

COMMUNICATION

Capillary Isoelectric Focusing Separation Combined with Mass Spectrometry Using Sonic Spray Ionization for Protein Analysis

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(Received September 19, 2001; Accepted October 26, 2001)

Capillary isoelectric focusing (CIEF) separation combined with mass spectrometry for protein analysis has been demonstrated by using a sonic spray ionization (SSI) interface, which has a buffer reservoir between the ion source and the separation capillary. Since the buffer reservoir was used as an outlet reservoir for CIEF, on-line and one-step CIEF/MS was possible. In SSI, since a sample solution is sprayed at any solution-flow rate from a sample-introduction capillary with a high-speed gas flow, high-polymer ampholytes for CIEF can be used without clogging up the spray nozzle of the interface. Also, additives such as ampholytes significantly decrease ion intensity of analyte molecules; however, the SSI interface whose reservoir is filled with an acetic acid solution minimizes the decrease in ion intensity of the analyte. We combined CIEF with an ion trap mass spectrometer and were able to detect 160 fmol of myoglobin and cytochrome-c.

Introduction

Separation and identification of proteins and peptides from cell-extracts are becoming important in the field of proteomics. The isoelectric point (*pI*) of proteins is a useful parameter for identifying proteins as well as their molecular weights. For this identification, isoelectric focusing separation in a capillary,¹⁾ *i.e.*, capillary isoelectric focusing (CIEF), is a powerful method for separation of proteins according to *pI*. Coupling CIEF and mass spectrometry²⁾⁻⁹⁾ are available for faster-protein characterization and higher reproducibility than two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). In these techniques, sheath-flow electrospray ionization (ESI) is used as an interface. However, they are not simple procedures; that is, they involve either of two types: after CIEF separation between the inlet and outlet reservoirs, one end of the separation capillary is removed from the reservoir and inserted into a sheath-flow interface^{3), 5), 7)-9)}; or the outlet catholyte is exchanged immediately for an acidic sheath liquid in the sheath-flow interface.⁴⁾

We have recently developed a sonic spray ionization (SSI) interface for capillary electrophoresis/mass spectrometry (CE/MS): it has a buffer reservoir between the sample-introduction capillary of the ion source and the electrophoresis-separation capillary.¹⁰⁾ In SSI, a sample solution is sprayed from a sample-introduction capillary by a high-speed nitrogen-gas flow that is coaxial to the capillary, and ions of the chemicals in the solution, as well as charged droplets, are produced at room temperature and atmospheric pressure.¹¹⁾⁻¹⁴⁾ Since the solution is sprayed only by the gas flow, it can be used with a wide range of buffer solutions and solution-flow rates,^{10), 14)} and high-polymer ampholytes for CIEF can be used without clogging up the spray nozzle of the

interface. In the SSI interface for CE/MS, an acetic acid solution in the buffer reservoir is pumped into the sample-introduction capillary by the pressure reduction around the tip of this capillary due to the high-speed gas flow; thus, the mobile-phase buffer, containing nonvolatile compounds such as phosphate in the sample-introduction capillary, is mixed with the acetic acid.¹⁰⁾ This mixing increases the ion intensity of the sample 100-fold by enhancing the evaporation of charged droplets.¹⁰⁾ Furthermore, since the buffer reservoir is also used as the outlet reservoir, one-step CIEF/MS analysis is possible. Using this SSI technique, we performed CIEF/MS analysis of a protein mixture.

Experimental

Materials

Carbonic anhydrase II (CA II, *pI* 5.4 and 5.9) and myoglobin (Mb, *pI* 6.8 and 7.2) for *pI* makers were purchased from Sigma. Cytochrome-c (Cyt-c, *pI* 10.1) was also purchased from Sigma. Pharmalyte (*pI* 3-10) was purchased from Amersham Pharmacia Biotech.

CIEF/SSI-MS system

Figure 1 is a cross-sectional view of the SSI interface. The details are described elsewhere.¹⁰⁾ The sonic spray ion source, which is a part of a three-dimensional quadrupole (3DQ) ion-trap mass spectrometer (M-8000, Hitachi, Ltd., Tokyo), is modified. Namely, one end of a fused-silica sample-introduction capillary (200- μm o.d., 100- μm i.d., 10 cm long) is inserted into an orifice (0.4-mm i.d.) in the housing of the ion source. Pressurized nitrogen gas flows into the housing, causing a gas flow through the orifice to the atmosphere, which thereby generates a spray. A voltage of -1.2 kV is applied to the housing, which is isolated from the solution by the sample-introduction capillary.¹⁰⁾ The other end of the sample-introduction capillary is inserted into a Teflon tube (0.25-mm i.d., 1.7-mm o.d.) that passes through a buffer reservoir. The buffer reservoir is filled with an analyte, so that the pinhole in the Teflon tube is submerged and the acetic acid solution flows through the

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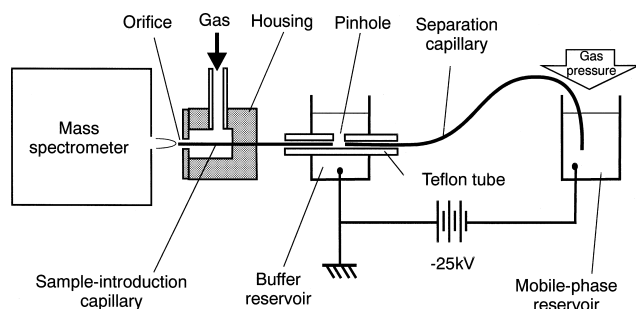


Fig. 1. Cross-sectional view of the sonic spray interface and CIEF-SSI/MS system.

pinhole into the sample-introduction capillary because of pumping effect.¹⁰ The electrical potential of the solution in the sample-introduction capillary and a buffer reservoir is held at ground through the electrode of the buffer reservoir. A fused-silica capillary for separation (*i.e.*, the separation capillary: 50- μm i.d., 375- μm o.d., 80 cm long) taken from a CAPI-3200 electrophoresis system (Otsuka Electronics Co., Ltd., Osaka) is inserted into the Teflon tube from the opposite end. The capillaries are set opposite each other near the pinhole in the tube. The other end of the separation capillary goes into a mobile-phase reservoir filled with the catholyte in CAPI-3200. A voltage is applied to the catholyte in the mobile-phase reservoir to generate a pH gradient for isoelectric focusing. After focusing, the samples pushed out from the separation capillary are mixed with the acetic acid solution in the area beneath the pinhole in the tube, and pumped into the sample-introduction capillary. The sample solution is then sprayed from the sample-introduction capillary by the sonic gas flow. The ions produced in the atmosphere are introduced into the vacuum region of the M-8000 mass spectrometer through a sampling orifice.

CIEF separation

The separation capillary coated with acrylamide is filled with protein mixture containing 1% v/v of Pharmalyte and 0.1% hydroxyethyl cellulose, then a voltage of -25 kV is applied between the mobile-phase reservoir filled with catholyte (20-mM sodium hydroxide, pH 12.0) and the buffer reservoir filled with an anolyte, 8% acetic acid solution (water-methanol-acetic acid, 50:42:8% (v/v/v); pH 3.0), for 15 or 20 minutes for focusing. Next, gas pressure is applied to the cathodic end, *i.e.*, mobile-phase reservoir, while maintaining the voltage, then the focusing proteins are pushed into the sample-introduction capillary and ionized.

Results and Discussion

Figure 2 shows an electrophorogram of the protein mixture (Fig. 2(a)) and mass spectra corresponding to the peaks of the electrophorogram (Figs. 2(b) and (c)). The sample mixture contained 0.6- μM CA II (pI 5.4), 0.6- μM CA II (pI 5.9), and 1.2- μM Mb (mixture of pI 6.8 and 7.2). And 940 fmol of each protein was injected into the separation capillary. A voltage of -25 kV was applied to the mobile-phase reservoir for 15 minutes, then a gas-pressure of 0.11 kgf/cm² was applied to its surface. We scanned the intensities at an m/z range from 600 to 1,900 and monitored the intensity at m/z 847 and

1,035, which correspond to the 21-protonated molecule of Mb and the 29-protonated molecule of CA II, respectively, in Fig. 2(a). After 9.2, 9.7, 11.7, and 12.3 minutes of pressuring the protein samples, clearly separated peaks corresponding to the CA II (pI 5.4), CA II (pI 5.9), Mb (pI 6.8), and Mb (pI 7.2) ions appeared, respectively. The noises observed from 8 to 14 minutes were due to Pharmalyte-containing compounds with a wide range of molecular weights. Mass spectra of CA II and Mb obtained at 9.2 and 12.3 minutes are shown in Figs. 2(b) and (c), respectively. Multiply-charged ions of proteins are clearly detected.

Figure 3 is an electrophorogram of the Mb and Cyt-*c* (pI 10.1) mixture. We injected 160 fmol of Mb and Cyt-*c* (concentration of each solution was 0.1 μM), and -25 kV was applied to the mobile-phase reservoirs for 20 minutes, then a gas pressure of 0.20 kgf/cm² was applied. We monitored the intensities at m/z 892 and 814 corresponding to the 20-protonated molecule of Mb and the 15-protonated molecule of Cyt-*c*, respectively. After 5.6 and 9.3 minutes, peaks corresponding to the Mb and Cyt-*c* ions appeared, respectively. Since Pharmalyte molecules were concentrated around neutral in the pH gradient, the signal/noise ratios of the

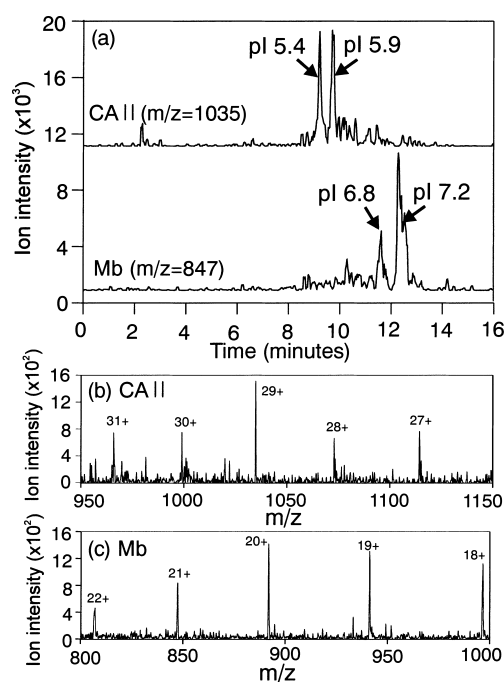


Fig. 2. (a) Electrophorogram of carbonic anhydrase II (pI 5.4 and 5.9) and myoglobin (pI 6.8 and 7.2) and mass spectra of (b) carbonic anhydrase II at a peak of 9.2 minutes and (c) myoglobin at a peak of 12.3 minutes in the electrophorogram. The mobile-phase (inlet) reservoir and the buffer (outlet) reservoir were filled with a 20-mM sodium hydroxide (pH 12.0) in water and 8% acetic acid solution (water-methanol-acetic acid: 50:42:8%, v/v/v; pH 3.0), respectively, and the amount of injected samples was 940 fmol. The separation capillary coated with acrylamide was 80 cm long and -25 kV was applied between the two reservoirs for 15 minutes, then a gas pressure of 0.11 kgf/cm² was applied to the inlet reservoir.

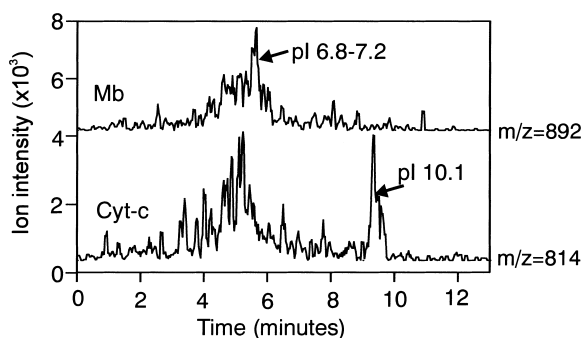


Fig. 3. Electrophorogram of myoglobin and cytochrome-c (*pI* 10.1). The mobile-phase (inlet reservoir and the buffer (outlet) reservoir were filled with 20-mM sodium hydroxide (pH 12.0) in water and 8% acetic acid solution (water-methanol-acetic acid=50:42:8%, v/v/v, pH 3.0), respectively, and the amount of injected samples was 160 fmol. The separation capillary coated with acrylamide was 80 cm long and -25 kV was applied between the two reservoirs for 20 minutes, then a gas pressure of 0.20 kgf/cm² was applied to the inlet reservoir.

Cyt-c peak on the alkali side were higher than the Mb peak.

In conclusion, CIEF-SSI/MS is valuable as a simple and fast method for protein analysis. Furthermore, use of other types of mass spectrometers, for example, time-of-flight (TOF) and Fourier-transform ion cyclotron resonance (FT-ICR), as a detector of CIEF-SSI/MS, will provide a powerful tool with high resolution and sensitivity.

Acknowledgments

We thank Prof. Satoko Akashi of Yokohama City University, Prof. Koji Takio and Dr. Hisashi Kato of RIKEN for their valuable discussions. We also thank Dr. Keiko Kitagishi and Mr. Kazushi Sasa of Otsuka Electronics Co., Ltd. for their valuable assistance and providing the tools used in our study. We also thank

Mr. Yoshinobu Kohara, Mr. Tomoyuki Sakai, Mr. Masao Kamahori, Dr. Tsuyoshi Sonehara, Dr. Kyoko Kojima, Mr. Toshihiro Ishizuka, and Mr. Atsushi Nemoto for their help in our experiments.

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Keywords: Capillary isoelectric focusing separation/mass spectrometry (CIEF/MS), Sonic spray ionization, Protein, Proteomics