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Pre-column derivatization of proteins to enhance detection sensitivity for sodium dodecyl sulfate non-gel sieving capillary electrophoresis

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Abstract

Pre-column derivatization of proteins with fluorescamine, naphthalene-2,3-dicarboxyaldehyde, and *o*-phthaldialdehyde was used to enhance absorption and fluorescence detection after separation by SDS non-gel sieving capillary electrophoresis. When compared with underivatized proteins, absorption sensitivity increased by as much as a factor of 22 at 280 nm, and 1.74 and 4.71 at 200 and 220 nm, respectively. Under favorable conditions, absorption detection limits with the labeled proteins at 280 nm were approximately equivalent to the detection limits of underivatized proteins at 200 nm. Fluorescence detection provided attomole detection limits with the best results being obtained with high-molecular-mass proteins. Pre-column labeling decreased the efficiency of the separation, but did not give rise to multiple peaks from heterogeneous labeling. The migration velocity of labeled proteins was slightly different from the unlabeled molecules, but did not significantly degrade molecular mass determinations. Pre-column derivatization with fluorescence detection allowed the proteins in a fertilization membrane isolated from a single amphibian embryo to be easily characterized.

1. Introduction

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a popular method for the separation of biopolymer mixtures. When SDS–protein complexes electromigrate in a sieving medium (e.g., gel), the mobility of the complex is proportional to the log of the effective molecular radius and thus to the molecular mass [1,2]. Performing these analyses in acrylamide gel-filled capillaries decreases the analysis time and enhances quantitation capabilities with on-column detection [3–5]. Despite these advantages, capillary gel electrophoresis suffers from several significant problems. These include defect formation during gel polymerization, the break down of the gels in high electric fields, and fouling of the matrix with "dirty" samples [3,6,7]. However, replacing the gel with a solution containing a sieving linear polymer [6,8–20] provides a size-based separation media that easily adapts to high potential field environments and can be easily replaced between analyses [6,14,18].

One problem associated with SDS non-gel sieving capillary electrophoresis (NGSCE) is detecting analyte zones isolated in the capillary. Absorption detection is most commonly used

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with capillary electrophoresis, but demonstrates relatively poor sensitivity due to the short optical pathlengths in the capillary. When used with SDS-NGSCE, absorption detection demonstrates comparable if not superior mass detection limits, but inferior concentration detection limits relative to SDS-PAGE utilizing silver or coomassie blue stains. One means of enhancing detection sensitivity is to react the analyte with a reagent to form a product which is more easily detected. Previous attempts to increase absorption sensitivity for free zone capillary electrophoresis by pre-column derivatization have met with some success [21], but can also have detrimental effects on the separation. Specifically, the availability of multiple reaction sites on the analyte can give rise to a multiplicity of labeled states that diminishes separation efficiency, or in extreme cases produces multiple peaks in the electropherogram [5,22]. For these reasons, pre-column derivatization reactions are not commonly utilized to enhance absorption detection in capillary zone electrophoresis, and are only used for detection for techniques in which the detector sensitivity would normally be very low (i.e., fluorescence) [5,23–26].

Pre-column derivatization of proteins may ultimately prove more useful when used in conjunction with SDS-NGSCE. These gel-based separations isolate molecules based on their physical dimensions, and so the addition of a low-molecular-mass label to the large protein molecule should not dramatically alter its migration velocity. Furthermore, low-molecular-mass impurities and excess labeling reagent should be easily separated from the analyte because of the large difference in size, as has been previously noted for labeled proteins separation by SDS-PAGE [27,28].

This paper investigates the use of pre-column labels to enhance absorption and fluorescence detection sensitivity for SDS non-gel sieving capillary electrophoresis of proteins. Three derivatizing agents will be examined with respect to their ability to enhance this detection and for their influence on separation efficiency and the mass determinations. Finally, the utility of these pre-column labeling techniques will be demonstrated by analyzing the proteins in a single fertilization membrane isolated from the embryo of the frog *Lepidobatrachus laevis*.

2. Experimental

2.1. Instrumentation

Capillary electrophoresis separations utilizing UV absorbance detection were performed with a BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) controlled with a MS-DOS computer running BioFocus 3000 control software (version 3.10, Bio-Rad Laboratories). Separations were performed in 24-cm long capillaries (19.4 cm inlet to detector) with an inner diameter of 50 μm and an outer diameter of 360 μm (Polymicro Technologies, Phoenix, AZ, USA). The electric field was maintained at -774 V cm^{-1} for all experiments. Forced liquid cooling was used to maintain the capillary at 20°C, while the sample carousel and the buffer reservoirs were maintained at 10°C.

Capillary electrophoresis with laser-induced fluorescence detection was performed on an instrument constructed in our laboratory. A regulated high-voltage DC power supply (Model EH50R02, Glassman High Voltage, White House Station, NJ, USA) generated an electric field of -486 V cm^{-1} in the separation capillary. Platinum wire electrodes were used to establish electrical contact between the high-voltage supply and the 0.4-ml inlet buffer reservoir, and the outlet reservoir and ground. The inlet reservoir and high-voltage end of the capillary were enclosed in a Plexiglass box to protect the operator from accidental shock. The outlet buffer reservoir was placed in a stainless-steel bomb so that the polymer solution could be forced through the capillary with nitrogen gas under a pressure of 700 kPa. Current passing through the separation capillary was measured by grounding the outlet buffer reservoir through a 1 kΩ resistor and monitoring the voltage developed across this resistor. Separations were performed in fused-silica capillaries (50 μm I.D., 360 μm O.D.,

Polymicro Technologies) which had a total length of 37 cm and an inlet to detector distance of 25 cm. The capillary and buffer reservoirs were maintained at ambient temperature (approximately 20°C) by natural radiative processes.

A schematic of the fluorescence detector used in these studies is shown in Fig. 1. The excitation beam for the fluorophore was provided by an argon-ion laser (Innova 90-5, Coherent, Palo Alto, CA, USA) operating at 457.9 nm for naphthalene-2,3-dicarboxyaldehyde (NDA), 363.8 nm for fluorescamine, and 351.1 nm for *o*-phthaldialdehyde (OPA). When ultraviolet excitation was used to excite fluorescence, a bandpass filter centered at 330 nm and a spectral bandpass of 80 nm (330WB80, Omega Optical, Brattleboro, VT, USA) was used to remove the broadband emission signal from the plasma tube in the spectral regions corresponding to fluorescence emission. A fused-silica lens (100-mm focal length, Newport Research Corporation, Irvine, CA, USA) was used to focus the 20-mW excitation laser beam into the separation capillary through a window formed by removal of the polyimide coating. The fluorescence emission from the capillary was collimated with an aluminum parabolic reflector (24 mm diameter, 5 mm focal length) and imaged onto a bandpass filter positioned approximately 30 cm away. Specular reflection of the excitation beam from the walls of the capillary was blocked by a thin strip of metal mounted across the front face of the parabolic reflector. For OPA and fluorescamine fluorescence measurements, a filter with a central pass wavelength of 450 nm and spectral bandpass of 100 nm (400EFLP with cutoff at 500 nm, Omega Optical) isolated the analytical signal. For NDA labeled proteins, a band pass filter centered at 500 nm and a bandpass of 40 nm (500DF40, Omega Optical) was used in conjunction with a 490-nm long-pass filter (490EFLP, Omega Optical) to select for the fluorescence signal. This signal was converted to a photocurrent with a photomultiplier tube (R1527-03, Hamamatsu Corp., Bridgewater, NJ, USA) biased at values between 650 and 1000 V, amplified with a current-to-voltage amplifier (Model 428, Keithley Instruments, Cleveland, OH, USA), and digitized with a 16-bit analog-to-digital converter (Model XL-1900 mainframe with XL-ADC2 ADC, Elexor Associates, Morris Plains, NJ, USA). An Objective C program developed in our laboratory and running on a NeXTstation computer (NeXT computer, Redwood City, CA, USA) recorded the data from the analog-to-digital converter and displayed the resulting electropherogram.

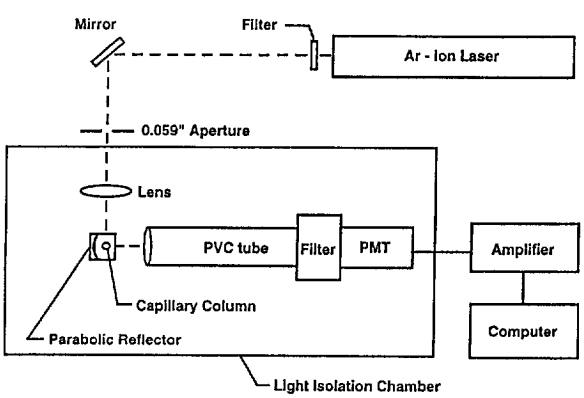

Fig. 1. Schematic of the laser-induced fluorescence detector used with non-gel sieving experiments.

2.2. Data processing

Peak areas and peak heights for data collected on the BioFocus 3000 instrument were determined with commercial integration software (BioFocus 3000 Integrator, Version 3.01, Bio-Rad Laboratories). Data collected with the NeXTstation computer were digitally low-pass filtered (Fourier filter, cutoff frequency of 0.55 Hz) with the program SciPlot (version 3.9; M. Wesemann; Berlin, Germany) to enhance the signal-to-noise characteristics of the electropherogram. The separation efficiency (N, expressed as theoretical plates) was estimated with the following formula:

$$N = 5.54(t_m / w_{1/2})^2$$

where t_m is migration time and $w_{1/2}$ is the peak width at half height.

2.3. Reagents

Horse heart myoglobin, α-chymotrypsinogen A type II (from bovine pancreas), conalbumin type I (from chicken egg white), fluorescamine, tris(hydroxymethyl)aminomethane (Tris) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), 2-mercaptoethanol, and *o*-phthaldialdehyde in the form of phthalic dicarboxaldehyde were obtained from the Aldrich (Milwaukee, WI, USA). Certified A.C.S. grade sodium hydroxide, boric acid, and HPLC grade acetone and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Protein molecular mass standards, SDS sieving buffer and SDS sample buffer were obtained from Bio-Rad Laboratories (Hercules, CA, USA), while naphthalene-2,3-dicarboxyaldehyde (NDA) was purchased from Molecular Probes (Eugene, OR, USA).

Stock solutions containing 2 mg ml^{-1} of protein were prepared in doubly deionized filtered water and 100 m*M* sodium borate buffer (pH 9.0) as appropriate. Dithiothreitol solutions were prepared by mixing 5.7 mg DTT, 6.2 μl of 3 *M* NaOH, and 118 μl of water. Solutions of the derivatizing reagents were prepared just prior to use by dissolving the reagent in an appropriate solvent to achieve the desired concentration. Specifically, fluorescamine (5.0 mg ml^{-1}) was dissolved in acetone, NDA (4.0 mg ml^{-1}) was dissolved in methanol, and OPA (5.0 mg ml^{-1}) was prepared in a solution composed of 75 μl of methanol, 924 μl of water and 5 μl of 2-mercaptoethanol.

2.4. Procedures

Capillary preparation

Prior to each analysis on the BioFocus 3000, and at the beginning of each day for the fluorescence detection experiments, the capillary was sequentially purged with 0.1 *M* NaOH for 90 s, 0.1 *M* HCl for 60 s, and Bio-Rad CE SDS run buffer for a minimum of 120 s. To insure that all the run buffer had been washed from the outside surface of the capillary prior to injection, the capillary inlet was dipped into two reservoirs containing a solution of 0.4 *M* boric acid, 0.4 *M* Tris, and 0.1% SDS.

Protein derivatization

Prior to analysis, protein samples were suspended in a 1:1 (v:v) solution containing Bio-Rad CE SDS sample buffer and 15 m*M* DTT, and boiled for 10 min to denature the protein. To react the sample with OPA, 40-μl aliquots of the denatured protein mixture were mixed with 4 μl of 5 mg ml^{-1} OPA solution and allowed to react for at least 5 min. For NDA derivatization, 35 μl of protein stock solutions (pH 9) were mixed with 2 μl of 0.1 *M* sodium cyanide and 5 μl of 4 mg ml^{-1} NDA solution. After approximately 3 min, the solution was mixed with 5 μl of DTT solution (15 m*M*) and 50 μl of Bio-Rad CE SDS sample buffer. Fluorescamine-labeled samples were prepared by mixing 85 μl of protein stock solutions (pH 9) with 5 μl of 5 mg ml^{-1} fluorescamine reagent. After 5 min, 10 μl of DTT stock solution and 100 μl of Bio-Rad CE SDS sample buffer were added to the mixture. These procedures utilized a large molar excess of the derivatizing reagent to simulate realistic analysis conditions and exhaustively label the analyte. Just prior to analysis, the solutions containing the labeled proteins were centrifuged for 2 min at 16 000 *g* to remove any particulates.

Preparation of molecular mass standards

Molecular mass standards were prepared by combining 10 μl of concentrated Bio-Rad SDS-PAGE standards, 40 μl of water, and 50 μl of Bio-Rad CE SDS sample buffer. After denaturing the mixture in a boiling water bath for 10 min, the sample was diluted to the desired concentration with a solution of 55% Bio-Rad CE SDS sample buffer and 45% water. Aliquots of this mixture were derivatized as previously described. The aprotinin standard was ignored for these experiments and was not used for molecular mass calibration.

Preparation of fertilization envelopes

Large quantities of fertilization envelopes from the frog *Lepidobatrachus laevis* were isolated by a previously described procedure [29], then individual membranes manually selected under a microscope with watchmakers forceps. This structure was suspended in 20 μl of a 1:1 (v:v) mixture of Bio-Rad CE SDS sample buffer and a 15 m*M* DTT solution and boiled in a water bath for 10 min. After allowing the sample to cool to room temperature, aliquots from this mixture were derivatized as previously described.

3. Results and discussion

3.1. Absorption sensitivity enhancement

OPA, NDA and fluorescamine were evaluated for their utility as pre-column derivatization reagents to enhance absorption detection by measuring the enhancement factor, the ratio of the peak area (or height) of the derivatized molecule to the unlabeled molecule, for proteins separated by capillary electrophoresis. Table 1 summarizes this enhancement as a function of detection wavelength for two representative proteins, myoglobin and conalbumin. Although all labels provided strong signal enhancement at 280 nm, the greatest signal enhancement and migration time reproducibility were observed for NDA labeled proteins. Typical %R.S.D. values observed for peak areas and heights for these measurements ranged between 5 and 10% for the three derivatizing agents. Fig. 2 shows the

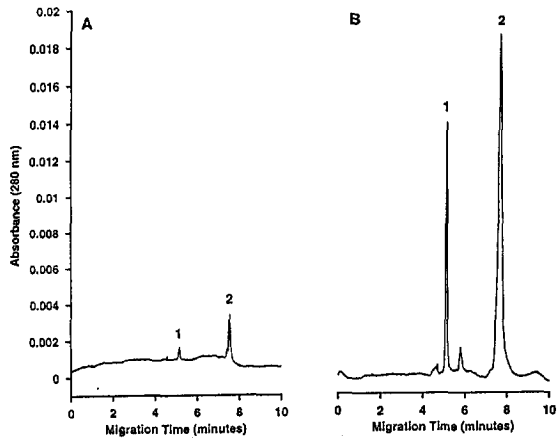

Fig. 2. Separation of (A) unlabeled and (B) NDA-labeled proteins. The peaks have been identified as follows: 1 = myoglobin (90 femtomoles), and 2 = conalbumin (48 femtomoles). Electrophoresis conditions: capillary length, 24 cm (19.4 cm from the inlet to the detector); electrokinetic injection, −10 kV for 10 s; detection, 280 nm; capillary temperature, 20°C; carousel temperature, 10°C; running buffer, Bio-Rad CE SDS sample buffer.

Table 1
Enhancement of UV absorption by pre-column derivatization[a]

Labeling reagent	Absorption					
	200 nm		220 nm		280 nm	
	Area	Height	Area	Height	Area	Height
Myoglobin						
NDA	1.57	1.36	2.69	2.36	22.5	19.5
OPA	1.71	0.79	4.71	2.16	4.14	2.03
Fluorescamine	1.74	0.93	2.16	1.14	14.6	7.88
Conalbumin						
NDA	1.21	0.82	2.09	1.23	12.0	7.24
OPA	1.52	0.70	3.48	1.56	3.03	1.41
Fluorescamine	1.48	0.77	1.64	0.84	7.51	3.80

[a] These data represent the average of six or more independent measurements. Peak areas and peak heights are expressed in arbitrary units.

electrophoretic separation of labeled and unlabeled myoglobin and conalbumin. Although a dramatic enhancement of signal intensity is evident from these data, it is also important to note that the retention time and peak efficiency are not greatly altered by the presence of the derivatizing reagent. This suggests that significant gains in detection sensitivity are possible without significantly altering analysis time. It is also interesting to note that the signal enhancement for myoglobin is nearly twice that observed for conalbumin. This difference may result from the greater preponderance of accessible derivatization sites on myoglobin relative to conalbumin [30].

The data in Table 1 also suggests a trend toward smaller signal enhancements as the detection wavelength is decreased. For the underivatized proteins, only the aromatic amino acids exhibit a significant absorbance at 280 nm, whereas the entire peptide backbone contributes to the signal at shorter wavelengths. Since the initial signal is much stronger at the shorter wavelengths, the enhancements are smaller. Even greater enhancements were recorded in spectral regions in which no significant protein adsorption was observed. For example, NDA-labeled myoglobin and conalbumin demonstrated excellent sensitivity at 320 nm. By choosing protein labels with specific spectral characteristics, the absorption signal can be tailored to wavelength regions where few sample concomitants absorb, and thus obtain greater signal discrimination.

Although peak area and peak heights were both observed to increase with the derivatized molecules, this enhancement is not necessarily consistent for both myoglobin and conalbumin. In general, when measurements were made at lower wavelengths, large increases in peak area were observed in good agreement with observations made for fluorescamine labeling of proteins in free-zone capillary electrophoresis [21]. However, peak heights were sometimes greater for the unlabeled molecules. At 200 nm, proteins derivatized with all three reagents showed a decrease in the peak heights of both proteins, while at 220 nm only fluorescamine-labeled proteins demonstrated this reduction. The signal improvement is small at the lower wavelengths because the increase in signal intensity is not able to compensate for the reduction in peak height caused by the reduced separation efficiency. At 280 nm, large enhancements of the absorbance signal resulted in peak heights greater than observed for the unlabeled species.

The separation efficiency for labeled and unlabeled conalbumin and myoglobin are summarized in Table 2. These data clearly indicate that pre-column derivatization of the proteins introduces additional zone broadening, but that the extent of this broadening is dependent on the reagent. NDA labeled proteins consistently demonstrated the highest efficiency of the reagents investigated. Fluorescamine and OPA produced nearly equivalent reductions in efficiency.

Electrophoretic analysis of proteins treated with SDS is typically used to estimate the mass of the molecule. To determine what impact pre-column labeling might have on molecular mass estimates, NDA-labeled and unlabeled solutions containing myoglobin, α-chymotrypsinogen A,

Table 2
Separation efficiency of labeled and unlabeled proteins[a]

Labeling reagent	Myoglobin		Conalbumin	
	Labeled	Unlabeled	Labeled	Unlabeled
NDA	20.6	25.6	11.0	30.7
OPA	6.9	31.0	6.0	36.4
Fluorescamine	9.3	28.0	7.3	35.7

[a] Efficiencies are expressed as thousands of theoretical plates.

ovalbumin, and conalbumin were analyzed by SDS-NGSCE. Unlabeled protein molecular mass standards were analyzed immediately preceding and following these samples. The logarithm of the molecular mass of each standard was plotted against its averaged migration time to construct a mass calibration plot to estimate the mass of both labeled and unlabeled proteins. These data are summarized in Table 3 along with the accepted mass of these molecules. Errors in molecular mass estimates were approximately equivalent for both samples, with the unlabeled proteins having a maximum error of 5.6% whereas the NDA-labeled species had a maximum error of 6.8%. While slightly poorer mass accuracy was observed for the labeled proteins, these errors are still within the 10% accuracy usually quoted for SDS sieving analysis [2,31].

With the data from Table 3, it is interesting to note that all of the molecular mass estimates for the derivatized proteins were lower than the actual molecular mass. This is surprising since the addition of the derivatizing reagent should increase the molecular mass of the protein SDS complex. There may be several explanations for these observations. The addition of hydrophobic label groups to the protein may have caused increased SDS binding to the protein, and therefore increased the negative charge on the complex. This would result in faster migration through the capillary and an apparent decrease in molecular mass. Similarly, the reaction of positively charged amine groups on the protein with the labeling reagent could reduce the positive charge intrinsic to the protein. Again, the more negative charge on the complex would give rise to lower molecular mass estimates. Whether one or both effects are responsible for this change in migration velocity has not been determined.

Our observations indicate that detection limits for derivatized molecules detected at 280 nm are similar to those for underivatized molecules at 200 nm. For the reagents studied, derivatization is unlikely to provide significant improvement in detection limits. The most useful application of these reagents is to shift the region of detection to avoid spectral interferences from buffer components and sample concomitants. In favorable cases, very dramatic shifts in detection wavelengths can be observed with a minimum penalty in absorption sensitivity or separation efficiency. Even though enhanced absorptivities are observed for derivatized proteins at the 200 to 220 nm range, these improvements are usually not sufficient to justify the loss of separation efficiency and the additional complexity associated with the sample preparation. New reagents that demonstrate enhanced extinction coefficients in this spectral region, or which simplify sample preparation may overcome these limitations.

3.2. Fluorescence detection

Analyte derivatization can be used to improve the absorption signal for the detection of proteins, but even greater sensitivity enhancements can be obtained using fluorescence detection. While this mode of detection has been used for both SDS-PAGE and capillary zone electropho-

Table 3
Molecular mass determinations for labeled and unlabeled proteins

Protein	Actual M_r[a]	Unlabeled proteins		NDA-labeled proteins	
		Estimated M_r ($\times 10^{-3}$)	Percent error	Estimated M_r ($\times 10^{-3}$)	Percent error
Myoglobin	16 950	17.9	5.6	15.9	6.2
α-Chymotrypsinogen A	25 656	24.8	3.3	23.9	6.8
Ovalbumin	43 300	44.0	1.6	42.9	0.9
Conalbumin	77 500	77.2	0.4	72.3	6.7

[a] Molecular masses obtained from Ref. [36].

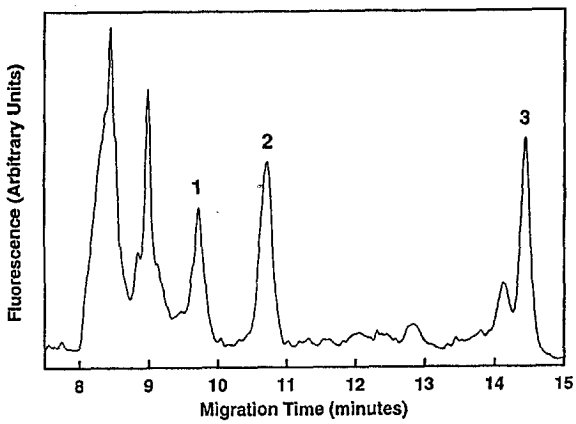

Fig. 3. Separation of fluorescamine-labeled: 1 = myoglobin (3.2 femtomole), 2 = α-chymotrypsinogen A (2.9 femtomole), and 3 = conalbumin (2.1 femtomole). Detection was by monitoring the laser-induced fluorescence signal. Electrophoresis conditions: capillary length, 37 cm (25 cm from the inlet to the detector); electrokinetic injection, −5 kV for 27 s; temperature, 20°C; running buffer, Bio-Rad CE SDS sample buffer.

resis, fluorescence detection may prove better suited to NGSCE. On-column detection provides improved quantitation and detection limits when compared with SDS-PAGE. In addition, SDS sieving separations minimize multiple peak formation from heterogeneous labeling of single protein species. Furthermore, low-molecular-mass impurities and excess labeling reagents are easily separated from the proteins of interest.

Fig. 3 shows the separation of a 500 n*M* mixture of fluorescamine-labeled myoglobin (3.2 femtomole), α-chymotrypsinogen A (2.9 femtomole), and conalbumin (2.1 femtomole) monitored by laser-induced fluorescence detection. The large broad peak from 8 to 8.5 min results from excess labeling reagent and unidentified low-molecular-mass species present in the sample and analysis buffer. The peak at 9 min is also an impurity and was present in blank samples. Impurity peaks in this region were common for all of the fluorophores. The numbers of these peaks and their position in the electropherogram are dependent on the derivatization reagent. Also noticeable are noise spikes throughout the electropherogram. These spikes were identified as small particulates generated by aggregation of material in the analysis buffer. The high viscosity of this buffer prevented these particles from being removed by filtration or centrifugation, but their presence could be minimized by heating the mixture in a boiling water bath for several minutes at the start of each day. The signal contribution of any particles remaining in the sieving buffer was suppressed with a digital low-pass filter. NDA-labeled protein solutions generated fewer artifacts in the electropherogram, but also caused a noticeable shift in the baseline approximately 10 to 12 min after the analysis was initiated.

Table 4 summarizes the limits of detection for myoglobin, conalbumin and α-chymotrypsinogen A labeled with the three fluorophores. OPA provided the lowest limit of detection for conalbumin ($3.0 \cdot 10^{-18}$ moles) but NDA provided nearly equivalent results. NDA provided better sensitivity than OPA for both myoglobin and α-chymotrypsinogen A, and both reagents provided consistently superior detection limits when compared with fluorescamine. As with absorption detection, there was a very significant reduction in detection limits with increasing mass of the analyte.

Labeling conditions for proteins were chosen to provide good detection limits and for their

Table 4
Fluorescence detection limits of pre-column labeled proteins[a]

Protein	NDA	OPA	Fluorescamine
Myoglobin	27	57	190
α-chymotrypsinogen A	59	100	160
Conalbumin	9.5	3.0	96

[a] Defined as a signal-to-noise of 3. Detection limits are expressed in attomoles.

ease of derivatization. While an attempt was made to use consistent conditions, the reaction characteristics of individual labels often dictated procedural changes. NDA and fluorescamine were used to derivatize protein samples prior to complexation of proteins with SDS. The NDA derivatization reaction is favored above pH 9, whereas the SDS sample buffer used in these studies was pH 8.3. Protein solutions were adjusted to pH 9.0 to insure good derivatization conditions, with the subsequent addition of the SDS buffer only after labeling had occurred. This was particularly important for fluorescamine as derivatization of proteins after SDS complexation was very difficult and resulted in low signal intensity. Fluorescamine hydrolyzes with a reaction half-time of only a few seconds [32], so we hypothesize that a large fraction of the fluorescamine is destroyed before it can interact with the SDS–protein complexes. Similarly, the slow change in color associated with the formation of the NDA–amine reaction product suggests that the NDA reaction is also slowed by the presence of SDS on the target molecule. It is possible that the SDS sterically hinders access to the derivatization sites on the protein or can interact with the labeling reagent to limit the accessibility of its reactive site. The OPA reagent was only reacted with the analyte after SDS complexation because the reaction product is relatively labile and would not tolerate the extreme conditions used for SDS complexation.

One advantage of SDS non-gel sieving CE is the ability to obtain molecular mass estimates of separated components. Fig. 4 shows the separation of commercial molecular mass standards derivatized with OPA. Detection limits for the protein molecular mass standards were determined to be 270 attomoles for lysozyme, 320 attomoles for trypsin inhibitor, 41 attomoles for carbonic anhydrase, 130 attomoles for ovalbumin, 240 attomoles for serum albumin, 51 attomoles for phosphorylase b, 39 attomoles for β-galactosidase, and 8.3 attomoles for myosin. Molecular mass calibration plots generated for labeled standards with fluorescence detection were nearly identical to similar plots generated with unlabeled standards and absorption detection. Even for separations of molecular mass standards near the detection limit (signal-to-noise ratio ≈ 5), linear regression provided a correlation coefficient (r) of 0.994, suggesting that effective mass calibration can be carried out at very low concentrations.

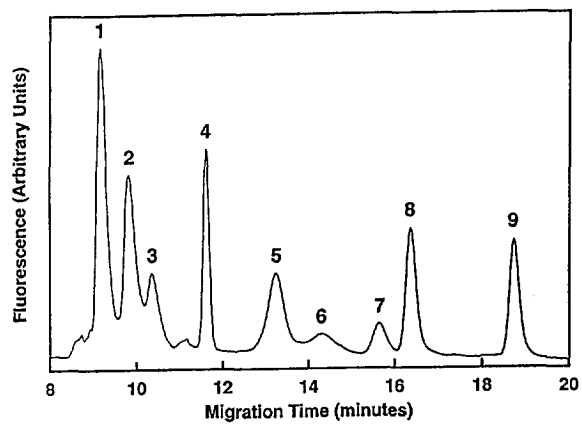

Fig. 4. Separation of molecular mass standards by non-gel sieving capillary electrophoresis. The peaks have been identified as follows: 1 = aprotinin, 2 = lysozyme, 3 = trypsin inhibitor, 4 = carbonic anhydrase, 5 = ovalbumin, 6 = bovine serum albumin, 7 = phosphorylase b, 8 = β-galactosidase, 9 = myosin. The amount of each protein injected on column was 25, 8.6, 5.8, 2.8, 2.9, 0.83, 0.57, 1.6, and 0.38 femtomoles, respectively. Electrophoresis conditions: capillary length, 37 cm (25 cm from the inlet to the detector); electrokinetic injection, −5 kV for 27 s; temperature, 20°C; running buffer, Bio-Rad CE SDS sample buffer.

3.3. Analysis of membrane proteins

To demonstrate the utility of pre-column derivatization techniques for the analysis of complex samples, the fertilization envelope from a single *Lepidobatrachus laevis* embryo was subjected to analysis by SDS-NGSCE with fluorescence detection. The resulting electropherogram of the OPA modified proteins is shown in Fig. 5. Previous investigations of this structure by SDS-PAGE required isolation of multiple envelopes for each analysis [29]. The detection sensitivity afforded by the pre-column derivatization reaction allowed a single fertilization envelope to be diluted in 21 μl of solution and still provides data with excellent signal-to-noise characteristics. This larger volume also simplifies sample hand-

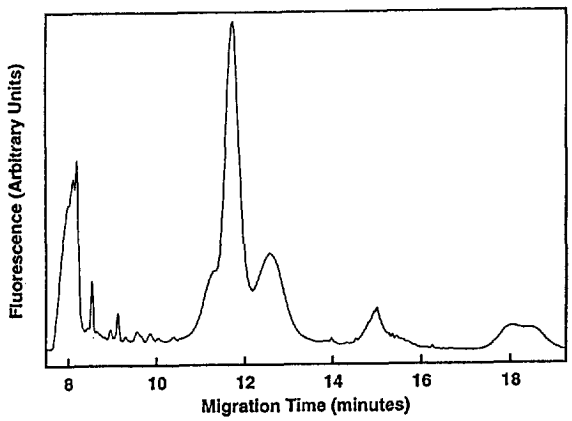

Fig. 5. Non-gel sieving capillary electrophoresis separation of a fertilization envelope from a single *Lepidobatrachus laevis* embryo. Electrophoresis conditions: capillary length, 37 cm (25 cm from the inlet to the detector); electrokinetic injection, −5 kV for 27 s; temperature, 20°C; running buffer, Bio-Rad CE SDS sample buffer.

ling and is sufficient for more than two thousand analyses by SDS non-gel sieving capillary electrophoresis.

The molecular masses of the protein and glycoproteins separated by SDS non-gel sieving CE are comparable to data obtained from the previous SDS-PAGE study. The peak at 11.7 min in Fig. 5 was estimated to have a mass of 44 600, as compared with 39 900 by SDS-PAGE [29]. Similarly, SDS non-gel sieving molecular masses were larger than those observed by SDS-PAGE for other membrane components. This discrepancy in molecular mass may have resulted because glycosylated proteins tend to bind lower quantities of SDS, which causes them to migrate anomalously. Higher sieving gel concentrations minimize these errors [33]. Since polyacrylamide gels have a greater effective gel concentration than sieving polymer solutions, it is logical to assume that these two mass estimates might be different. Fortunately, these errors in mass assignment can be partly compensated with Ferguson analysis [34]. Ferguson analysis by SDS-NGSCE can be automated and completed in approximately the same amount of time required for one SDS-PAGE analysis, and so provide even more accurate mass estimates [19,35].

4. Conclusions

Pre-column labeling of proteins prior to separation by SDS non-gel sieving capillary electrophoresis provides an effective means of enhancing sensitivity for absorption and fluorescence detection. The greatest absorption sensitivity enhancements were observed at 280 nm with NDA as the derivatizing reagent although enhancements were also observed at 200 and 220 nm. Derivatized molecules monitored at 280 nm provided similar limits of detection as those measured for unlabeled proteins at 200 nm and 220 nm. This would allow detection to be shifted to longer wavelengths, thus avoiding spectral interferences from buffer components and sample concomitants without significant loss of sensitivity. Derivatization of the analyte produces a small but measurable reduction in separation efficiency, but this had a minimal impact on the accuracy of the molecular mass determinations. Formation of multiple peaks from single analytes due to derivatization was not observed with either UV absorbance or fluorescence detection, which suggests that pre-column derivatization of macromolecules may be more routinely applicable to this separation technique. When pre-column derivatization was utilized with fluorescence detection, the resulting detection limits were comparable to those observed for free zone capillary electrophoresis and allowed the analysis of microscopic samples.

It may be possible to further enhance detection sensitivity by developing labeling reagents which more readily react with target analytes, and which have enhanced absorptivity in specific spectral regions. Continued improvements in sieving polymer matrices which generate smaller background signals should also decrease detection limits. The high sensitivities provided by fluorescence detection coupled with the effectiveness of SDS electrophoresis techniques for separation of complex mixtures promises to be extremely useful for many applications of biological analysis. As previously demonstrated, the ability to characterize sub-cellular structures from individual cells may be advantageous when these structures cannot be isolated in larger quantities.

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