Journal of Chromatography A, 1217 (2010) 179–182

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Capillary isoelectric focusing coupled offline to matrix assisted laser desorption/ionization mass spectrometry

Noah G. Weiss, Nicole L. Zwick, Mark A. Hayes*

Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ, USA

ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
13 November 2009
Accepted 17 November 2009
Available online 27 November 2009

Keywords:
Capillary IEF
MALDI-MS
MS
Syringe pump

ABSTRACT

This work presents several critical details for making cIEF-MALDI-MS a robust technique which will allow for more routine application and aid in automation. This includes emphasis on the hardware necessary for syringe pump mobilization and proper protocol for preventing disruption from gas bubbles. Following these guidelines, excellent elution time reproducibility is demonstrated for six p*I* markers (RSD <5%). Additionally, the p*I* markers are used to calibrate the pH gradient and determine experimental p*I*s of proteins detected offline by mass spectrometry. This was demonstrated using a standard protein mixture of myoglobin and two forms of β-lactoglobulin. Experimental determination of protein p*I*s and molecular weights were found to be in agreement with literature values. The technical details discussed provide a sound foundation for applying the offline coupling of MALDI-MS with cIEF.

1. Introduction

Understanding protein variance across the proteome, or subsets of the proteome, can provide insight into the health and disease state of humans. Protein analytics often requires the examination of complex samples and heterogeneous expression patterns, thus highly selective tools are critical. A key candidate that can contribute to the analysis of protein variance is capillary isoelectric focusing (cIEF) coupled offline to matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). The coupling of cIEF with MALDI-MS has been only demonstrated a few times [1–6] and is in need of further development in order to reach its full potential. The technical details presented here provide a basis for expanding the use of the technique to solve greater proteomic/analytical problems.

In cIEF, carrier ampholyte mixtures are used to establish a stable pH gradient inside a capillary suspended between an acid and base reservoir. Amphoteric analytes, typically proteins, will migrate with their local electrophoretic mobility until reaching the pH where their net charge is zero, defined as the isoelectric point (p*I*). Once steady state is achieved in cIEF, the focused analytes are typically mobilized for detection and/or fraction collection. Most commonly this is either through chemical, electroosmotic, or hydrodynamic mobilization [7]. Of these, hydrodynamic mobilization is arguably the best choice for interfacing with fraction collection for MALDI-MS detection. It does not cause significant band broadening when the potential field is maintained [8], allows for predictable elution profiles unlike cathodic mobilization [9], and is compatible with internally coated capillaries known to improve cIEF performance [7]. In this work the syringe pump mobilization method is investigated.

Even though the syringe pump is designed to improve the ease of cIEF coupling to MALDI-MS, the additional hardware can cause failed runs if not handled properly. These malfunctions are most often attributed to a disruption of the electric field or fluid flow by a large particle or gas bubble. Such events can have detrimental effects to the electric and/or flow fields resulting in poor separations and null results. Particles arise from sample contamination or precipitation during the focusing process, and resolving this problem has been discussed elsewhere [7]. Gas bubbles are a consequence of electrolysis, joule heating, and hardware handling issues at the site of liquid/air interfaces. This is easily avoided in other mobilization modes since the capillary is suspended between reservoirs open to atmosphere. Air bubbles are more frequent and problematic in syringe pump mobilization due to the additional capillary lines, dynamic junctions, and the need for airtight pressure seals. The work here demonstrates the proper hardware and protocol necessary to make syringe pump mobilization compatible for cIEF and MALDI-MS interfacing. Following careful protocol, reproducible elution times of markers (RSD <5%) and accurate p*I* determination of proteins is demonstrated.

* Corresponding author at: Department of Chemistry and Biochemistry, PO Box 871604, Tempe, AZ 85287, USA. Tel.: +1 480 965 2265; fax: +1 480 965 2747.
E-mail address: mhayes@asu.edu (M.A. Hayes).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.11.047

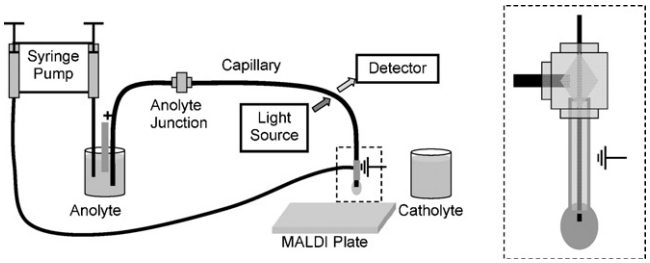

Fig. 1. Schematic illustration of experimental set up to perform syringe pump mobilization in cIEF coupled to MALDI-MS detection. The anode and cathode are marked by the positive and ground symbols respectively. The inset illustrates the sheath flow arrangement allowing for fraction collection while maintaining the potential field.

2. Experimental

2.1. Chemicals and materials

Sinapinic acid, myoglobin (from equine skeletal muscle), β-lactoglobulin A and B mixture (from bovine milk), acetonitrile, trifluoracetic acid, fluorescent IEF markers (p*I*: 7.6, 6.8, 6.2, 5.5, 5.1, and 4), and BioChemika ampholyte pH 5-8 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Pharmalyte pH 3–10 was obtained from Amersham Biosciences (Poscataway, NJ, USA). Fused silica capillaries with an electroosomotic flow suppression coating were obtained from Microsolv (Long Branch, NJ, USA). A Nanopure UV Ultra water system from Branstead/Thermolyne (Dubuque, IA, USA) was used to provide deionized nanopure water.

2.2. Capillary isoelectric focusing

cIEF was performed on an in-house system utilizing a 50 cm × 75 μm (id) coated capillary (proprietary) for electroosmotic flow suppression (Fig. 1). Absorbance detection (325 nm) was carried out 7 cm from the capillary end using a capillary flow cell, DH-2000 deuterium light source, and USB 4000 bench top spectrometer (Ocean Optics, Dunendin, FL, USA). For assessment of p*I* marker reproducibility, the sample solution was composed of 2% (w/v) Pharmalyte 3–10 and six fluorescent p*I* markers (p*I*: 4.0, 5.1, 5.5, 6.2, 6.8, and 7.6) adjusted between 6 and 50 μg/mL. For validation of cIEF-MALDI-MS, the sample solution contained 2% (w/v) carrier ampholytes of BioChemika 5–8, five fluorescent p*I* standards (p*I*: 5.1, 5.5, 6.2, 6.8, and 7.6) adjusted between 6 and 50 μg/mL, myoglobin (70 μg/mL), and a mixture β-lactoglobulin A and B (70 μg/mL). The capillary was filled with the sample solution using a pressure of 20 psi for approximately 25 s. Isoelectric focusing was run at 15 kV for 20 min in all experiments, using 10 mM phosphoric acid as the anolyte and 20 mM ammonium hydroxide as the catholyte and sheath flow. For the mobilization step, a syringe pump was used for capillary elution and sheath flow delivery at flow rates of 0.75 μL/min and 2.6 μL/min respectively. At the start of mobilization, the catholyte reservoir was removed to allow for fraction collection at the capillary tip. Fractions were collected in 30-s intervals, corresponding to 1.4 μL, by contacting a ground steel MALDI plate (96 well) to the developing droplet where the sheath and capillary flows combined. This configuration allowed for absorbance detection of p*I* markers and MALDI-MS detection of standard proteins. It took 25.3 min for mobilization to the online detector (43 cm) and 30 min to elute the entire sample out of the capillary (50 cm).

2.3. Hardware components for syringe pump mobilization and sheath flow elution

After sample injection, the focusing capillary is connected to the anolyte junction using a microtee joint and microferrules (Upchurch Scientific, Oak Harbor, WA, USA). The anolyte junction provides an airtight seal to the anolyte vial while allowing the focusing capillary to be easily removed for rinsing and sample injection. Initially it is filled with anolyte solution and is coupled to the anolyte vial by an 8 cm connecting capillary. The anolyte vial consists of a 3 mL vial with an airtight cap ensured by sealing with epoxy. It has an embedded platinum electrode, a capillary line connected to the syringe (in-line), and a capillary line connected to the anolyte junction (out-line).

The sheath flow configuration allows for fraction collection while maintaining an electrical connection across the capillary to minimize hydrodynamic flow band broadening. The focusing capillary is threaded coaxially through an 18 G stainless steel tube, and a micro tee fitting allows for allows for delivery of the sheath flow from the syringe pump. A ground wire attached to the outer part of the tube allows for the electrical circuit to be completed while performing syringe pump mobilization.

2.4. Matrix assisted laser desorption/ionization mass spectrometry

For cIEF-MALDI-MS experiments, a saturated solution of sinapinic acid in 70/30 0.1% trifluoracetic acid/acetonitrile was used as the MALDI-MS matrix. Immediately after collecting a fraction, 2 μL of matrix solution was added. The droplets were allowed to evaporate at room temperature and pressure until completely dry (~15 min). Mass spectral data was collected on a Bruker Daltonics (Billerica, MA, USA) Autoflex MALDI-TOF spectometer. The instrument was operated in linear, positive ion mode and the data was collected with a 20 kV extraction voltage and 250 ns delay time. Excitation of the SA matrix was achieved using a 337 nm nitrogen laser. Samples were evaluated at a m/z range of 4000–40,000 and 500 shot spectrums were accumulated for each sample.

3. Results and discussion

At the start of a cIEF separation it is critical to ensure that no bubbles become trapped inside the junctions or capillaries. Bubbles can disrupt the applied field and/or prevent capillary elution causing poor cIEF reproducibility. To avoid this problem the anolyte vial and the connecting capillaries are completely filled with anolyte solution. Similarly, the junctions are filled with excess anolyte solution to prevent air entrapment upon connection of the fittings. The time of atmosphere exposure should be minimized for each air/liquid interface to prevent any significant height induced hydrodynamic flow or evaporation which could introduce air into the system. It is also important to vent the anolyte vial after multiple trials to remove any gas generation due to long periods of electrolysis or Joule heating. Establishing these protocols is critical since applying pressure to a system with bubbles can cause compression rather than fluid transport; thus, capillary contents will fail to elute so long as the applied pressure is compensated by a compressible bubble. The collapsing bubble phenomenon can be identified by unusually long or inconsistent elution times. Chaotic changes in the current can also signal interference in the focusing or mobilization steps due to bubbles.

Following this procedure, reproducible cIEF separations of p*I* markers are observed over several trials. A representative electropherogram shows well defined peaks ($A_{325\,nm}$) of six fluorescent p*I* markers in pH 3–10 (Fig. 2A). The poor resolution between p*I* 5.5 and 5.1 is not indicative of resolution loss due to hydrodynamic mobilization since the β-lactoglobulin species exhibit better resolution although having a smaller p*I* difference (Δp*I* ~0.2). The relative standard deviation of elution times for the p*I* markers are less than 5% for all peaks over ninety trials (Fig. 2B),

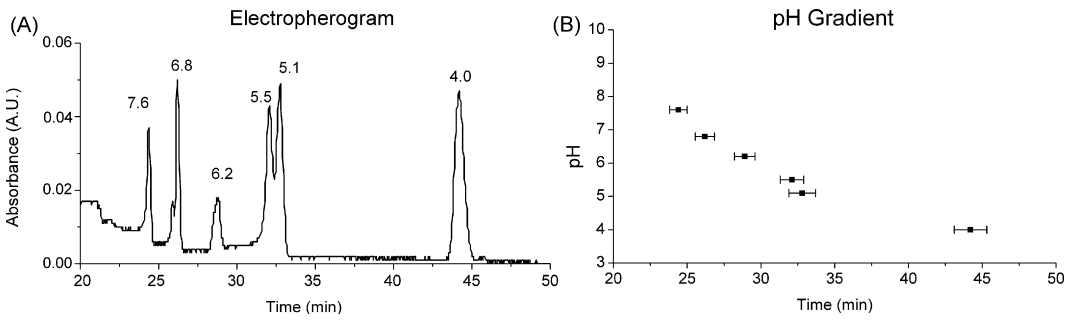

Fig. 2. (A) cIEF elution UV absorbance trace (315 nm) showing the separation of six p*I* markers (p*I* labeled above each peak) in pH 3–10. (B) Corresponding pH gradient plot with standard deviation over several trials ($n = 90$) as error bar.

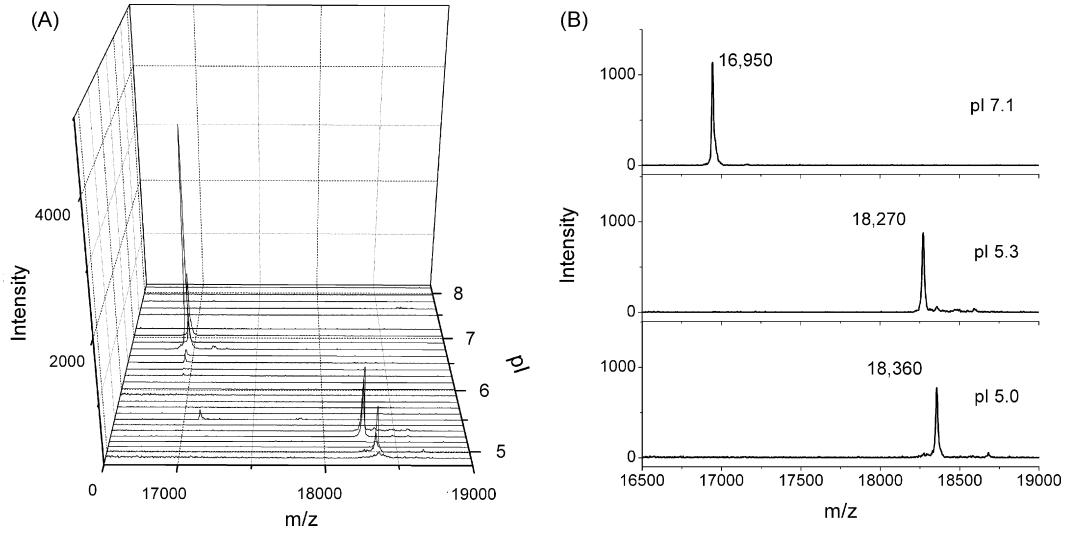

Fig. 3. (A) All MALDI-MS spectra of fractions collected from a cIEF separation of myoglobin (p*I* 7.1; MW 16,950 Da), β-lactoglobulin A (p*I* 5.0, 18,360 Da), and β-lactoglobulin B (p*I* 5.3, 18,270 Da). (B) Individual spectra of the main p*I* fractions for each protein.

which is comparable to non-coupled cIEF literature data [10]. However, the reproducibility of proteins would likely be less due to stronger surface interactions with the capillary wall [11]. It can be seen in the plot that the pH gradient does not exhibit uniform linearity over the entire range, and thus care must be taken in determining experimental p*I*s [12]. The elution times of the standard proteins was determined by MALDI-MS detection after correcting for the 4.1 min delay between absorbance detection and fraction collection. The fraction having the highest MS signal was used for determining the experimental protein p*I* after the five p*I* marker elution times were fitted with a second order polynomial ($R^2 = 0.99$). Using this approach, the p*I* of myoglobin, β-lactoglobulin B, and β-lactoglobulin A was found to be 7.0, 5.3, and 5.0 respectively. These p*I* determinations are in good agreement with literature values [1–2].

The resultant MALDI-MS spectra obtained for all of the collected fractions reveal good separation between each protein species (Fig. 3A and B). Each of the proteins was found in two to three fractions and thus the peak widths are 1–1.5 min which is comparable to the p*I* markers. The myoglobin signal (p*I* 7.1; MW 16,950 Da) is well resolved from the two β-lactoglobulin species. Baseline resolution between β-lactoglobulin A (p*I* 5.0; MW 18,360 Da) and β-lactoglobulin B (p*I* 5.3; MW 18,270 Da) was only observed when using the shallower pH gradient 5–8. The MS signals likely are subject to ampholyte suppression although the extent to which was not examined [6,13]. Overall, MALDI-MS protein detection extends the applicability of cIEF by improving detection limits relative to absorbance measurement.

4. Conclusions

We report that syringe pump mobilization is compatible in coupling cIEF with MALDI-MS for protein analysis. In our investigation, problematic or null cIEF experiments were primarily the result of bubbles entrapped in the system. Through careful attention to hardware components and interfaces, bubbles were eliminated and the syringe pump mobilization method was shown to have competitive reproducibility of p*I* markers and accurate p*I* determination of standard proteins. There does not appear to be significant cIEF resolution loss due to the fraction collection as the β-lactoglobulin A and B species were baseline resolved although differing by only two amino acids.

References

[1] F. Foret, O. Muller, J. Thorne, W. Gotzinger, B.L. Karger, J. Chromatogr. A 716 (1995) 157.
[2] A. Chartogne, M. Gaspari, S. Jespersen, B. Buscher, E. Verheij, R. van der Heijden, U. Tjaden, J. van der Greef, Rapid Commun. Mass Spectrom. 16 (2002) 201.
[3] T.A. Crowley, M.A. Hayes, Proteomics 5 (2005) 3798.
[4] W. Yu, Y. Li, C. Deng, X. Zhang, Electrophoresis 27 (2006) 2100.
[5] M. Lechner, A. Seifner, A.M. Rizzi, Electrophoresis 29 (2008) 1974.
[6] L.H.H. Silvertand, J.S. Toraño, G.J. de Jong, W.P. van Bennekom, Electrophoresis 30 (2009) 1828.

[7] R. Rodriquez-Diaz, T. Wehr, M. Zhu, Electrophoresis 18 (1997) 2134.
[8] M. Minarik, F. Groiss, B. Gas, D. Blaas, E. Kenndler, J. Chromatogr. A 738 (1996) 123.
[9] Q. Tang, A.K. Harrata, C.S. Lee, J. Mass Spectrom. 31 (1996) 1284.
[10] T.L. Huang, M. Richards, J. Chromatogr. A 757 (1997) 247.
[11] P.G. Righetti, J. Chromatogr. A 1037 (2004) 491.
[12] A. Suratman, H. Wätzig, J. Sep. Sci. 31 (2008) 1834.
[13] L.H.H. Silvertand, J.S. Toraño, G.J. de Jong, W.P. van Bennekom, Electrophoresis 29 (2008) 1985.