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Journal of Chromatography A, 664 (1994) 271–275

JOURNAL OF CHROMATOGRAPHY A

Isolation of hydrophobic lipoproteins in organic solvents by pressure-assisted capillary electrophoresis for subsequent mass spectrometric characterization ☆

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(First received January 26th, 1993; revised manuscript received December 17th, 1993)

Abstract

Two capillary electrophoretic (CE) separation techniques with either simultaneous solvent flow induced by hydrostatic pressure or CE followed by low pressurization with helium were developed for the analysis of extremely hydrophobic proteins, such as the lung surfactant protein SP-C. For both related procedures, buffer solutions containing up to 70% of 2-propanol were used for the capillary electrophoretic separation. This high concentration of organic co-solvent, needed to solubilize the protein, dramatically reduces the electroosmotic flow (EOF) in aminopropyltrimethoxysilane-treated fused-silica capillaries. Because the EOF was insufficient to elute the separated analytes from the capillary, two "pressure-assisted" CE techniques were developed. An additional flow to elute the separated analytes was produced either by raising the inlet of the capillary or by helium pressure. Using the pressurization procedure a baseline separation of the SP-C protein and its dimeric complex was obtained in a 55-minute electrophoretic run, followed by pressure elution of the analyte to the detector. The present combination of pressurization and capillary electrophoresis does not require any detergents or involatile buffer additives, which are usually needed to solubilize extremely hydrophobic lipoproteins. It is therefore applicable to on-line coupling with electrospray mass spectrometry for the direct structural characterization of hydrophobic proteins.

1. Introduction

Separation by capillary electrophoresis (CE) combined with desorption–ionization mass spectrometry such as ^{252}Cf plasma desorption (PD-MS) or electrospray mass spectrometry (ES-MS) has been developed as an efficient method for the characterization of primary structure and molecular homogeneity of proteins [1–4].

Highly hydrophobic proteins and many lipoproteins are poorly soluble or insoluble in aqueous buffers without the use of detergents or organic solvents. Combining CE with PD-MS or ES-MS, however, requires the use of volatile

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☆ Presented at the *5th International Symposium on High Performance Capillary Electrophoresis, Orlando, FL, January 25–28, 1993.* The majority of the papers presented at this symposium were published in *J. Chromatogr. A*, Vol. 652 (1993).

0021-9673/94/$07.00
SSDI 0021-9673(93)E0069-7

buffers containing minimal amounts of inorganic salts or detergents. To avoid the use of detergents in CE–MS, buffers containing, *e.g.*, 20% of 2-propanol or 25% of acetonitrile in 10 m*M* acetic acid have been employed for the separation and identification of water-insoluble lipopeptides, both by the "off-line" combination of CE and PD-MS and by "on-line" CE–ES-MS [5].

Even higher concentrations of organic solvents, *e.g.*, 50–70% of 2-propanol, are required for solubilizing extremely hydrophobic proteins such as the lung surfactant protein SP-C [6]. Also, for the separation and mass spectral analysis of hydrophobic proteins, acidic conditions are desirable because of the instability of these compounds in basic buffers; further, the separation of peptides as negative ions, followed by analysis by positive-ion ES-MS, can lead to reduced mass spectrometric sensitivity. The use of untreated fused-silica capillaries with acidic mobile phases, however, leads to significant peak broadening for strongly hydrophobic proteins due to adsorption on the capillary wall. Capillary treatment with aminopropyltrimethoxysilane (APS) produces a positively charged capillary wall, repelling positively charged proteins and leading to reduced wall adsorption [2,7]. In CE with APS-treated capillaries, where reversed polarity is used, there are two competing driving forces: (i) the movement of the positively charged analyte upstream towards the cathode and (ii) downstream movement resulting from the electroosmotic flow (EOF). With aqueous acidic buffers the EOF predominates, whereas with organic modifiers the EOF is strongly decreased, as demonstrated for peptides using buffers containing up to 25% of acetonitrile [5]. Owing to its high viscosity, 2-propanol has an even greater effect on decreasing the EOF [5,8]. At a concentration of 50% of 2-propanol highly hydrophobic proteins migrate very slowly and the analysis times become very long, resulting in severe peak broadening and reduced mass flow to the detector.

Two procedures applying the same principle, called "pressure-assisted capillary electrophoresis", are described here which avoid these problems and significantly shorten the analysis times of extremely hydrophobic proteins even in high concentrations of organic solvents.

2. Experimental

2.1. Capillary electrophoresis

A 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) derivatized with APS (Aldrich, Milwaukee, WI, USA) [7] was cut to a length of 71 cm. A small area of the polyimide coating was burned off to form a window for UV detection by an SSI-500 UV detector (Applied Science Labs., State College, PA, USA). The CE conditions were as follows: APS capillary, 71 cm $\times$ 75 μm I.D. (UV detection at 44 cm); hydrostatic procedure, buffer, 50% propanol in 10 m*M* acetic acid *ca.* 5 nl of protein solution (2.6 ng of SP-C) were loaded by a 7-s hydrostatic injection; separation voltage, -20 kV (282 V/cm). The hydrostatic solvent flow was obtained by a 20-cm height difference between the cathode and anode ends of the capillary.

For the determination of the hydrostatic flow-rate, a solution of 2% acetone and 50% 2-propanol in 10 m*M* acetic acid was loaded and the elution of acetone was monitored by measuring the UV absorbance at 279 nm. The hydrostatic flow-rate was 1 cm/min (20-cm height differential), which is *ca.* 44 nl/min for a 75 μm I.D. capillary. The pressurized procedure was carried out with buffer, 10 m*M* acetic acid–70% 2-propanol. A protein solution of 0.5 μg/μl in 70% 2-propanol in 10 m*M* acetic acid was loaded by pressure injected for 1 s from a pressurized injection vessel (1 bar) [9]. The sample was pushed on to the capillary for 3 min.

The inlet end of the capillary was then removed from the injection system and placed in a buffer solution maintained at 20 cm above the anode end, and a voltage of -25 kV (350 V/cm) was applied for 55 min. After disconnecting the high voltage, the capillary inlet was placed in the buffer solution in the injection system, which was then pressurized to 0.5 bar for elution of the

separated proteins. Using 70% instead of 50% of 2-propanol reduced the hydrostatic flow-rate by an order of magnitude to *ca.* 4 nl/min (20-cm height differential, no voltage).

2.2. Lung surfactant protein SP-C

Human-identical, biscysteinylpalmitoylated lung surfactant SP-C protein was obtained by chemical palmitoylation of recombinant SP-C, expressed in *Escherichia coli* [10,11]. The structure and homogeneity of the protein were established by PD-MS and ES-MS [6,12]. The lyophilized protein was dissolved in 50% 2-propanol in 10 m*M* acetic acid to give a solution of 0.5 μg/μl prior to CE separation. Approximately 5 nl of sample solution (2.6 ng of protein) were loaded in a 7-s hydrostatic injection with a height difference of 20 cm.

2.3. Electrospray mass spectrometry

The ES mass spectrum was obtained on a Vestec-A201 single-quadrupole electrospray mass spectrometer (Vestec, Houston, TX, USA) by direct injection of 20 μl of protein solution (0.1 μg/μl of protein in 50% 2-propanol + 50% acetic acid).

3. Results

The analysis of the homogeneous human identical recombinant SP-C protein by ES-MS (Fig. 1) shows a triply protonated molecule (*m/z* 1343) as the base peak and also a quadruply protonated molecule (*m/z* 1007) (M_r 4025) together with the $[M + 5H]^{5+}$ ion of an SP-C "leucine-zipper" analogue dimer complex (*m/z* 1612) [12]. As SP-C is a highly surface-active compound of therapeutic importance, the specific dimerization has recently attracted major interest and is thought to be biologically significant. The separation and characterization of such supramolecular structures requires the development of a capillary electrophoresis procedure which can eventually be combined with mass spectrometry.

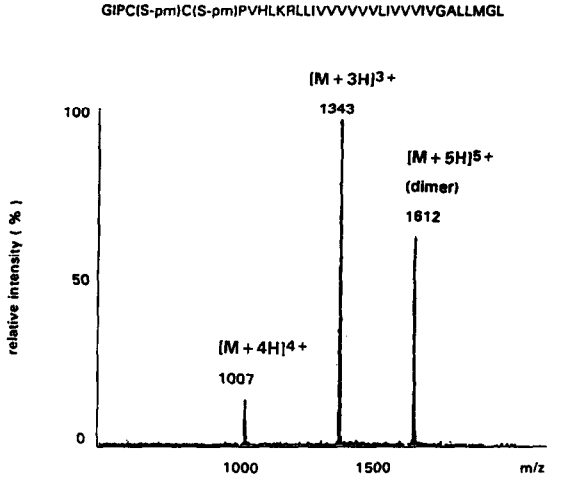

Fig. 1. ES mass spectrum of recombinant palmitoylated SP-C showing the triply and quadruply protonated molecular ions of the SP-C monomer (M_r 4025) and the $[M + 5H]^{5+}$ ion of the dimeric SP-C complex (M_r 8048).

The separation of the SP-C protein was achieved by applying two different procedures. In the first procedure, the protein (0.5 μg/μl in 50% 2-propanol–10 m*M* acetic acid) was loaded hydrostatically and the sample pushed *ca.* 22 cm on to the capillary by applying a low pressure (the total time to the detector was determined in this way to be *ca.* 65 s). The capillary (cathodic end) was then disconnected from the pressure system and placed in the CE buffer reservoir, which was raised 20 cm above the anode buffer reservoir. This mode produced a hydrostatic flow of 44 nl/min. As a result of the combination of hydrostatic pressure and electrophoretic mobility, two major components could be separated in less than 30 min (Fig. 2A).

In the second pressure-assisted procedure, with 70% 2-propanol buffer, the hydrostatic flow-rate was dramatically decreased as determined in a separate experiment. After 55 min of hydrostatic pressure-assisted CE the analyte was still on the capillary. The high voltage was then switched off and the analyte was eluted from the capillary at low pressure (*ca.* 0.5 bar). This mode resulted in a nearly baseline separation of the two SP-C components, as shown in Fig. 2B. As SP-C has been established to have a homogeneous chemical structure [11,12], the electro-

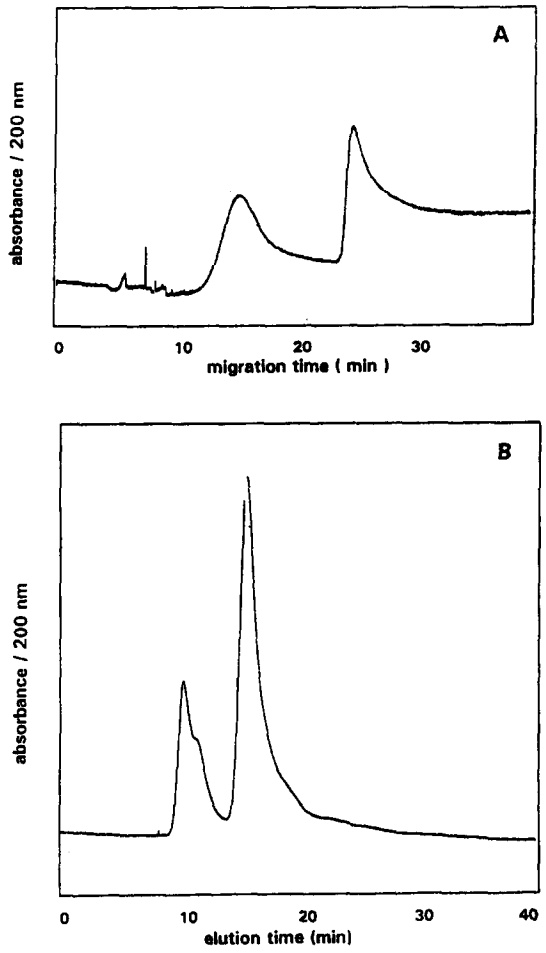

Fig. 2. (A) Capillary electropherogram of recombinant palmitoylated SP-C obtained by applying −20 kV and simultaneous hydrostatic pressure with a 50% 2-propanol buffer. (B) Capillary electropherogram of recombinant palmitoylated SP-C obtained by applying −25 kV for 55 min for CE separation and subsequent pressure elution of the analyte to the detector with 70% 2-propanol.

pherograms in Fig. 2 probably represent the separation of monomeric SP-C and its dimeric complex; confirmation of the latter by CE–ES-MS of the separated peaks is in progress.

4. Discussion

The "pressure-assisted" CE method allows the application of CE to highly hydrophobic analytes in buffers with high organic solvent concentrations without the use of any detergent by first forcing the analyte into the capillary, followed by (i) slow elution by hydrostatic pressure during CE separation at high voltage, or (ii) CE separation and subsequent elution from the capillary by pressurization. It will thus permit the on-line combination with MS at a controllable flow-rate. Without the pressure elution, the mass flow would be too low for subsequent MS analyses. The use of a shorter capillary might decrease the peak broadening caused by the parabolic pressure-induced flow profile and increase the separation efficiency, but the minimum length with our CE–ES-MS system is limited to 70 cm. Although a baseline separation is desirable, it is not required for CE–ES-MS, because discrete m/z values are registered even for overlapping compounds (a significant advantage over absorbance measurements). No effort has been made so far to determine the reproducibility of migration times, because the main goal was to develop a method of the CE separation of lipoproteins. Although the pressurization induces a hydrodynamic flow profile leading to peak broadening, it is necessary to shorten analysis times and increase the mass flow for coupling with mass spectrometry. The effects on separation efficiencies will be the subject of further investigations, as also will be the on-line coupling of the procedure with ES-MS.

5. Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn. We thank the German Academic Exchange Service (DAAD, Bonn, Germany) for providing a fellowship to one of us (W.W.).

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