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Pressurized Electrochromatography Coupled with Electrospray Ionization Mass Spectrometry for Analysis of Peptides and Proteins

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Pressurized capillary electrochromatography (pCEC) was coupled with electrospray ionization mass spectrometry (ESI-MS) using a coaxial sheath liquid interface. It was used for separation and analysis of peptides and proteins. The effects of organic modifier and applied voltage on separation were investigated, and the effects of pH value of the mobile phase and the concentration of the electrolyte on ESI-MS signal were investigated. The resolution and detection sensitivity with different separation methods (pCEC, capillary high-performance liquid chromatography) coupled on-line with mass spectrometry were compared for the separation of a peptide mixture. To evaluate the feasibility and reliability of the experimental setup of the system, tryptic digests of cytochrome *c* and modified protein as real samples were analyzed by using pCEC-ESI-MS.

Capillary electrochromatography (CEC) combines the best features of capillary electrophoresis (CE) and capillary high-performance liquid chromatography (cHPLC): high separation efficiency of CE and the versatile selectivity and large sample capacity of cHPLC.[1−2] However, in practice, when CEC was used without pressure, often on a commercial CE instrument, there were problems and difficulties associated with bubble formation and column dry-out. These problems can be solved by a pressurized CEC (pCEC) system, in which a mobile phase is driven by both a pressurized flow and an electroosmotic flow (EOF). In such a system, pressure is applied to the capillary column to suppress bubble formation. Quantitative sample introduction in pCEC can be achieved through a rotary-type injector. The EOF can either be in the same direction as, or against, the pressurized flow. Therefore, the sample elution order may be manipulated. Most importantly, it is amenable for a solvent gradient mode, similar to that in HPLC, by programming the composition of eluents.[3,4] With pCEC, the promises of CEC can be fully exploited.

Mass spectrometry (MS) provides high sensitivity and reliable detection for various samples, particularly in biochemical applications where the sample amount is very limited.[5] Moreover, it offers significant information on the structure and identity of the analytes. Therefore, pCEC−MS plays an important role in the analysis of biological samples. However, the interface between pCEC and MS might affect the performance of such hyphenation. Among all the developed interfaces, an electrospray ionization (ESI) interface permits the mass spectrometric analysis of large biomolecules with masses up to several hundreds of thousands within the low-mass scan range, and it proves to be suitable for the coupling with HPLC, CE, CEC, and pCEC.[6−20]

Bayer et al.[19,20] coupled pCEC with MS by using an ESI interface for analysis of a crude extract of ergot fungus and enantiomers. They demonstrated convincingly the usefulness and power of pCEC using the laboratory-made modular system. In our report, cHPLC-ESI-MS and pCEC-ESI-MS were carried out using the same experimental setup. The effects of the pH and the concentration of the electrolyte in pCEC on ESI-MS signal intensity were studied experimentally and theoretically, and the effects of applied voltage and the organic modifier on separation

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of peptides were also investigated. The tryptic peptides, protein, and modified proteins were analyzed to illustrate the capability of the pCEC-ESI-MS system.

EXPERIMENTAL SECTION

Materials. Methanol (HPLC-reagent grade), acetonitrile (HPLC-reagent grade), formic acid (80%, analytical-reagent grade), and ammonium acetate (analytical-reagent grade) were purchased from Yuwang Ltd. Co. (Shandong, China). Capillary columns (100-μm i.d., 375-μm o.d., 30 cm, 20 cm packed with ODS) were purchased from Unimicro Technologies Inc. (Pleasanton, CA). TPCK-treated trypsin, cytochrome *c*, Gly-His, Gly-Leu-Tyr, Pro-Phe-Asp, Leu-Ala-Tyr, and Ala-Trp were obtained from Sigma (St. Louis, MO). Insulin standard and modified insulin were gifts from Prof. Huang at Huazhong Institutes of Technology (Wuhan, China). High-purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA). The ammonium acetate−formic acid electrolyte was prepared from the ammonium acetate solution with subsequent adjustment of pH with formic acid.

pCEC Operation and Hyphenation with ESI-MS. All pCEC and cHPLC experiments were performed on TriSep-2010GV capillary electrochromatography system (Unimicro Technologies, Inc., Pleasanton, CA). ESI-MS was performed on a Finnigan LCQ^{DUO} ion trap mass spectrometer (Finnigan MAT, San Jose, CA).

The mass spectrometer was equipped with an electrospray ion source and calibrated by direct infusion of a solution of methyl reserpate 3,4,5-trimethoxybenzoic acid ester (10 pg/μL, Finnigan) in methanol−water (50:50, v/v) containing 1% acetic acid. An electrospray voltage of 4.5 kV was employed. The temperature of the heated transfer capillary was set to 200 °C. The sheath-liquid flow comprising 0.5% formic acid in methanol−water (50:50, v/v) was provided at a flow rate of 3 μL/min by using a syringe pump (Mode 22, Harvard Apparatus, South Natick, MA). Electrochromatograms and mass spectra were recorded on a LCQ DUO workstation with Core data system software, version 1.2 (Finnigan). Peptide searches were performed using the SEQUEST software.[21−24]

Figure 1 shows the schematic diagram of the pCEC-ESI-MS system with the coaxial sheath flow interface. On-line ESI-MS was performed in the positive-ion full-scan mode and selected ion monitoring scan mode, typically using an ESI voltage of 4.5 kV. The flow rate of sheath gas was set at 12 mL/min. For pCEC-MS experiments, the sample injection volume was 20 nL for each scan. For the MS/MS experiments, the maximum sample injection volume was 50 nL for each scan. The relative collision energy for CID was set at 35%.

Preparation of Tryptic Protein Digest. The protein digest mixture was prepared as follows: first, the sample was dissolved in 100 mM ammonium bicarbonate to a concentration of 1 mg/mL. TPCK-treated trypsin dissolved in 100 mM ammonium bicarbonate was then added at a substrate-to-enzyme ratio of 20:1, and the solution was incubated overnight at 37 °C

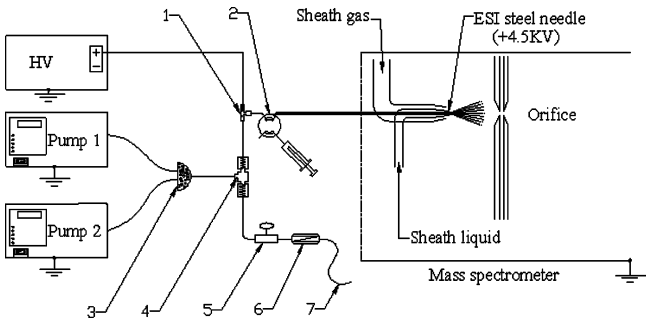

Figure 1. Schematics of pCEC coupled with ESI-MS: (1) tee/electrode; (2) nanoinjector; (3) micromixer; (4) microsplitter; (5) on/off valve; (6) back pressure regulator; (7) waste.

RESULTS AND DISCUSSION

pCEC Separation of Peptides. Good separation resolution for the five selected peptides could not be achieved in isocratic solvent condition. However, programmed solvent gradient can be carried out conveniently with the binary solvent pCEC system. The ESI-MS was performed in selected ion monitoring mode with a 40-min solvent gradient from 10 to 70% acetonitrile in 30 mM ammonium acetate−formic acid at pH 3.0. Baseline separation of the peptides was achieved under the gradient condition (see Figure 2).

cHPLC and pCEC. As we can see from Figure 1, the actual electrical potential across the column is the result of the applied voltage from the pCEC subtracting the ESI voltage. Therefore, for pCEC-ESI-MS to be realized, the applied voltage needs to be bigger or less than the ESI voltage. For pure cHPLC-ESI-MS to be realized, the applied voltage has to be the same as the ESI voltage. In other words, one would actually be performing pCEC when coupling a cHPLC instrument to this ESI-MS system because the 4.5-kV electrospray voltage is applied on the outlet end of the capillary column. In this case, the EOF in the capillary is against the pressurized flow. The differences of the cHPLC and pCEC separation of two peptides (1, Gly-His; 2, Leu-Ala-Pro) were investigated, and the results are shown in Figure 3. The two peptides were positively charged under the pH value of 3.0. In Figure 3b, the applied voltage and ESI voltage was both set at the same value (4.5 kV) and they compensated each other; there was no electrophoretic mobility and no EOF. In fact, this is actually a cHPLC-ESI-MS case and the two peptides were not separated. In Figure 3a, the applied voltage was higher than the ESI voltage so that the electrophoretic mobility and EOF in the column were in the same direction as the pressurized flow. Consequently, the speed of the separation is much higher and the two peptides were baseline separated due to the difference in their electrophoretic mobility. In Figure 3c, the applied voltage was set at zero. However, the ESI voltage of 4.5 kV was applied to the outlet of the column simultaneously when electrospray was carried out. The inlet of the column is partially grounded through the solution in the tubings that are connected to the pumps. In this case, the direction of the electrophoretic mobility and EOF in the capillary column were against the pressurized flow. Accordingly, two peptides were separated in a reverse order and the migration times of the two peptides were much longer.

Effects of Applied Voltage on Separation of Peptides. The effects of the applied voltage on separation of four peptides were investigated, and the results are shown in Figure 4. The separation

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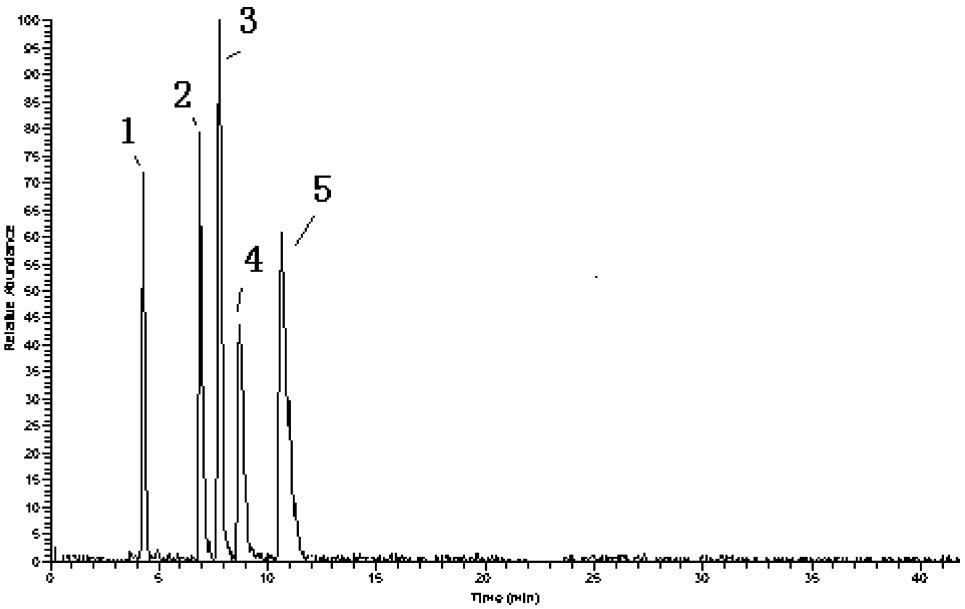

Figure 2. pCEC-ESI-MS analysis of five peptides. pCEC conditions: column, 100−μm i.d., 375−μm o.d., 30-cm total length, 20 cm packed with ODS; mobile phase, (A) acetonitrile; (B) 30 mM formic acid−ammonium acetate; gradient, 10% A to 70% A in 40 min.; pressure, 7000 kPa; Applied voltage, 10 kV; injection volume, 20 nL. MS conditions: sheath gas, 12 unit/min; spray voltage, 4.5 kV; sheath liquid, 0.5% formic acid in methanol−water (50:50, *v/v*), 3 μL/min; scan range, 200−2000 u. Sample: 1, Gly-His; 2, Leu-Ala-Pro; 3, Pro-Phe-Asp; 4, Ala-Trp; 5, Gly-Leu-Tyr.

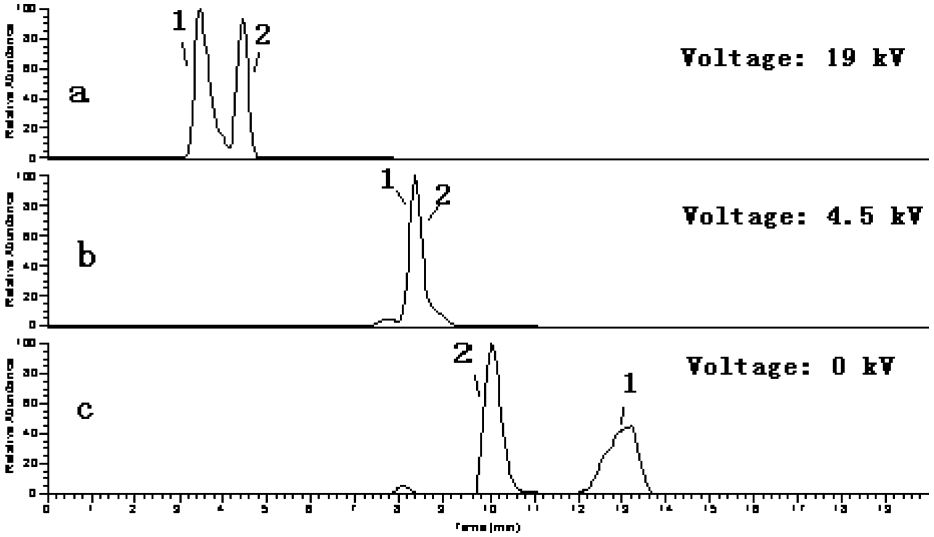

Figure 3. Comparison of cHPLC and pCEC for separation of peptides. Conditions: mobile phase, 70% acetonitrile in 30 mM ammonium acetate−formic acid; sample, 1, Gly-His; 2, Leu-Ala-Pro; 20−50 μg/mL each. Other conditions as in Figure 2.

of four peptides was improved by changing the applied voltage. When the applied voltage increased from 4.5 to 15 kV, the resolution of the separation of the four peptides became better. When the applied voltage decreased from 4.5 to 0 kV, the resolution of the separation of the four peptides also became better. We could make a conclusion that the separation of samples would improve with the increase of the separation voltage. The resolutions of peak pairs 1 and 2 and 2 and 4 were calculated, and the results are listed in Table 1.

Effects of pH Value of the Mobile Phase on ESI-MS Signal. In ESI, the number of ions that escape from a droplet is related to the charge on the droplet, which can be derived from the spray current measurement. Formic acid−ammonium acetate buffer should be considered as a trinary-electrolyte system. We have[25] proposed equations for a trinary-electrolyte system.

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (1)$$

$$I_H = fp\frac{k_H[H^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (2)$$

$$I_{NH_4} = fp\frac{k_{NH_4}[NH_4^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (3)$$

where I_S is the S^+ ion signal at MS detection; I_H is the H^+ ion

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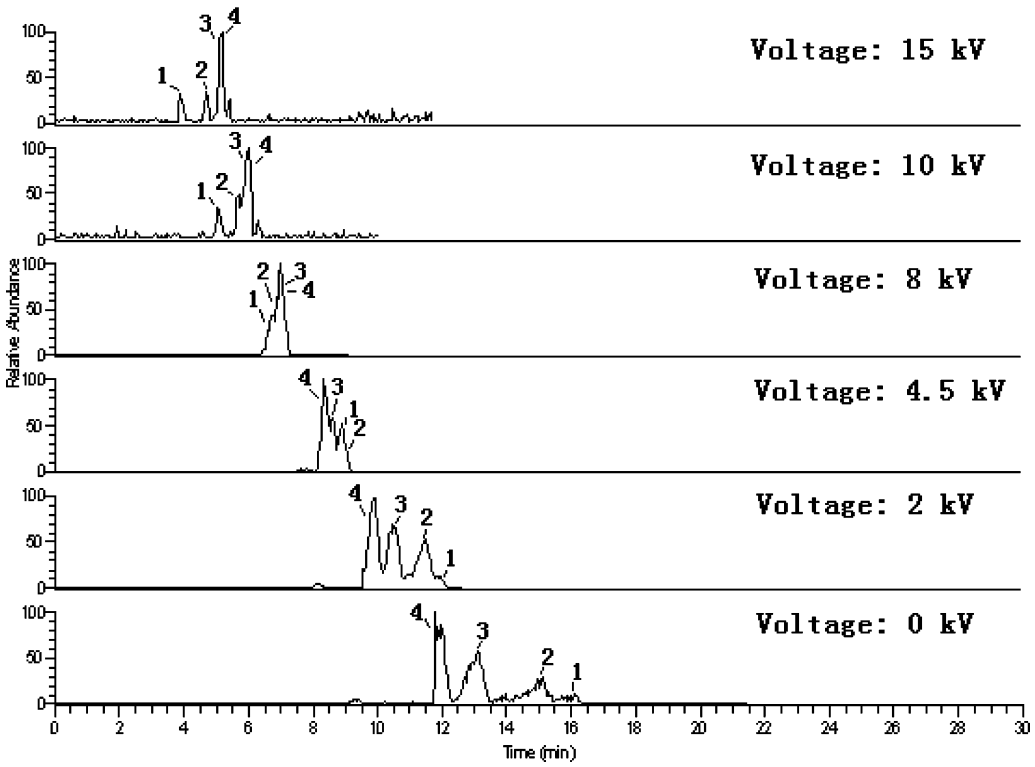

Figure 4. Influence of applied voltage on separation of peptides. Conditions: mobile phase, 30 mM formic acid−ammonium acetate/70% acetonitrile; sample, 1, Gly-His; 2, Leu-Ala-Pro; 3, Gly-Leu-Tyr; 4, Pro-Phe-Asp; 20−50 μg/mL each. Other conditions as in Figure 2.

Table 1. Effects of Applied Voltage on Resolution of Peptides

	applied voltage (kV)					
	0	2	4.5	8	10	15
$Rs_{1,2}$	0.58	0.36	0.18	0.43	1.71	2.72
$Rs_{2,4}$	2.22	1.17	0.81	0.15	0.65	1.70

signal at MS detection; I_{NH_4} is the NH_4^+ ion signal at MS detection; f represents the fraction of charges on the droplet that are converted to the gas-phase ions; p is the fraction of gas-phase ions transported into the mass analyzer; k_S, k_H, and k_{NH_4} are respectively the sensitivity coefficients for S^+, H^+, and NH_4^+; I_{spray} is total droplet current (spray current); and $[S^+]$, $[H^+]$, and $[NH_4^+]$ are the concentrations of S^+, H^+, and NH_4^+. For an increasing concentration of electrolytes in solution, the spray current increases weakly with conductivity,[26,27]

$$I_{spray} \propto (\text{conductivity})^n \qquad (4)$$

where $n < 1$.

The effects of pH value of the electrolyte on the sample ions signal intensity have been investigated, and the results are shown in Figure 5. It can be seen that the sample ion signal intensity of Gly-His increases with the increase of electrolyte pH value from 2.5 to 2.7 and reduces as the increase of the electrolyte pH value from 2.7 to 3.3. This phenomenon can be explained by eq 1. When

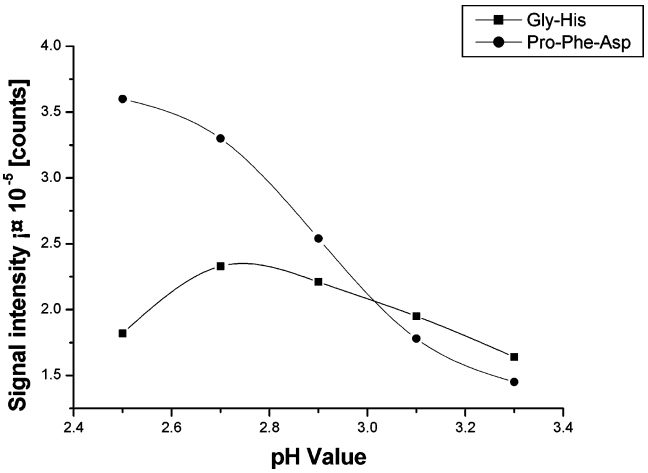

Figure 5. Effects of pH value of the mobile phase on ESI-MS signal. Conditions: applied voltage, 19 kV; mobile phase, 50 mM ammonium acetate−formic acid/70% acetonitrile, pH 2.5−3.3. Other conditions as in Figure 2.

the electrolyte pH increases from 2.5 to 2.7, the sample ion has been fully ionized and there is residue $[H^+]$ in the system. In this pH range, $[H^+]$ is the dominant factor on the intensity of the sample ions signal. Accordingly, eq 1 can be further modified as the following.

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_H[H^+]}I_{spray} \qquad (5)$$

When the pH of the electrolyte increases from 2.5 to 2.7, $k_H[H^+]$ decreases. Accordingly, I_S increases. When the electrolyte pH

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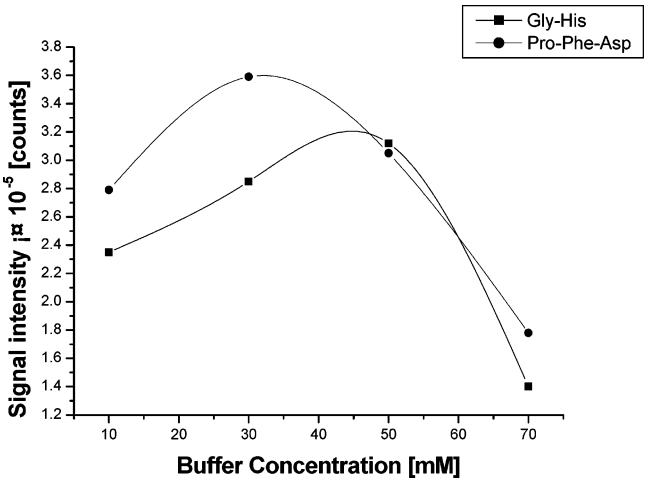

Figure 6. Effects of the concentration of the mobile phase on ESI-MS signal. Conditions: applied voltage, 19 kV; mobile phase, 10−70 mM ammonium acetate−formic acid/70% acetonitrile, pH 3.0. Other conditions as in Figure 2.

increases from 2.7 to 3.3, the sample ions are not fully ionized, and $[NH_4^+]$ is the dominant factor on the sample ion signal intensity. Consequently, eq 4 can be further modified as the following.

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (6)$$

In this pH range, $K_S[S^+]$ decreases and $k_{NH_4}[NH_4^+]$ increases. I_S is reduced with the increase of $[NH_4^+]$ concentration. For Pro-Phe-Asp, the sample ions signal intensity reduces in the investigated pH range, which could also be explained by eq 6.

Effect of Electrolyte Concentration on ESI-MS Signal. The effect of the electrolyte concentration on the ESI-MS signal has been investigated, and the results are shown in Figure 6. For Gly-His, maximum sample signal intensity was achieved with 50 mM formic acid−ammonium acetate at pH 3.0. At lower concentrations of 10 and 30 mM formic acid−ammonium acetate, the sample signal intensity decreased. One possible explanation for this behavior is that at lower concentration the ζ potential of the capillary surface increases and therefore enhances the adsorption of peptides to the capillary surface, which leads to band broadening. Accordingly, the sample ions signal intensity is reduced. When the electrolyte concentration increased from 50 to 70 mM, the sample ions signal intensity decreased too. This might be caused by the high NH_4^+ concentration, which competed with the sample ions in the conversion process from solution to gas-phase ions.

pCEC-ESI-MS Analysis of Tryptic Peptides. Separation and identification of a tryptic digest of cytochrome *c* were chosen to test the applicability of pCEC-ESI-MS. A background mobile phase composed of 5% acetonitrile in 50 mM ammonium acetate−formic acid at pH 3.0 was selected as the initial condition, where all of the peptides were protonated. The direction of the electroosmotic flow and electrophoretic mobility in the mobile phase was the same. ESI-MS detection conditions of the tryptic peptides should be divided into three sections. First, ESI-MS was performed in the positive-ion, full-scan mode to detect all the ions from the heated capillary to the ion trap. Then, it was automatically changed

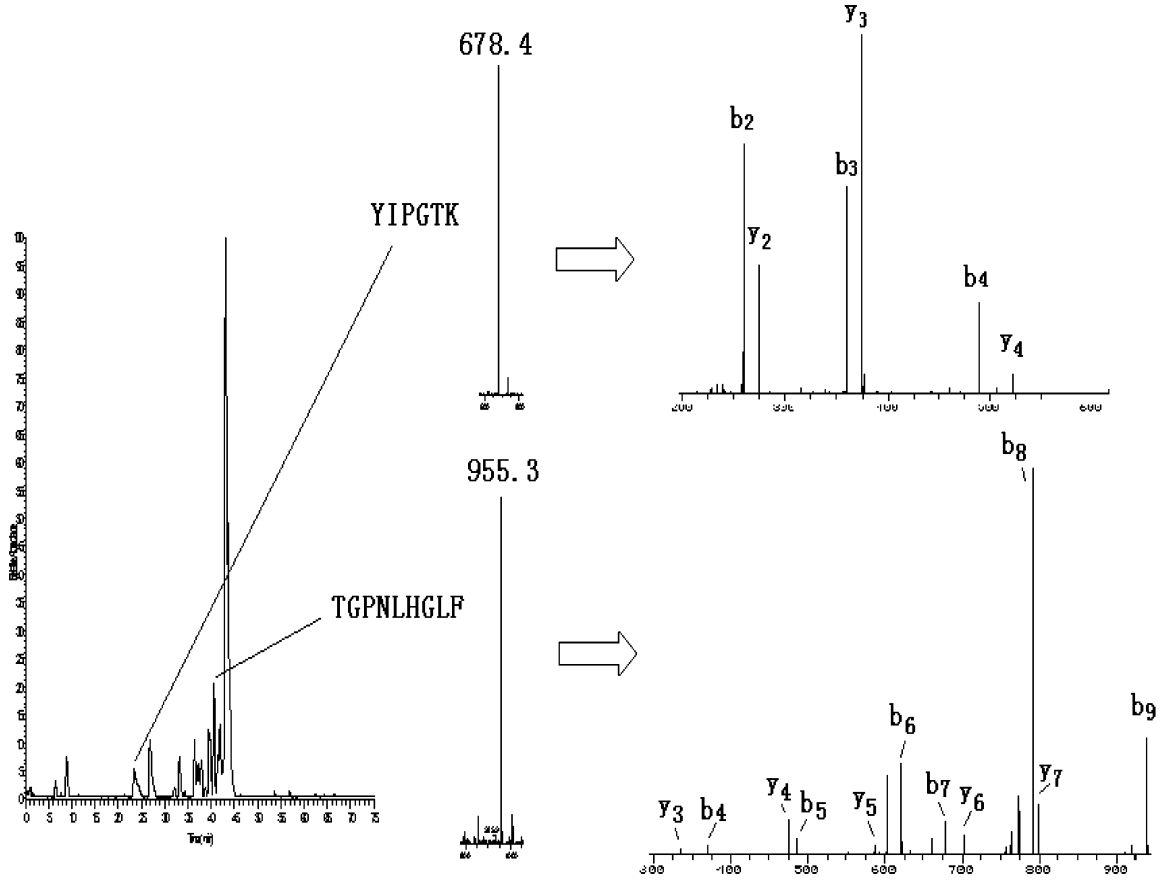

Figure 7. pCEC-ESI-MS analysis of tryptic digests of cytochrome *c*. Conditions as in Figure 2.

Table 2. Results of SEQUEST Database Search

position	sequence	retention time (min)	charge	measured mass	calculated mass
9−13	IFVQK	26.91	+1	634.4	633.8
9−22	IFVQKCAQCHTVEK	43.22	+1	1633.2	1633.9
28−36	TGPNLHGLF	40.46	+1	955.3	955.4
28−38	TGPNLHGLFGR	41.39	+2	585.1	585.1
39−53	KTGQAPGFTYTDANK	36.38	+2	800.1	799.4
40−53	TGQAPGFTYTDANK	37.26	+2	736.1	736.0
56−60	GITWK	33.13	+2	604.1	603.3
61−72	EETLMEYLENPK	42.50	+1	1495.3	1495.7

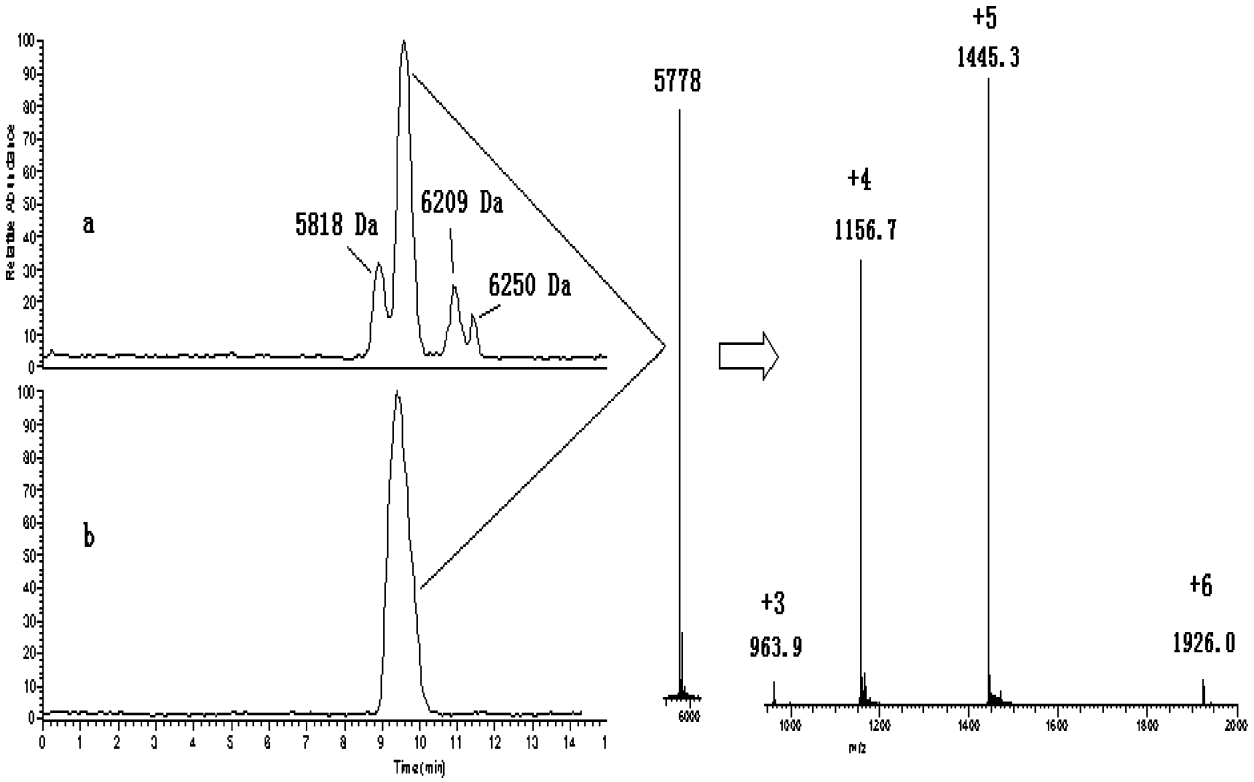

Figure 8. pCEC-ESI-MS analysis of modified proteins. Conditions: applied voltage, 10 kV; mobile phase, 50 mM ammonium acetate−formic acid/50% acetonitrile, pH 3.0. (a) Chromatogram of modified insulin; (b) chromatogram of insulin. Other conditions as in Figure 2.

to ZoomScan mode detection of the charge of the ion when the ion signal intensity on MS was bigger than 5×10^{-4} counts. Finally, it automatically changed to MS/MS mode analysis of the ion. Figure 7 shows the full-scan electrochromatogram by using pCEC-ESI-MS of the tryptic peptides where a total of 13 of the 23 peaks could be directly identified from the database as digested peptides of cytochrome *c*. Table 2 shows the results of a SEQUEST database search. Additional structure information could be obtained by tandem MS/MS analysis of selected peaks performed on the digest peaks, as shown in Figure 7 (right).

pCEC-ESI-MS Analysis of Modified Protein. Analysis of modified protein was carried out to prove the capability of the pCEC-ESI-MS. Insulin (molecular weight 5778) was modified by using cholic acid (molecular weight 408). A mobile phase comprising 50% acetonitrile in 50 mM ammonium acetate−formic acid at pH 3.0 was used for the pCEC separation of the modified insulin. The sheath liquid was methanol−water (50:50, v/v) with 0.5% formic acid added. Figure 8a shows the electrochromatogram of insulin with modification. Figure 8b shows the electrochromatogram of insulin (from pig) without modification. It can be seen that modified insulin separated to four peaks, but insulin without modification was only a single peak. According to the full-scan mass spectra of insulin and the molecular weight information, the second peak in Figure 8a was clearly identified to be insulin. The molecular weights of other peaks were calculated to be 5818, 6209, and 6250, respectively. These results show that the insulin was indeed modified. Detailed identification of these peaks is in progress and will be discussed in future work.

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