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Capillary Electrophoresis/Tandem Mass Spectrometry for Analysis of Proteins from Two-dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

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Capillary electrophoresis/time-of-flight mass spectrometry(CE/TOFMS) has been used for analysis of in-gel digests of protein spots excised from two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-D SDS-PAGE). An off-line purification and preconcentration procedure with a Zip Tip is used before CE/TOFMS analysis which allows for detection of protein spots with <1 picomole of material from 2-D gels. The off-line procedure provides sufficient purification for analysis while maintaining the quality of the CE separation. Using this procedure, several proteins from Coomassie Blue and zinc negatively stained gels are identified by the peptide maps generated and database searching. CE/TOF tandem mass spectrometry is used for the confirmation of database searching results and structural analysis of peptides that do not match the expected peptide maps obtained from the database in order to identify structural modifications. Several modifications were pinpointed and identified by this method. Copyright © 1999 John Wiley & Sons, Ltd.

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Two-dimensional (2-D) gel electrophoresis has become an essential tool for the study of the protein content of a cell. This method can resolve thousands of proteins in cell lysates based upon separation along a pH gradient in one dimension followed by an electrophoretic separation, which is related to the molecular weight in a second dimension. 2-D gel electrophoresis has become a convenient method for the study of protein synthesis regulation in normal and tumor cells and as a means for detection of the differential expression of proteins in response to external cell stimuli. The recent development of mass spectrometry based peptide mapping methods combined with database searching has resulted in a means for rapid identification of proteins from 2-D gel separations.[1–14]

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and liquid chromatography/mass spectrometry (LC/MS) have become methods for analysis of proteins separated by 2-D gel electrophoresis. Proteins are digested with an enzyme either via in-gel digestion or by being electroblotted onto a membrane for on-membrane digestion. MALDI-MS can provide identification based upon the peptide maps generated and the match obtained against a database. However, often a limited coverage of the protein sequence is obtained due to suppression effects in the MALDI-MS process, which is highly dependent on sample preparation. HPLC/MS has been used as an alternative for protein identification based upon peptide mapping,[7] the sequence tag method[14] and correlation of peptide fragmentation data with a database using the SEQUEST method.[15] HPLC/MS provides a much more extensive coverage of the protein sequence by elimination of suppression effects and MS/MS structural information can be obtained using an ion trap or quadrupole mass spectrometer.

An alternative method for analysis of protein spots from 2-D gel separations is capillary electrophoresis mass spectrometry (CE/MS).[16] Capillary electrophoresis can provide much improved resolution of peaks in complex mixtures in a much shorter time than HPLC. A CE separation of a tryptic digest can be performed in 5–10 min compared to 30–40 min for an equivalent HPLC separation, although in recent work Lee and co-workers have developed a novel integrated microscale LC column electrospray ionization (ESI) interface and variable flow solvent delivery system so that digests of gel-separated proteins can be analyzed in <10 min.[17] In addition, CE consumes only nanoliter amounts of sample which is important when dealing with limited amounts of material from 2-D gel electrophoresis. The concentrated bands generated by the high resolution and the slow flow rate make CE ideal for high sensitivity in detection by mass spectrometry, which is a concentration dependent detector. Recently time-of-flight (TOF) mass analyzers have become popular for CE since CE/TOF can achieve a high duty cycle due to its nonscanning properties.[18–24]

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The main disadvantage of CE relative to capillary HPLC for analysis of peptide digests from 2-D gel separations is the lack of preconcentration in the CE separation, which limits the concentration detection capabilites. The volume of a 50 cm long capillary with 50 μm i.d. is about 1 μL. Since the optimal analyte resolution and separation efficiency are achieved with less than 2% of the total capillary column, the amount of sample injected into a 50 cm, 50 μm i.d. capillary will be less than 20 nL. Several approaches have been developed to increase the amount of sample injected onto the CE separation. On-column isotachophoresis (ITP),[25] sample stacking,[26] and field amplified polarity switching[27] have been used to obtain a preconcentration factor of 100. In other work,[28] on-line coupling capillary ITP has been used to achieve a concentration factor of 1000.

A second problem in the use of CE for analysis of protein digests from 2-D gels is that CE requires sample cleanup prior to injection. Complex samples containing salts and detergents lower the sensitivity and affect the CE separation. Solid phase extraction devices have been used for sample cleanup and preconcentration. Several groups have used an on-line coupling preconcentrator with an impregnated membrane,[29] or a mini-column packed with reversed phase absorbent.[16,30] Recently, the inlet of a capillary packed with C_{18} has been used to achieve sample stacking and cleanup.[31] Although these methods improve the preconcentration of the sample, the nonhomogenous solid phase and high organic content of eluting solvent degrades the separation.

In this work, we use off-line solid phase extraction with CE/TOFMS to identify 2-D gel-separated proteins from cancer cell lines. The in-gel protein digest is cleaned with a commercial ZipTip C_{18} (Millipore) for CE analysis. In this off-line procedure, the sample is reconstituted from the ZipTip in a couple of microliters of deionized water where the preconcentration effect is achieved by minimizing the final sample volume. It is shown that protein digests can be detected from femtomole levels of material injected and that a relatively broad coverage of the protein sequence can be obtained. The use of the off-line purification method maintains the high resolution of the CE separation, which is especially important in cases where on-line MS/MS may be required for further detailed structural analysis.

EXPERIMENTAL

2D PAGE

Proteins from Esophageal Adenocarcinoma and Thymocyte cell extracts were separated by 2-D gel electrophoresis, with pH 4–8 carrier ampholytes isoelectric focusing (IEF) as the first dimension and SDS polyacrylamide as the second dimension. The 2-D gels used in this study were run at the Department of Pediatrics, the University of Michigan, according to a procedure described previously.[32]

Gel staining

After separation of proteins from Esophageal Adenocarcinoma and Thymocyte cells by 2-D gel electrophoresis, the gels were stained with either Coomassie Blue R-250 or zinc negative staining. If Coomassie Blue staining was used, gels were fixed with 5% acetic acid and 50% ethanol for two hours, then stained with 1% Coomassie Blue R-250, 10% acetic acid, 40% methanol for 30 min and destained with 10% acetic acid, 40% methanol for 30 min. The zinc staining method was used to avoid interference of the dye[33] in the analysis. Gels were first equilibrated in 1% Na_2CO_3 solution for 5 min, stained with 200 mM imidazole, 0.1% SDS for 15 min, washed with deionized water for 1 min and then developed with 0.1 M $Zn(Ac)_2$ until sufficient contrast was obtained. The gels were rinsed with water three times before gels were destained with 1% citric acid twice for 20 min by vortexing.

In-gel digestion

The digestion procedure basically follows that of Williams *et al.*,[34] but with some modifications. Gel spots were cut into 0.5×0.5 mm pieces and washed with buffer B (50% CH_3CN, 50% 100 mM NH_3HCO_3) twice for 30 min. After removing the washing buffer, gel pieces were covered with buffer B and 45 mM dithiothreitol (DTT) was then added to bring the final concentration to 1mM. Reduction was complete after 20 min incubation at 37 °C. Twice the volume (as compared to the volume of 45 mM DTT added) of 100 mM iodoacetamide was added and the pieces were incubated for 40 min at 37 °C for the alkylation. After the supernatant had been removed, the gel pieces were washed again with buffer B for 30 min once and then twice for 15 min all with vortexing. After washing, the gel pieces were dried under speedvacuum for 20 min. In order to perform digestion, 10 μL aliquots of trypsin solution (1–2 μg in 100 mM NH_4HCO_3) were gradually added to the dried gel pieces until they were fully swollen, and more digestion buffer was added to sufficiently cover them. Digestion was performed at 37 °C for 24 hr. After digestion, the peptides were extracted with 1% trifluoroacetic acid (TFA), 60% CH_3CN by sonication three times for 30 min. The combined extracts were dried by speedvac.

Solid phase extraction

The digests were restored with 10 μL 0.1% TFA and cleaned using $ZipTip_{C18}$ from Millipore.[35] A ZipTip is a 10 μL pipette tip packed with approximately a half microliter of silica C_{18}. Briefly, the ZipTip was first wet with 50% acetonitrile/H_2O and equilibrated with 0.1% TFA. The protein digests were bound to the ZipTip by aspirating and dispensing the sample for 10 cycles. The remaining liquid was dispensed to waste. The tip was washed with buffer (0.1% TFA/H_2O) and was dispensed to waste. Elution was achieved with 4 μL of 0.1% TFA/ 60% acetonitrile/ H_2O by aspirating and dispensing the eluant through the tip four times. The eluate was dried under speedvacuum again and dissolved in 4 μL of deionized water.

On-line sheathless CE/MS system

The digests were separated using a capillary electrophoresis apparatus constructed in-house, which includes a 30 kV high voltage power supply (model CZE 1000R, Spellman High Voltage Electronics Corp., Plainview, NY, USA) and a Polybrene (positive) coated capillary column (37 μm i.d.) prepared according to the procedure of Li.[36] The detector used was an ion trap storage reflectron time-of-flight (IT/reTOF) mass spectrometer described in previous work.[36,37] The apparatus consists of a quadrupole ion trap storage device (model 1251,R.M. Jordan Co., Grass Valley, CA, USA) interfaced to a reflectron TOF mass analyzer (model

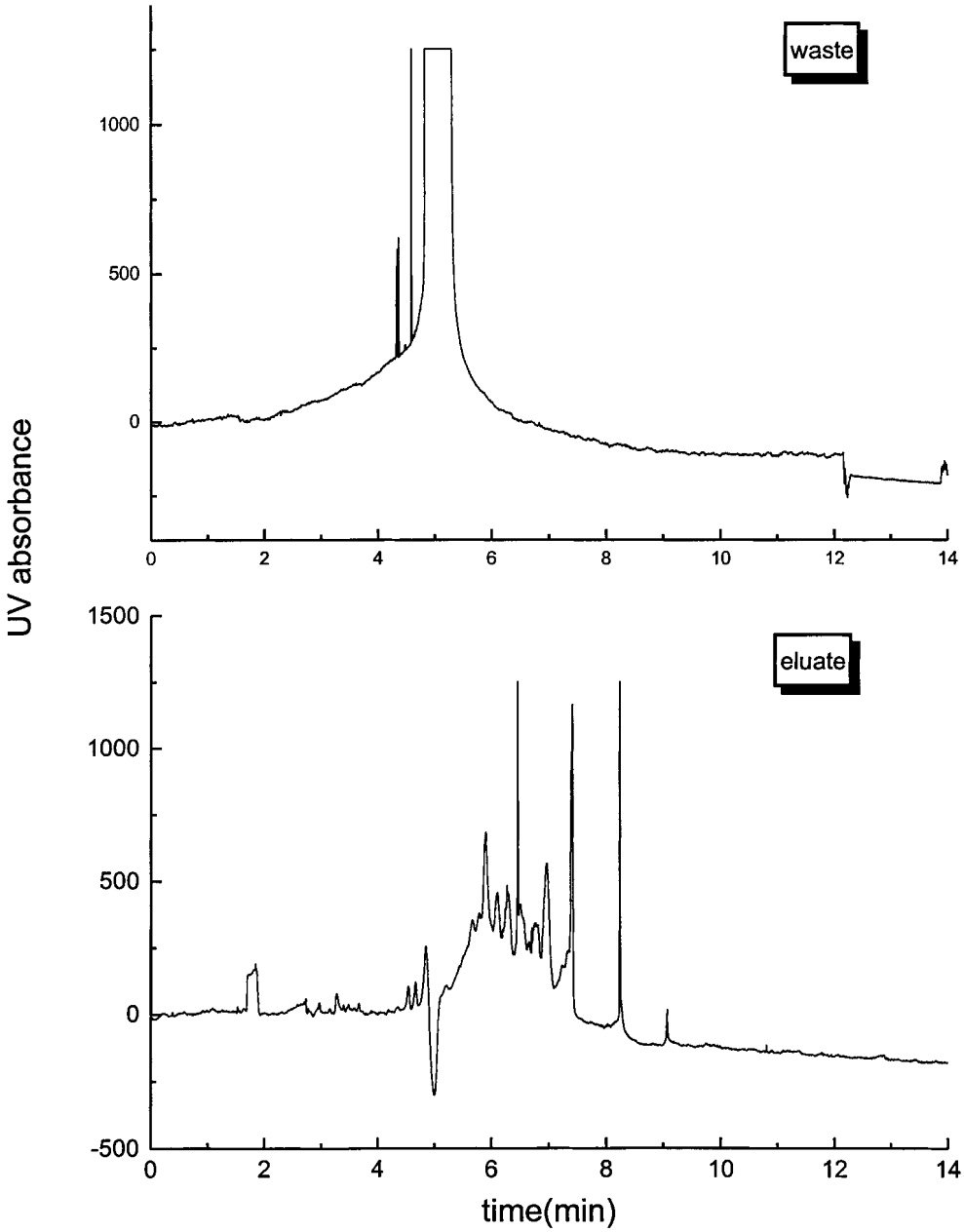

Figure 1. CE/UV electrophorograms of waste and eluate of tryptic digests of C2 after ZipTip cleanup. CE conditions: 70 cm long, 75 μm i.d. fused capillary, injection: 8000 V × 20 s, separation: 26 kV.

D1450). Argon buffer gas (1 mTorr) was introduced into the ion trap to enhance the trapping. Detection was achieved using a 40 mm triple microchannel plate (MCP) detector (Model C-2501, R.M. Jordan Co.).

The anodic end of the CE capillary was used as the sheathless microelectrospray tip. This configuration was achieved by coating the capillary tip with silver.[38] Electrokinetic injection was performed with 5000 V for 20 s. The power supply of the capillary electrospray was set at ca. −12 kV, while the microelectrospray needle was set at 3 kV, so that the total voltage across the capillary was −15kV.

The mass data were collected and processed using a 250 MHz transient recorder (Model 9846, Precision Instruments Inc., Knoxville, TN, USA) connected to a Dell Pentium II 400 Mz processor. Data analysis was accomplished using custom 2-D false color image software as described in previous work, with the *m/z* spectrum on one axis, TIE elution time as the other axis and *m/z* intensity as the image color.[39] A cursor on this image map (or on the mass spectra and TIE plot) could be used to display the corresponding individual *m/z* spectrum on the x-axis and also integrate multiple spectra over an elution time range to increase the signal-to-noise (S/N) ratio. This integration capability allowed peaks of low intensity to be identified.

On-line CE/MS/MS

On-line CE/MS/MS with stored waveform inversed Fourier transform (SWIFT) technology has been developed in the ion trap reflectron time-of-flight mass spectrometer.[40] Briefly, as a peak elutes it is detected and digitized by a PI9845 digitizer board in the computer. The computer interacts with an arbitrary waveform generator board

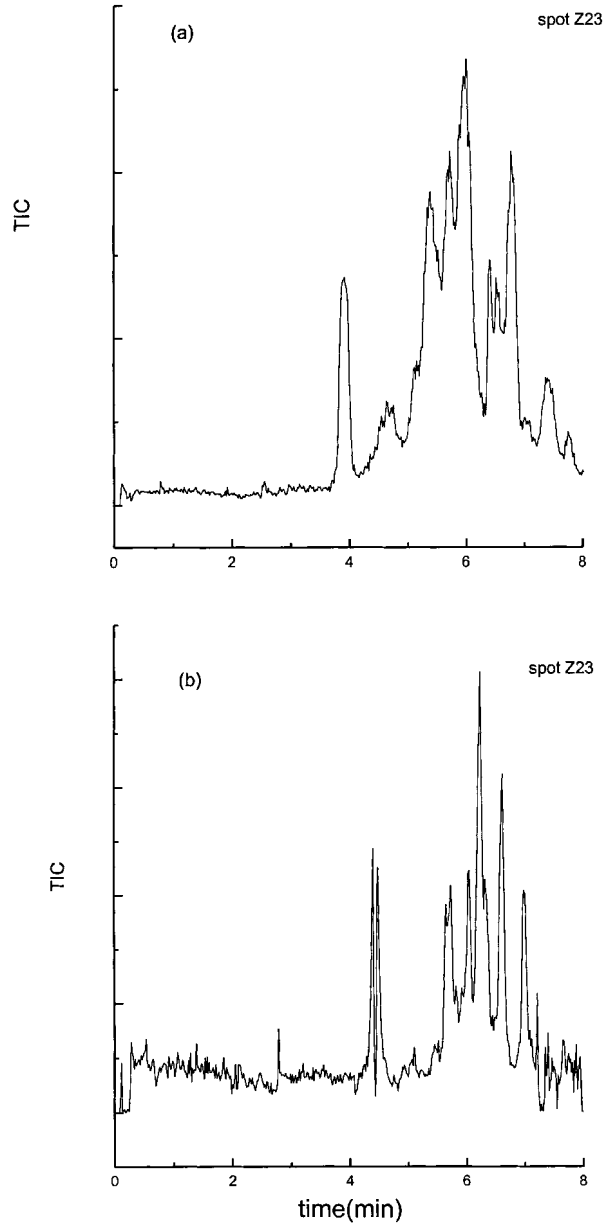

Figure 2. CE/TIC of tryptic digests of spot Z23 with capillaries of (a) 75 μm i.d. (b) 37 μm i.d. Injection: 5000 V × 20 s, separation: 300 V/cm.

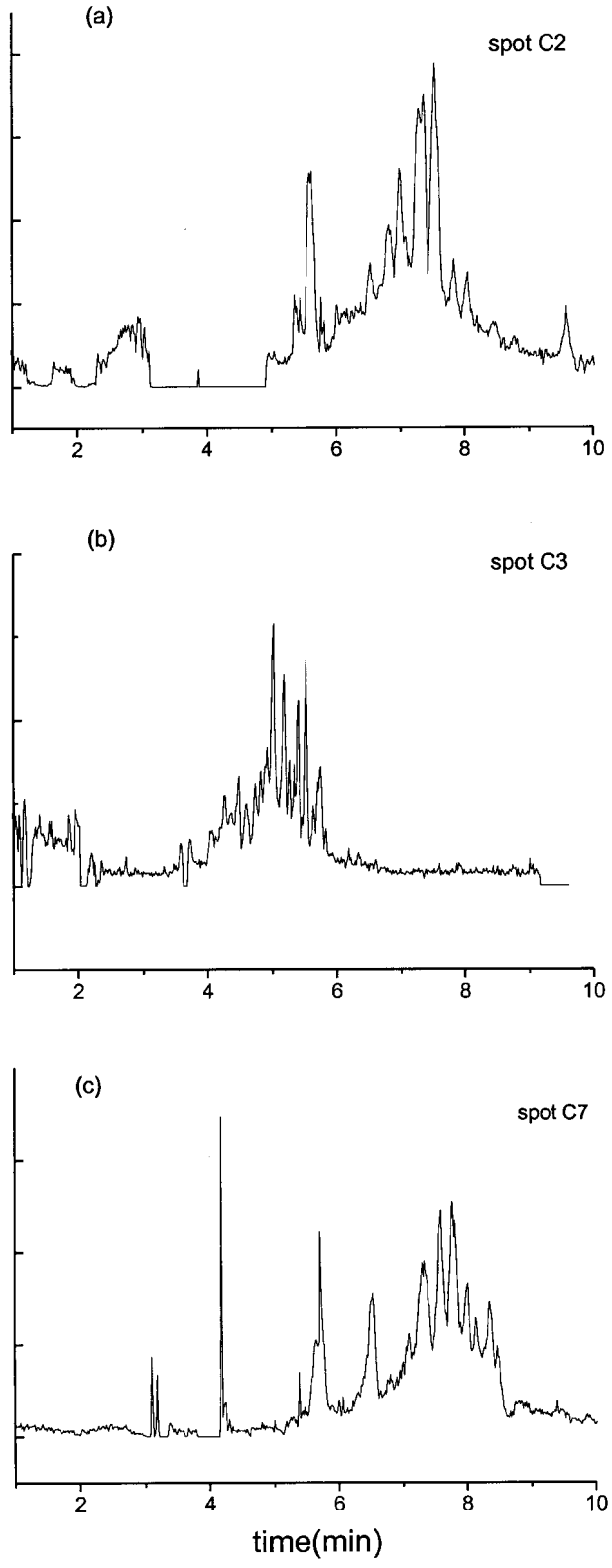

Figure 3. CE/TICs of tryptic digests of three identified proteins from Coomassie Blue stained gel (a) spots C2, (b) spots C3 and (c) spots C7. Injection: 5000 V × 20 s, separation: 3000 V/cm.

(AWGB) (model WSB-100-10, Quatech, Akron, OH, USA) which creates a notch in the frequency domain and parent ions are isolated and accumulated in the ion trap while other ions are ejected out of the ion trap. A TICKLE waveform is applied to induce the collision between the parent ions and buffer gas ions. Fragment ions are ejected by a DC pulse and detected by a reflectron TOF mass spectrometer. The TICKLE voltage is optimized on-line and MS/MS spectra are achieved at a sampling rate of 4 Hz. MS/MS can be achieved at a sampling rate up to 8 Hz[40] at a compromise of the S/N of the spectrum; 4 Hz was chosen in this work as the best compromise between the speed of MS/MS acquisition and the quality of the mass spectra. The sequence of events for acquiring a single MS/MS spectrum and the timing of these events on the ion trap instrument using SWIFT and TICKLE waveforms and corresponding waveform time scales for each event at a 4 Hz rate, as used in this work, include: (1) Accumulate ions in the trap (136 ms). (2) Stop ions from entering the trap using an ion gating voltage. (3) Apply the SWIFT isolation waveform (32 ms). (4) Cool (25 ms). (5) Apply the TICKLE activation waveform (32 ms). (6) Cool (25 ms). (7) Eject ions from the trap. (8) Digitize the mass spectra. (9) Permit ions to begin accumulating in the trap again. (10) Transfer digitized data

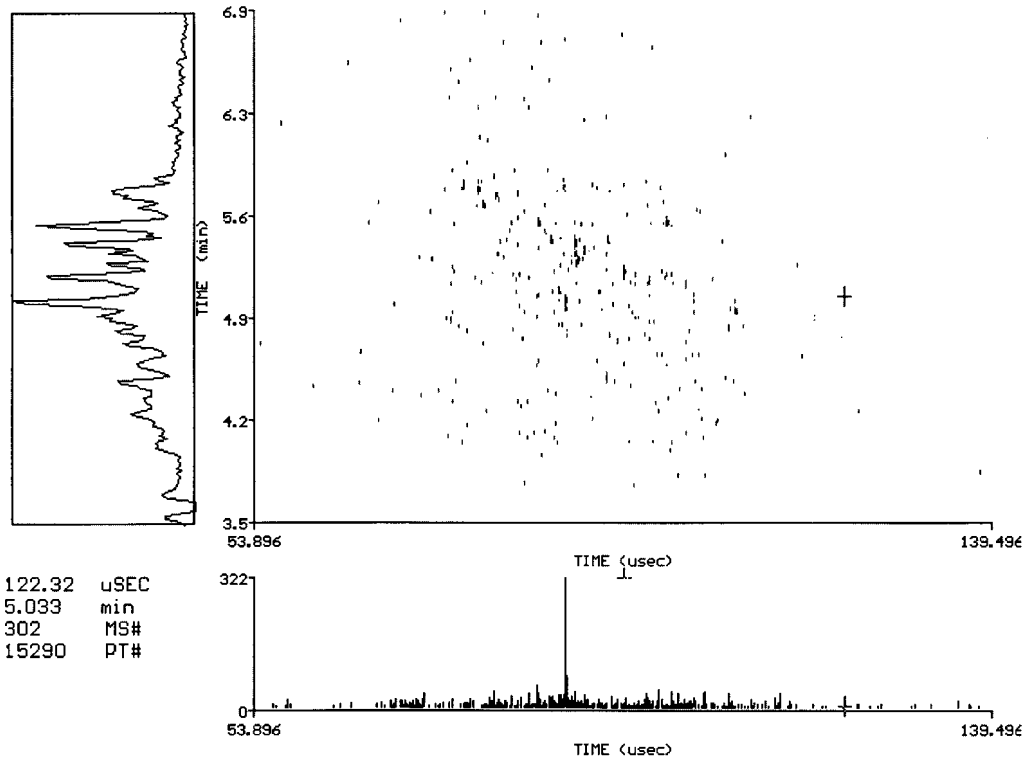

Figure 4. 2-D false color map of tryptic digests of C3. The x-axis is flight time and y-axis is CE elution time.

to the computer. (11) Save mass spectra data in the computer. (12) Analyze mass spectra to determine appropriate TICKLE voltage to apply on the next cycle.

RESULTS AND DISCUSSION

Method development

The use of CE/MS as a method for analyzing complex sample mixtures often encounters the two problems of sample cleanup and concentration limit of detection (CLOD). In order to overcome these problems, previous work has been performed with on-line coupling of solid phase extraction (SPE)[16,31] or membrane preconcentration to on-line CE/MS.[30] Although on-line SPE/CE/MS has high sensitivity and sample recovery, the heterogeneity of the SPE/CZE coupling and the use of organic solvents for eluting the material may result in deterioration of the CE separation. Thus, we have investigated the CE/MS of gel-separated protein digests using an off-line SPE procedure for sample cleanup and preconcentration.

The digests of gel-separated proteins always contain salts, detergents, water-soluble polymers and tiny gel particles. If the sample is not purified before injection into the CE capillary, the capillary can be easily clogged and the separation degraded where contaminants may suppress the various peaks in mass spectrometric detection. In other work, HPLC has been used to clean and separate protein digests from gels since LC is more tolerant toward salts and detergents compared with CE.[41] Thus in this work SPE is used to clean the protein digests obtained from 2-D gels. The ZipTip is used because it contains a microliter bed of C_{18} absorbent which leads to improved sample recovery. Figure 1 shows the CE/UV electrophorograms of eluate and waste after treatment with the ZipTip$_{C18}$. The protein spots used were cut from 2-D gels of an Esophageal Adenocarcinoma cell line stained with Coomassie Blue. The i.d. of the capillary was 75 μm and the sample was injected with 8000 V for 20 s. In order to improve the separation with relatively large amounts of injected material, an on-line stacking technique was used. After sample injection, 50 mM NH_4OH was injected with 8000 V for 10 s and then separation was completed with −26 kV. The CE separation of the waste material (Fig. 1(a)) contains a large saturated peak eluting around 4–6 min, while CE of the eluate (Fig. 1(b)) contains the peptide peaks which elute after 4 min. If the sample is not cleaned before CE separation, contaminants and peptide peaks overlap and the signal is suppressed.

A narrower capillary (37 μm i.d.) was used to replace the 75 μm i.d. capillary column to improve the separation. The amount of sample introduced by electrokinetic injection into a 37 μm i.d. capillary is only one quarter that of the sample injected into a 75 μm i.d. column under the same conditions. Figure 2 shows the separation of Spot Z23 from a Thymocyte cell with negative zinc staining by capillaries of two different diameters. The CE separation with 37 μm i.d. provides much improved results compared with the separation with 75 μm i.d., while peak intensities were still sufficiently strong for identification of proteins. Decreasing the amount of sample injected and increasing the separation voltage also improved the CE separation. The use of a 37 μm i.d. capillary provides sufficient sensitivity to analyze protein digests from 2-D gels with improved separation.

Figure 3 shows the CE/TIE of spots C2, C3, C7 and Fig. 4 shows a 2-D false color image map of protein spot C3. The protein spots from eight pieces of gel which were obtained from eight 2-D gels run simultaneously were combined to perform digestion for CE/MS analysis. Each spot from the gel of C2 was estimated to contain 0.5–1 picomole before in-gel digestion. The digests was dissolved in 4 μL of the

Table 1. Proteins identified from 2-D SDS-PAGE separation of cancer cell line by CE/MS

Spot	Protein identified	Acc#	MW kDa	PI	Sequence coverage	Considering modification?
C2	HSP-60	P10809	61055	5.7	54%	yes
					46%	no
C3	Actin, Cytoplasmic1,2	P02570/P02571	41737	5.29	44%	yes
					38%	no
C7	Adenosine kinase	P55263	37491	6.28	41%	yes
					41%	no
Z23	Tubulin beta-1Chain	P07437	49759	4.75	33%	yes
					19%	no

solution where more than 40 CE/MS experiments could be performed. It is estimated that around ten femtomoles of sample are injected on each CE/MS experiment where ~20–30 nanoliters are injected on each run. However at least 2 μL of the liquid must be available in the 0.5 mL microcentrifuge tube in order to perform the electrokinetic injection properly so that only half the sample is actually used. Since CE/MS only consumes nanoliter amounts of sample, multiple experiments are possible, including on-line CE/MS/MS and, in principle, CE/MS/MS/MS which can be used to further confirm the identification of proteins and post-tranlational modifications of proteins obtained from 2-D gels. It should be noted that we could perform analysis of even a single gel piece using this method; however, since eight gel pieces were available, the entire sample was used since it was much easier to handle the larger amounts of material. The original eight pieces of gel provide a maximum of 2–4 picomoles of material for analysis since half of the sample can be used. Since 40 injections with 10 femtomole per injection amount to 400 femtomoles, the losses involved are still substantial. Further procedures for enhancing recovery in the purification procedure will be required.

Identification of proteins by database search combined with CE/MS/MS confirmation and analysis

The peptide mass peaks obtained from the 2-D false color maps were used for the database search. The integration capability of the 2-D program allows for detection of weak peptide peaks. MS-FIT created by UC San Francisco was chosen as the search engine because MS-FIT combines pI range, MW range, potential modifications, minimum matching peptide number and other features. The searching results with and without modification were compared. The modifications considered here include oxidation of methionine and peptide N-terminal Gln to pyro-Glu. The search can be performed in different protein databases. SWISS-PROT and NCBInr are two major databases used for the protein search.

Table 2. Database search results with and without modification considered The modifications considered here include N-terminal glutamine to pyro-Glu, oxidation of methionine and phosphorylation of S and T

Spot	Considering modification?	Rank	MOWSE score	#(%)Masses matched	Swiss-Prot Acc#
C2	Yes	1	4.67E + 07	43/51(88%)	P10809
	No	1	1.43E + 08	34/51(66%)	P10809
C3	Yes	1	5.57E + 03	17/26(65%)	P02570
		2	5.56E + 03	17/26(65%)	P02571
		3	791	12/26(46%)	P12718
	No	1	1.09E + 04	10/26(38%)	P02570
		2	1.09E + 04	10/26(38%)	P02571
C7	Yes	1	1.17E + 06	19/44(43%)	P55263
		2	1.73E + 03	16/44(36%)	P48775
		3	193	15/44(34%)	P14550
	No	1	4.86E + 05	14/44(31%)	P55263
Z23	Yes	1	2.15E + 04	21/35(60%)	P07437
		2	5.80E + 03	21/35(60%)	P05217
		3	1.53E + 03	18/35(60%)	P04350
	No	1	3.21E + 03	10/35(28%)	P07437

Table 1 lists four proteins identified by CE/MS. The spots C2, C3 and C7 were cut from Coomassie Blue stained 2-D gel separations of an Esophageal Adenocarcinoma cell line, while Z23 is from a 2-D gel separation of zinc negatively stained Thymocyte cells. It was found that performing database searching without considering possible modifications results in a lower peptide matching percentage; however, it generally provides one good match, as shown in Table 2. In the case of database searching with modifications considered, slightly lower MOWSE scores were obtained for samples C2 and C3 as compared with the case of C7 and Z23, where database searching with modifications increased the MOWSE score compared with those with no modification considered. It should be noted that there are two kinds of peptide match: scoring matches and non-scoring matches. Scoring matches include unmodified peptides and acrylamide modified cysteine and N-terminal glutamine to pyro-Glu and oxidation of methionine in the presence of the unmodified peptide. Non-scoring matches include pyro-Glu and oxidation of methionine in the absence of the unmodified peptide, acetylated N-terminal, phosphorylation of S, T and Y and single amino substitutions. In computing the MOWSE score unmatched masses are ignored. In the case of multiple matching masses the scores are multiplied together. The final MOSWE score is normalised to an average protein molecular weight of 50 kD.[42]

C2 has been identified as HSP 60. A high matching percentage and MOWSE score are obtained for this sample. Spot C3 has been identified as actin, β or γ chain, where the major difference between the β and γ chain is in the first tryptic fragment. We did not detect the first fragment, so we were unable to differentiate between these two proteins. Many parameters in the in-gel digestion and SPE procedure affect the peptide recovery. It is possible that some hydrophilic peptides are lost during the washing procedure. In order to improve peptide recovery, mixed absorbents have been used by one group.[4] C7 has been identified as adenosine kinase. Z23 has been identified as tubulin beta-1 chain.

Database searching in different databases will provide different results. In this case, a comparison between such

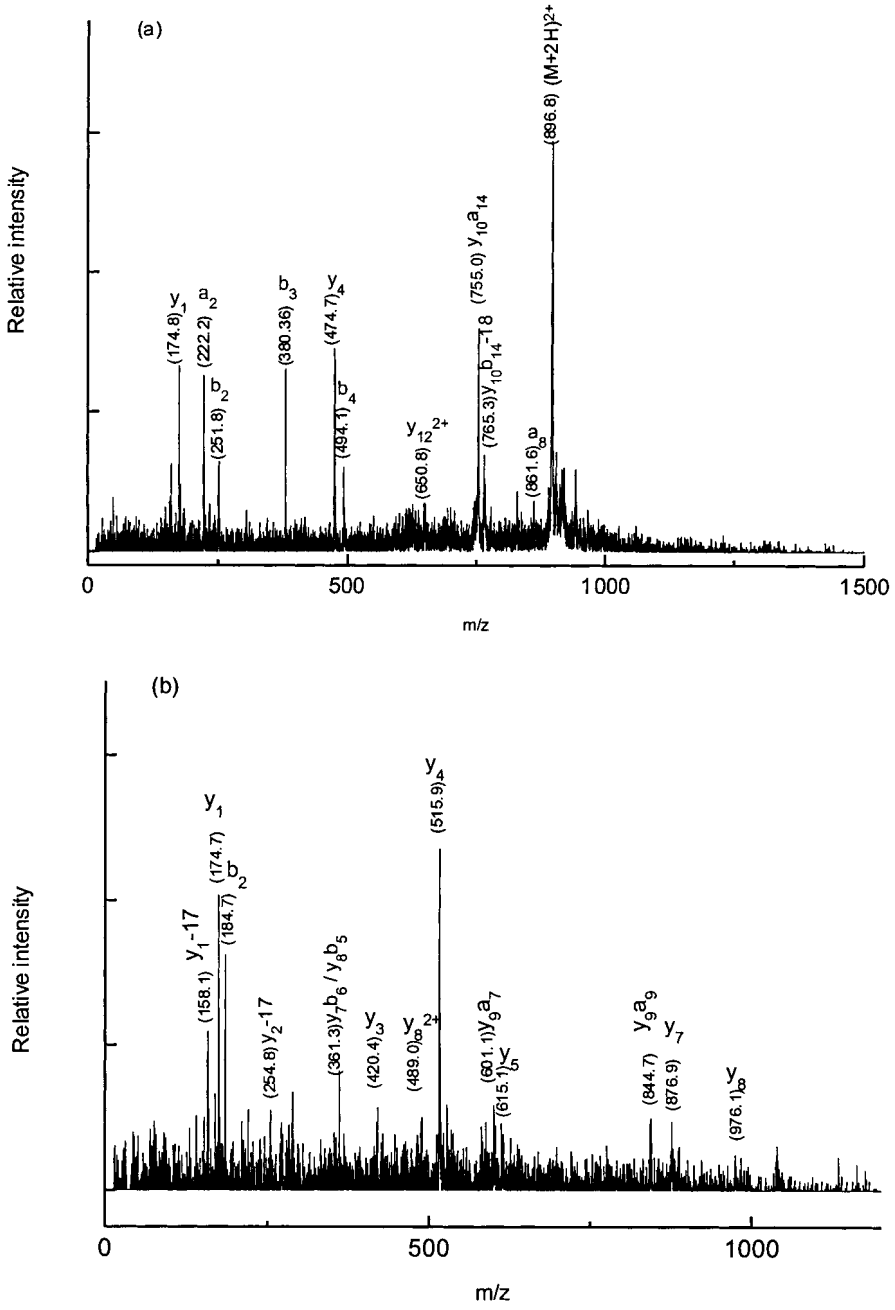

Figure 5. CID spectra of two peaks chosen from tryptic digests of (a) spots C3, *m/z* of parent ion 895.67, sequence SYELPDGQVITIGNER and (b) spots Z23, *m/z* of parent ion 580.58, sequence LAVNMVPFPR where M is oxidized.

results must be cautiously analyzed. In order to confirm the identification, tandem mass spectrometry is required. Because CE/MS only consumes nanoliters of sample per run, detailed information concerning post-translational modification of proteins can be obtained by CE/MS/MS. CE/MS/MS confirmed spot C3 as actin and Z23 as tubulin beta-1 chain. The collision induced dissociation (CID) spectra are shown in Fig. 5. The CID spectrum of parent ion 895.67 from C3 matched the sequence SYELPDGQVITIGNER, though the *m/z* of the sequence MQKEITALAPSTMKIK is similar to that of the parent ion too. Figure 5(b) shows the CID spectrum selected from the CE/MS mapping of the Z23 tryptic digest. The sequence is LAVNMVPFPR where methionine is oxidized.

CONCLUSIONS

Capillary electrophoresis/TOFMS has been used for the analysis of in-gel digests of protein spots separated by 2-D SDS-PAGE. The method was capable of analyzing spots from Coomassie Blue stained gels and zinc negatively stained gels where less than 1 picomole of material may be available for analysis. An off-line purification and preconcentration procedure using a ZipTip was used before CE/TOF mass spectrometric analysis for purification of the sample from detergents and salts and to achieve a concentration level allowing detection of the peptide digest products from gels. This off-line method was used since it provided sufficient purification of the sample while

maintaining the quality of the CE separation. The CE/TOFMS method was used to identify actual protein spots from 2-D gels in the *m/z* range from 40 to 60 kDa using peptide maps and database searching. The ion trap was used to perform tandem mass spectrometry on selected peptides to pinpoint the presence of modifications.

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