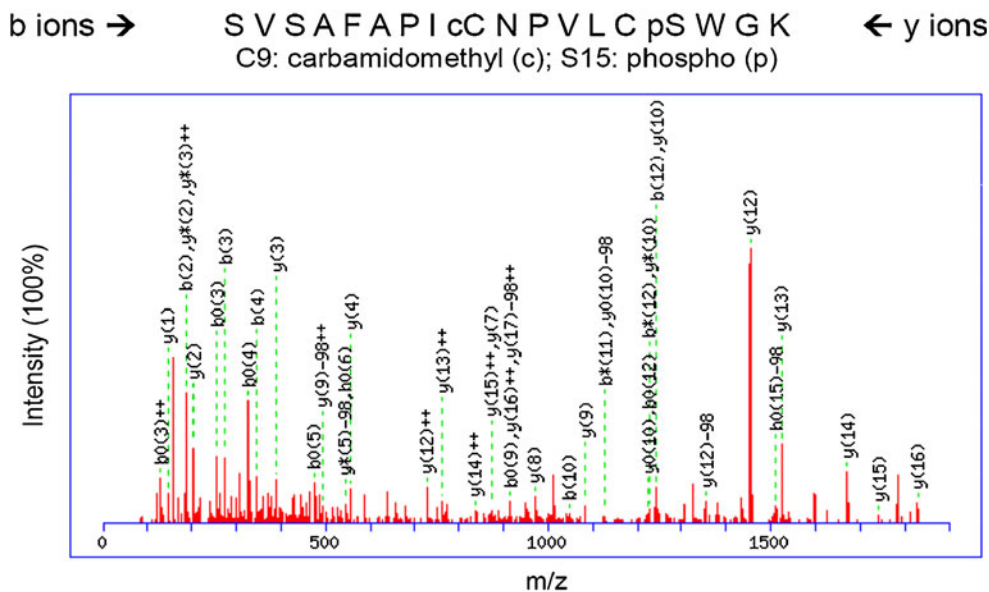
Fig. 4 A LC-MS/MS chromatographic record of peptides identified by this home-assembled device after digestion



of this device for protein identification of 2-DE, we have selected several protein spots from a 13 cm 2-DE gel of mesangial 13 cell. As shown in Fig. 3, fifteen spots with different intensity were chosen from this silver-stained gel. All these 15 spots were identified with adequate scores, and the results of identification are summarized in Table 4. These data demonstrated that satisfactory protein identification can be achieved by using this nanoscale system with nanoESI-MS/MS. The chromatographic record of tryptic peptide

separation by this home-assembled device is shown in Fig. 4. Furthermore, all these protein identifications were done within 10 min, so this is an efficient strategy for fast protein identification. Surprisingly, we also found a phosphorylated peptide from protein “esterase D” (spot 10 in Fig. 3) by this device. The spectrum of this phosphorylated peptide is shown in Fig. 5. The precursor ion $[M+2H]^+$ and sequence of this peptide are m/z 1008.12 and SVSAFAPICNPVLCpSWGK. In this sequence, C9 is

Fig. 5 The MS/MS spectrum of phosphorylated peptide identified by the nanoscale separation system coupled with Mascot search engine



also modified with iodoacetamide. Why is cysteine C9 modified with carbamidomethyl but the cysteine C14 is not? According to our speculation, the reasonable explanation is that cysteine modified with iodoacetamide is an artificial modification. This reaction is an S_N2 reaction where “S” of cysteine is a nucleophile and “I” of iodoacetamide is a leaving group. When this reaction occurs, not all the “S” of cysteine can be modified with iodoacetamide because this reaction is random. In theoretical situations, this sequence can be modified with 0, 1 or 2 iodoacetamide. A major factor affecting this reaction is steric hindrance. In this sequence, the S15 is phosphorylated so it is difficult for C14 to be modified with iodoacetamide because of the steric hindrance. The original idea of this nanoflow system was derived from the method reported by Williams and Tomer [16], this was a simple system but application of this method for protein identification in real 2-DE samples was absent. Our previous study used mini-capillary columns to set up a nanoflow system and identify proteins from 2-DE samples [17], this method was fast and workable but prepared the mini-capillary columns was time consuming. And protein identification by this method was achieved within 25 min. This new method is more convenient than our previous study because we did not have to prepare the mini-capillary columns, which requires extra time and labor. Furthermore, this method is isocratic chromatography so we can spend less time (< 10 min) for protein identification. This method also provides a simple, fast, and inexpensive platform for creating a nanoflow system when an expensive nano-LC system is not available.

Conclusions

In this article we have demonstrated an on-line nanoscale system, combined commercial ZipTip, syringe and syringe pump, for fast protein identification. Application of this device with a nanoESI-MS/MS successfully identified a variety of 2-DE proteins with varied intensity after tryptic digestion. This method is suitable for proteomic analysis by a home-made nanoscale device. Moreover, this on-line strategy is simple, fast, inexpensive and easy to handle. Finally, ZipTip is disposable and thus cross contamination is avoided.

Acknowledgements The authors are grateful to the National Science Council (NSC 98-2113-M-037-010 and 97-2113-M-037-008-MY2), Kaohsiung Medical University (Q-097021 and Q-098004) and Center of Excellence for Environmental Medicine (Kaohsiung Medical University) for financial support of this work. The authors are also grateful to the Center for Resources, Research and Development (Kaohsiung Medical University) for instrumental support of this work.

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