Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1022 (2004) 201–206
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Post-column fluorescence derivatization of proteins and peptides in capillary electrophoresis with a sheath flow reactor and 488 nm argon ion laser excitation

Mingliang Ye, Shen Hu, Wes W.C. Quigley, Norman J. Dovichi*

Department of Chemistry, University of Washington, Seattle, WA 98915-1700, USA

Received 10 June 2003; received in revised form 17 September 2003; accepted 19 September 2003

Abstract

We report the use of a sheath flow reactor for post-column fluorescence derivatization of proteins. The derivatization reaction employed naphthalene-2,3-dicarboxaldehyde (NDA) and β-mercaptoethanol, which were added in the sheath buffer. The labeled proteins were detected by laser-induced fluorescence with an argon-ion laser beam at 488 nm. The performance of this detection scheme was evaluated by separation of some protein standards. A column efficiency of 450,000 plates/m was obtained without stacking. The limits of detection for those standard proteins were determined to be from 8 to 32 nM. Excellent linear relationship was obtained with correlation coefficient of 0.9998 for α-lactalbumin concentration ranging from 3.91×10^{-7} to 1.25×10^{-5} M. Separation of protein standards at low pH was also demonstrated by reversing the electroosmotic flow (EOF) with addition of cetyltrimethylammonium bromide (CTAB) to the running buffer. Different separation selectivity was achieved, but the sensitivity is poorer than that at high pH. This post-column derivatization detection system was applied successfully to analyze the protein extract from HT29 human colon cancer cells as well as tryptic peptides.

Keywords: Derivatization, electrophoresis; Sheath flow reactor; Proteins; Peptides

1. Introduction

Capillary electrophoresis is a powerful tool for separation of proteins [1]. However, detection sensitivity remains an important issue, particularly when dealing with minor components in complex mixtures. Ultraviolet absorbance, while providing near-universal detection, suffers from limited sensitivity because of the short optical pathlength across the capillary. Laser-induced fluorescence (LIF) detection produces outstanding sensitivity [2–5]. Native fluorescence is useful for the detection of proteins that contain aromatic amino acids [6,7]. Those systems general provide nanomolar limits of detection (LODs) for proteins. However, the high cost and short lifetime of the ultraviolet lasers required to excite native fluorescence have discouraged its widespread use. Fluorescence derivatization of proteins before separation is another approach for highly sensitive detection of proteins [6,8–14]. However, loss of efficiency and resolution has been observed for separation of labeled proteins by capillary zone electrophoresis (CZE), which results from multiple labeling [15]. We have reported that the broad envelope of multiply labeled product peaks collapses to a single, narrow peak by addition of submicellar concentration of sodium dodecylsulfate (SDS) to the running buffer [8]. Unfortunately, the addition of surfactant is not compatible with all electrophoresis methods. Also, precolumn or on-column labeling introduces additional manipulations that may not always be desirable.

Post-column derivatization of proteins is an alternative approach for LIF detection. One of the major challenges for post-column derivatization is the construction of the post-column reactor. The ideal reactor should minimize band broadening and sample loss. Several type of reactors, including gap reactors [16–18], coaxial reactors [19,20], membrane reactor [21] and sheath flow cuvette reactor [22,23] have been developed for post-column derivatization. Among these, the sheath flow reactor may be the most convenient. A typical LIF sheath flow cell can be used as the reactor without modification [22,23]. This reactor has been employed

* Corresponding author. Tel.: +1-2065437835; fax: +1-2065437835.
E-mail address: dovichi@chem.washington.edu (N.J. Dovichi).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.09.037

for LIF detection of amino acids [22] and proteins [23] with the use of *o*-phthaldialdehyde and β-mecaptoethanol. The LOD for glycine was 100 nM, and that for proteins varied from 0.7 to 10 nM. The sheath flow reactor, compared with other reactors, offers the important advantage of maintaining the high separation efficiency of CE [22,23]. The highest column efficiency obtained for proteins is 800,000 plates/m without use of stacking [23].

To eliminate creating a large background signal, the post-column derivatization reagents must only fluoresce after reaction with analytes. In order to maintain the high separation speed and efficiency of CE, an extremely rapid reaction must be used. Due to its fluorogenic nature and fast reaction kinetics, *o*-phthalaldehyde (OPA) with β-mercaptoethanol is the most commonly used reagent for post-column derivatizations [14,22–24]. The excitation wavelength for the reaction products of OPA-β-mercaptoethanol is 325 nm [20,22,23]. Lasers operated at this wavelength tend to be expensive and not particularly reliable. An analog of OPA, naphthalene-2,3-dicarboxaldehyde (NDA) has also been used for post-column derivatization of proteins. The extension of the aromatic-ring system by the replacement of OPA with NDA shifts the excitation maximum from 325 nm to 460 nm [25]. This shift in the excitation wavelength is important because it permits the argon-ion and helium–cadmium lasers to be used as excitation sources. The stability of the derivatives of NDA-β-mercaptoethanol is poorer than that of OPA-β-mercaptoethanol. However, this issue is less crucial in post-column reactions [17,21,25].

2. Experimental

2.1. Reagents and solutions

Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO, USA). Naphthalene-2,3-dicarboxaldehyde, was obtained from Molecular Probes (Eugene, OR, USA). The derivatization solution for post-column labeling was prepared each day by mixing appropriate volumes of NDA stock solution (20 mM), β-mercaptoethanol stock solution (200 mM), methanol, and borate buffer (100 mM, pH 9.5). The stock solutions for NDA and β-mercaptoethanol were prepared in methanol each day. The final derivatization mixture consists of 1 mM NDA, 8 mM β-mercaptoethanol, 30% methanol and 70% borate buffer (100 mM). If not otherwise stated, 50 mM borate buffer (pH 9.5) was used as the electrophoresis running buffer. The standard proteins solutions were prepared in running buffer, so no focusing or stacking effects occurred. The homogenate from HT29 human colon adenocarcinoma cell was prepared as in our previous report [12]. Before injection, 10 μl of the cell homogenate was mixed with 10 μl running buffer. Dephosphorylated β-casein (1 mg/ml) was digested in 0.02 mg/ml trypsin solution for 15 h at room temperature. The tryptic peptide mixture was injected without dilution.

2.2. Emission and excitation spectra of NDA-labeled bovine serum albumin (BSA)

Excitation, emission and absorbance spectra of BSA were performed as follows. The derivatization reaction employed a solution containing 1 mM NDA and 8 mM β-mercaptoethanol made into a 70% mixture of 100 mM borate buffer and 30% methanol. This solution was analyzed as a blank and then a small amount of sample solution was added to the cuvette. The excitation and emission spectra employed 600 μl of derivatization buffer, and 50 and 100 μl of sample solution, respectively.

Spectra were generated with a Perkin-Elmer LS 50B with a resolution of 0.5 nm. The emission spectra were collected with 420 (width of 5 nm) as the excitation wavelength. The excitation spectrum was collected with 550 nm emission (width of 5 nm). The collection process for the excitation and emission takes ∼45 s. Each spectrum is the average of two spectra.

2.3. Apparatus

All separations were carried out with a locally constructed capillary electrophoresis system. The high voltage was provided by a capillary zone electrophoresis 1000R high-voltage power supply (Spellman, Plainview, NY, USA). The schematic of the sheath flow post-column reactor is shown in Fig. 1. All the tubes in Fig. 1 are made of polymer. A polyether ether ketone (PEEK) tee with 0.25 mm bore was used for delivering the sheath fluid that contained the derivatization solution. The cuvette had a 200 μm square flow chamber and 800 μm thick walls. It was connected to two 1/16 in. tubes (1 in. = 2.54 cm) by inserting them in a heated poly(vinyl chlorides) (PVC) tube. The sheath flow was driven by the height difference between the sheath fluid reservoir and the waste reservoir. A 12 mW argon-ion laser

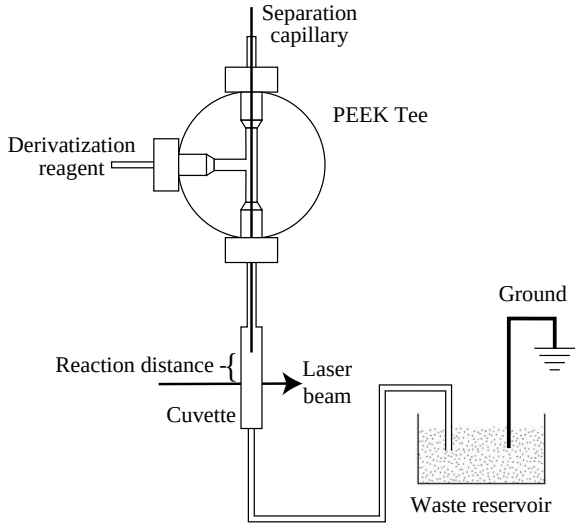

Fig. 1. Schematic of the sheath flow post-column reactor.

beam at 488 nm was used for excitation (Uniphase, San Joe, CA, USA). The beam was focused about 3 mm downstream from the capillary tip. Fluorescence was collected at right angles with a 60×, 0.7 N.A. microscope objective (Universe Kogaku, Oyster Bay, NJ, USA), filtered with a 560DF30 bandpass filter (Omega Optical, Brattleboro, VT, USA), and detected with a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ, USA) biased at 900 V.

Two Pt electrodes were used to complete the electrical circuit. One was put at the inlet of the capillary and the other was put in the waste reservoir (Fig. 1). The electrode in the waste reservoir was grounded. Fused-silica capillaries (50 μm i.d. × 178 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The separation potential was 300 V/cm. Electrokinetic injection was at 100 V/cm for 5 s.

3. Results and discussion

3.1. Spectral properties of NDA labeled protein

Lasers that operate in the visible portion of the spectrum are relatively inexpensive. It appears that only the helium–cadmium 325 and 442 nm lasers and the argon ion 457.9 nm lasers have been employed for fluorescence detection of post-column reaction products [17,21,25]. The helium-cadmium is notorious for having a limited lifetime and the 457.9 nm argon ion laser is relatively expensive. Although two review articles have pointed out that the excitation wavelength of NDA derivatives matches the 488-nm line of the argon ion laser [26,27], to the best of our knowledge there has not been a publication that has employed post-column derivatization with this laser.

Fig. 2 presents the excitation and emission spectra of the reaction product of NDA, β-mercaptomethanol, and bovine serum albumin. The excitation spectrum shows two absorbance bands, one in the near UV and the other in the blue portion of the spectrum. The long-wavelength excitation band shows hints of vibronic structure, with the 0-0 transition at 460 nm. The emission spectrum is featureless and centered at 540 nm. Emission spectra were generated with excitation at both 420 and 488 nm; the spectra were similar in shape.

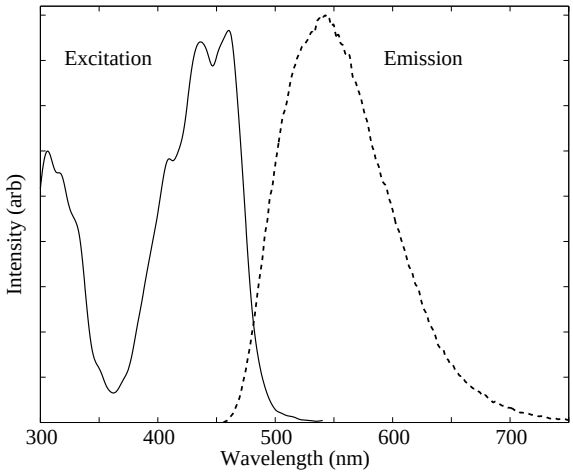

Fig. 2. Excitation (solid) and emission (dashed) spectra of NDA-labeled BSA. The excitation spectrum was taken with.

We also generated an absorbance spectrum for the labeled ovalbumin; as expected, that spectrum was similar to the excitation spectrum. The emission intensity was about five times higher with excitation at 420 nm compared with excitation at 488 nm.

In choosing an excitation wavelength for fluorescence measurements, it is important to consider both the intensity of the fluorescence signal and the noise associated with the background signal. The background is often dominated by Raman scatter from the solvent. The main Raman bands from water are a relatively weak band at 300 cm^{-1} and a much stronger band centered at 3500 cm^{-1}.

Consider the conventional excitation at 442 nm. In this case, the 300 cm^{-1} Raman band is at 448 nm, far removed from the emission wavelength. Unfortunately, the main Raman band at 3500 cm^{-1} falls at ∼520 nm, which overlaps with a significant fraction of the fluorescence spectrum. Excitation at 442 nm generates Raman scatter that interferes with the fluorescence signal. This interference either results in significant noise in the background signal or requires spectral filtering, with a concomitant decrease in fluorescence intensity.

In contrast, the argon ion laser's 488 nm line generates Raman scatter centered at 495 and 575 nm. This fortuitous situation results in an ∼80 nm wide spectral window for fluorescence detection that is centered at the emission maximum with minimal interference from Raman scatter. While excitation at 488 nm is less efficient that excitation at 442 nm, the background signal is also lower, so that the signal-to-noise ratio should not differ too much for the two excitation wavelengths.

These spectroscopic studies suggest that the 488 nm argon ion laser may prove useful for detection of NDA derivatives. The sealed-cavity, fixed wavelength, low-power argon ion 488 nm laser is quite reliable and is the most-common source employed for laser-induced fluorescence detection in capillary electrophoresis. We investigated the use of the laser for post-column fluorescence detection of NDA protein derivatives.

3.2. Detector design

Both Oldenburg and Xi [22] and Coble and Timperman [23] used a metal fixture to hold their post-column detectors. The fixture also acts as an electrode, which generated bubbles because of electrolysis. The cuvette was purged about once every 10 runs to flush the bubbles from the chamber [24]. Our initial work was also based on a sheath flow cuvette with a metal holder. It was found both the current and

the baseline were not stable at an electric field of 300 V/cm. Many bubbles accumulated in the metal chamber, which resulted in unstable current and baseline. To prevent bubble formation, we modified the cuvette fixture to eliminate metal components (Fig. 1). This fixture eliminated bubble formation and produced stable current and baseline.

A rapid reaction of the derivatization agent with the analyte is required to maintain the fast separation and high efficiency of CE. It has been reported that the labeling reaction with NDA and β-mercaptoethanol of lysine-containing peptides is very fast [21]. The fluorescence intensity maximized in less than 30 s. However, the products of this reaction were very unstable, and the fluorescence intensity decreased quickly. This disadvantage is less crucial in post-column reactions because of the short reaction time. Post-column derivatization of proteins with NDA and β-mercaptoethanol were successfully performed in HPLC and CE [17,25] using other reactors.

It is necessary to optimize the reaction time, which is determined by the reagent flow rate and the distance from the capillary tip to the observation region. One advantage of this reactor is that a very short reaction distance can be employed. However, high fluorescence intensity was not obtained when detection was performed immediately after the capillary tip due to the insufficient mixing of reaction solution with the proteins. We fixed the reaction distance at 3 mm, and we manipulated the reaction time by adjusting the linear flow rate of the sheath fluid, which is determined by the height difference between the derivatization solution reservoir and the waste reservoir. Fig. 3 presents the electropherograms of five standard proteins obtained at three different height differences. The highest signal-to-noise ratio was obtained at a height difference of 3 cm. Higher flow rates lead to shorter reaction times and incomplete reaction. Lower flow rates lead to decomposition of products and diffusion of the product, which resulted in band broadening.

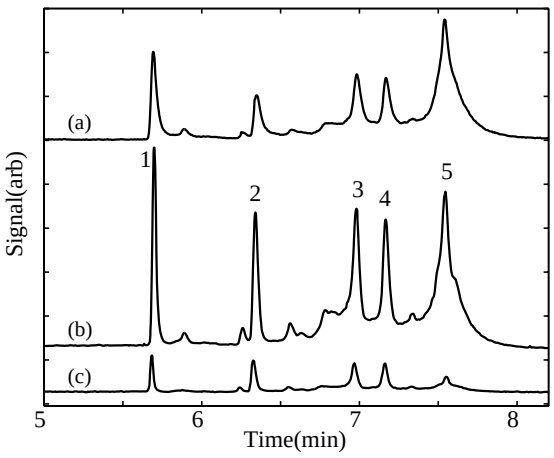

Fig. 3. Separation of protein standards at height difference of (a) 0.5 cm, (b) 3 cm, and (c) 6 cm between derivatization solution reservoir and waste reservoir Experimental conditions: 50 cm × 50 μm i.d. × 178 μm o.d.; separation, 300 V/cm; injection, 100 V/cm for 5 s; running buffer, 50 mM borate buffer (pH 9.5). Peaks: (1) myoglobin (2.5 μM), (2) α-lactalbumin (5.0 μM), (3) β-lactoglobulin B (5.0 μM), (4) β-lactoglobulin A (5.0 μM), (5) BSA (2.0 μM).

Table 1
Column efficiencies and limits of detection (LOD) ($S/N = 3$) of protein standards post-column labeled by NDA/β-mercaptoethanol with LIF detection at optimal conditions

Elution order	Proteins	Column efficiency (plates/m)	LOD (nM)	LOD (amol)
1	Myoglobin	450000	8.6	41
2	α-Lactalbumin	360000	26.0	112
3	β-Lactoglobulin B	310000	30.0	117
4	β-Lactoglobulin A	330000	31.9	121
5	BSA	90000	8.3	30

Experimental conditions are the same as Fig. 3b.

Table 1 gives the column efficiencies for proteins in this detection scheme at optimal condition. The efficiencies for the first four proteins varied from 300,000 to 450,000 plates/m. However, the efficiency for bovine serum albumin (BSA) is relatively poor, only 87,000 plates/m was obtained. The poor efficiency of BSA is likely due to the presence of a set of glycoforms that migrate as a broad envelope.

Our results are comparable to earlier reports with different reactors. With a gap reactor, Gilman and Ewing achieved a detection limit of 50 nM for transferrin [17]. Kostel and Lunte [21] reported a limit of detection of 100 nM for Substance P. As shown in Table 1, our detection limits for 5 protein standards varied from 8 to 30 nM. However, the detection limit of this system is poorer than a similar system that used OPA as the derivatization agent [23], which reported detection limits of 1.4, 10, and 7 nM for α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A, respectively. This poorer performance may reflect the instability of the derivatization products of NDA.

It is interesting that the best detection limit LOD was obtained for BSA even though it produced modest separation efficiency in this work. BSA has 60 lysine residues, so that the high sensitivity is likely due to labeling at multiple sites.

α-Lactalbumin was selected as a model protein to investigate the linear range of this approach. An excellent linear relationship was obtained with correlation coefficient of 0.9998 when the concentration of α-lactalbumin varied from 3.91×10^{-7} to 1.25×10^{-5} M ($n = 7$ points). However, at 2.5×10^{-5} M, the peak height decreased from the predicted trend, which may arise because the concentration of NDA is not high enough to completely derivatize the protein at high concentration.

Limits of detection are reported as the analyte concentration that generates a signal that is three times the standard deviation in the background signal. The limit of quantitation is the analyte concentration that generates a signal that is ten times the standard deviation of the background signal.

3.3. Low pH separation

OPA, fluorescamine, and NDA have been used for post-column derivatization for fluorescence detection [27]. These reagents react with lysine residues, and high pH is required for high reaction efficiency. Borate is often used at pH 9.5 to prepare the reaction solution and running buffer. However, the pH of the running buffer is an important parameter to adjust selectivity for separation of proteins in capillary electrophoresis. To the best of our knowledge, there is no report that describes separation at acidic pH with post-column derivatization-based detection.

Separation of standard proteins at pH 2.5 was achieved in this work by reversing the EOF with the addition of cetyltrimethylammonium bromide (CTAB) in the running buffer. Phosphate buffer (10 mM, pH 2.5) containing 5 mM CTAB was used as the running buffer. The NDA reaction solution was prepared with borate buffer at pH 9.5. Fig. 4 shows the electropherogram for separation of three proteins under this condition. The positively charged proteins migrated with the electroosmotic flow (EOF).

The running buffer and the proteins mix with the derivatization solution when they enter the cuvette. Because only 10 mM phosphate was used in the running buffer, this solution will be adjusted to high pH value by the high concentration of the borate buffer (75 mM) present in the reaction solution. The detection limits of lysozyme, BSA, and α-lactalbumin were determined to be 90, 15 and 180 nM, respectively. The LOD for BSA is slightly poorer than that obtained at pH 9.5, but the LOD for α-lactalbumin is about seven times poorer than that obtained at pH 9.5.

Although the sensitivity is not as good as that with the system using running buffer of high pH, this approach has its merits. First, the selectivity is different from that at high pH. As shown in Fig. 4, α-lactalbumin eluted before BSA at high pH, while it eluted after BSA at low pH; Second, basic proteins were separated with good peak shape. Strongly basic proteins, i.e. lysozyme, cannot be detected when using 50 mM borate buffer (pH 9.5) as the running buffer because of the strong adsorption by the negatively charged capillary wall. However, as shown in Fig. 4, lysozyme could be separated with column efficiency of 335,000 plates/m as a symmetric peak at low pH, which means the interaction between lysozyme and the capillary wall was reduced significantly at low pH and with the addition of CTAB. CTAB has also been used as an additive to improve the peak shape for separation of basic proteins in CZE [28].

3.4. Separation of HT-29 cancer cell homogenate

We have reported separation of FQ labeled proteins from HT29 human colon adenocarcinoma cells by capillary electrophoresis [12,29,30]. Either pre-column derivatization of cell homogenate or on-column derivatization of proteins from the single cell was conducted. About 30 protein components were typically resolved in these systems [12,29,30]. The post-column label technique described above was also applied to separate the proteins from HT29 human colon adenocarcinoma cells. Fig. 5 shows the electropherograms at two different pHs. At pH 9.5, some peaks experience severe tailing and column efficiency was poor, perhaps because of adsorption to the capillary wall. Although the pH increased only by 0.5 unit, the separation was much improved at pH 10. Around 15 components were resolved within 10 min.

3.5. Separation of tryptic digests

Peptide mapping is an important analytical technique widely used to study the primary structure of proteins. Fig. 6 presents peptide mapping with post-column detection for dephosphated β-casein digested by trypsin. More than 30 peaks were detected, and most peaks have high column

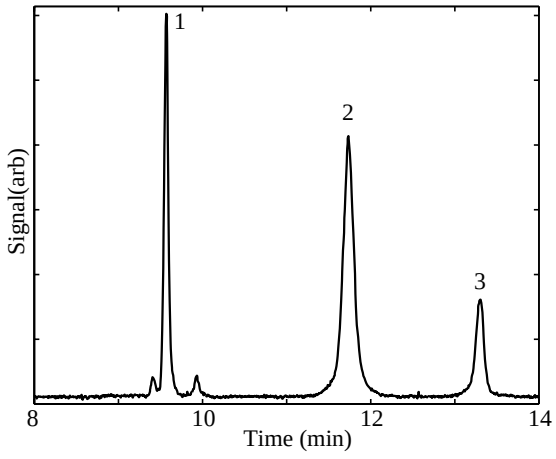

Fig. 4. Separation of protein standards at pH 2.5. Experimental conditions: running buffer: 10 mM phosphate buffer (pH 2.5) containing 5 mM CTAB. Other conditions are as in Fig. 3b. Peaks: (1) lysozyme (20 μM), (2) BSA (2 μM) and (3) α-lactalbumin (10 μM).

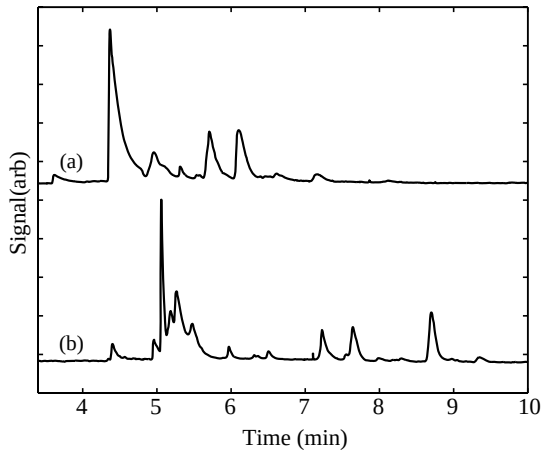

Fig. 5. Separation of proteins from HT29 cancer cells at (a) pH 9.5 and (b) pH 10. Experimental conditions are the same as Fig. 3b.

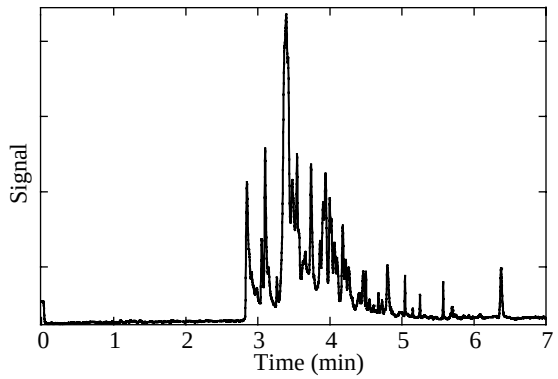

Fig. 6. Capillary electrophoresis tryptic mapping of dephosphated β-casein with post-column fluorescence derivatization. Experimental conditions are the same as Fig. 3b.

efficiencies, which demonstrated that post-column labeling is also useful for studying peptides.

4. Summary

We have described a post-column derivatization scheme with a sheath flow reactor. An analog of OPA, naphthalene-2,3-dicarboxaldehyde was used as the agent to label proteins. The long excitation wavelength of NDA labeled proteins permits the inexpensive argon-ion laser beam (488 nm) to be used as excitation sources. Detection limits as low as 8 nM and column efficiency as high as 450,000 plates/m were achieved. The separation of proteins with acidic buffer was demonstrated; the sensitivity is lower than that of high pH. This detection scheme was also successfully used to analyze proteins from HT29 human colon adenocarcinoma cells and tryptic peptides.

Acknowledgements

This work was supported by the Department of Energy Genomes to Life Program and by MDS-SCIEX Inc.

References

[1] T. Wehr, R. Rodriguez-Diaz, M. Zhu, Capillary Electrophoresis of Proteins, Chromatographic Science Series, vol. 80, Marcel Dekker, New York, 1999, p. 28.
[2] D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang, N.J. Dovichi, J. Chromatogr. 559 (1991) 237.
[3] S. Wu, N.J. Dovichi, J. Chromatogr. 480 (1989) 141.
[4] T.T. Lee, E.S. Yeung, J. Chromatogr. 595 (1992) 319.
[5] S. Nie, R. Dadoo, R.N. Zare, Anal. Chem. 65 (1993) 3571.
[6] D.F. Swaile, M.J. Sepaniak, J. Liq. Chromatogr. 14 (1991) 869.
[7] T.T. Lee, E.S. Yeung, J. Chromatogr. 595 (1992) 319.
[8] D.M. Pinto, E.A. Arriaga, D. Craig, J. Angelova, N. Sharma, H. Ahmadzadeh, N.J. Dovichi, C.A. Boulet, Anal. Chem. 69 (1997) 3015.
[9] D. Richards, C. Stathakis, R. Polakowski, H. Ahmadzadeh, N.J. Dovichi, J. Chromatogr. A 853 (1999) 21.
[10] M.D. Harvey, D. Bandilla, P.R. Banks, Electrophoresis 19 (1998) 2169.
[11] G. Hunt, W. Nashabeh, Anal. Chem. 71 (1999) 2390.
[12] S. Hu, L. Zhang, L.M. Cook, N.J. Dovichi, Electrophoresis 22 (2001) 3677.
[13] S. Hu, L. Zhang, N.J. Dovichi, J. Chromatogr. A 924 (2001) 369.
[14] B. Nickerson, J.W. Jorgenson, J. Chromatogr. 480 (1989) 157.
[15] J.Y. Zhao, K.C. Waldron, J. Miller, J.Z. Zhang, H.R. Harke, N.J. Dovichi, J. Chromatogr. 608 (1992) 239.
[16] A. Emmer, J. Roeraade, Chromatographia 39 (1994) 271.
[17] S.D. Gilman, A.G. Ewing, Anal. Methods Instrum. 2 (1995) 133.
[18] R. Zhu, W.T. Kok, J. Chromatogr. A 716 (1995) 123.
[19] A. Emmer, J. Roeraade, J. Chromatogr. A 662 (1994) 375.
[20] L. Zhang, E.S. Yeung, J. Chromatogr. A 734 (1996) 331.
[21] K.L. Kostel, S.M. Lunte, J. Chromatogr. B 695 (1997) 27.
[22] K.E. Oldenburg, X.I. Xi, Analyst 122 (1997) 1581.
[23] P.G. Coble, A.T. Timperman, J. Chromatogr. A 829 (1998) 309.
[24] S.D. Gilman, J.J. Pietron, A.G. Ewing, J. Mirocol. Sep. 6 (1994) 373.
[25] K.J. Dave, J.F. Stobaugh, T.M. Rossi, C.M. Riley, J. Pharm. Biomed. Anal. 10 (1992) 965.
[26] H.A. Bardelmeijer, H. Lingeman, C. Ruiter, W.J.M. Underberg, J. Chromatogr. A 807 (1998) 3.
[27] R. Zhu, W.Th. Kok, J. Pharm, Biomed. Anal. 17 (1998) 985.
[28] A. Cifuentes, M.A. Rodriguez, F.J. Garcia-Montelongo, J. Chromatogr. A 742 (1996) 257.
[29] S. Hu, Z.R. Zhang, L.M. Cook, E.J. Carpenter, N.J. Dovichi, J. Chromatogr. A 894 (2000) 291.
[30] Z.R. Zhang, S. Krylov, E.A. Arriaga, R. Polakowski, N.J. Dovichi, Anal. Chem. 72 (2000) 318.