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Argon-laser-induced fluorescence detection in sodium dodecyl sulfate-capillary gel electrophoretic separations of proteins

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Abstract

The use of argon-laser-induced fluorescence detection in sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) separations of proteins is described. A simple, rapid (10 min) precolumn labeling reaction for a series of standard proteins (molecular masses of 20 100–77 000) was developed which utilizes 4-fluoro-7-nitrobenzofurazan and 4-chloro-7-nitrobenzofurazan as fluorogenic labeling reagents. Detection limits of 25 ng/ml for labeled bovine serum albumin were obtained. These detection limits compare favorably with alternate detection techniques such as Coomassie blue staining (2.5 μg/ml) and UV–Vis absorption detection in SDS-CGE (0.5 μg/ml). Conditions were identified for the labeling reactions which produce no measurable zone broadening effects for labeled proteins relative to unlabeled species. By using relative migration data from labeled proteins and molecular mass calibration plots obtained exclusively from unlabeled proteins, molecular mass estimates of labeled proteins typically accurate to within 4.5% of literature values can be obtained. The labeling reaction thus maintains the molecular mass information inherent in the separation.

Keywords: Laser-induced fluorescence detection; Detection, electrophoresis; Derivatization, electrophoresis; Proteins; Fluoronitrobenzofurazan; Chloronitrobenzofurazan

1. Introduction

The use of ionic denaturing agents, such as sodium dodecyl sulfate (SDS), in conjunction with electrophoresis through polyacrylamide or other types of gels is commonly used for analysis of unknown mixtures of proteins and determinations of protein molecular masses [1]. This molecular mass information is often of substantial importance, especially in dealing with analytes of unknown origin, as it provides an important qualitative indication of the identity or origin of the protein. In conventional slab gel electrophoresis, detection of the resolved species is often problematic. Common methods of detection of separated proteins include Coomassie Blue staining [2] and silver staining [3]. These techniques involve laborious, time consuming protocols which typically are only semi-quantitative.

Capillary zone electrophoresis (CZE) offers the advantages of an instrumental approach to electrophoresis [4]. Detection of proteins separated via CZE can also be problematic, however. Online detection via UV–Vis absorption is available, eliminating the need for post-separation staining [5]. However, the concentration limits of detection available are poor due to the short optical pathlength and low molar absorptivities of proteins. Laser-induced fluorescence (LIF) offers the potential for improved detection

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limits for proteins separated via CZE. Unfortunately, precolumn fluorescent labeling of the proteins yields a statistical, heterogeneous charge distribution among individual protein molecules, which limits practical separation efficiencies [6]. Pre-column labeling can be avoided through the use of UV laser-excitation of the native fluorescence of the protein [7]. Unfortunately, the UV lasers required are not yet widely available due to their high cost. No commonly available fluorescence detection technique for CZE currently provides low detection limits, the ability to detect an unanticipated analyte species and molecular mass information in a single analysis.

Our interest is in the development of laser-induced fluorescence detection for use in SDS-capillary gel electrophoresis (CGE) separations. In contrast to zone electrophoresis separations, minor charge heterogeneity induced by incomplete labeling reactions is of little consequence to the separation. Most proteins bind a constant mass of SDS per unit mass of protein (1.4 g SDS/g protein) [8]. Thus, the total charge of the protein–SDS complex is largely due to SDS. The use of such precolumn labeling in conventional SDS-polyacrylamide gel electrophoresis (PAGE) has been reported [9,10]. Separations of precolumn labeled proteins in these studies have demonstrated separation properties similar to those of unlabeled proteins. In addition, LIF detection has been reported for the detection of labeled proteins in conventional SDS-PAGE slab gel systems [11]. These systems relied upon photographic detection of the fluorescence and are substantially less precise and less quantitative than the system we describe. In addition, these systems require substantially larger masses of sample for analysis. The ability to analyze small sample masses is often of importance in biological studies [12–14]. Other groups have recently reported the use of precolumn derivatization to enhance detection limits in CGE [15]. The reagents described here offer excitation wavelengths suitable for use with a low power argon-ion laser, such as those commonly found with commercial instrumentation.

This paper describes our recent work utilizing 4-fluoro-7-nitrobenzofurazan (NBD-F) and 4-chloro-7-nitrobenzofurazan (NBD-Cl) as fluorogenic precolumn labeling reagents for proteins separated via CGE and detected via LIF. NBD-F and NBD-Cl,

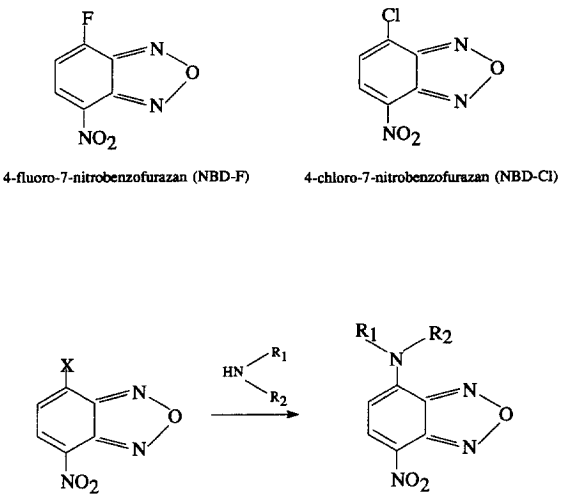

Fig. 1. Structures of the fluorogenic amine labeling reagents, NBD-F and NBD-Cl. Typical reaction between NBD reagent and primary or secondary amine.

whose structures are shown in Fig. 1, are fluorogenic amine labeling agents which have been used previously for labeling a variety of amines and proteins [16,17], but to the best of our knowledge, this is the first use of NBD labels in CGE of proteins. We also describe the effect of labeling conditions upon separation conditions and accuracy of molecular mass estimates. Specifically, we report that conditions can be found such that the fluorescent labeling has no significant effect upon protein separation efficiency or the accuracy of the molecular mass estimate. Enhancements in concentration detection limits due to the use of LIF consist of a 100-fold improvement over conventional Coomassie Blue staining and a 20-fold improvement over UV absorption detection in CGE systems.

2. Experimental

2.1. Apparatus

A capillary electrophoresis system was designed and constructed in a fashion similar to systems previously described [18]. A high-voltage power supply (Model PS/MJ30P0400-11, Glassman High Voltage, Whitehouse Station, NJ, USA or CZE1000R, Spellman, Plainview, NY, USA) with a

maximum d.c. voltage of 30 kV was used to drive the gel electrophoresis separation. A Plexiglass box was used to isolate the operator from the high-voltage lead. Fused-silica capillaries (360 μm O.D. and 75–50 μm I.D.) with a total length of 52–60 cm and a 30–40 cm separation length were used. The inner surface of the capillary was left uncoated.

The LIF detection system utilized was a modified version of one described previously [18]. The laser used was a Model 532-50BS argon-ion laser (Omnichrome, Chino, CA, USA) with single line output at 488 nm. The laser beam was initially passed through a 488 nm interference filter (Edmund Scientific, Barrington, NJ, USA) to block the plasma discharge of the laser. The incident beam was then focused by a 9 mm focal length biconvex quartz lens (Edmund) onto the detection window of a fused-silica capillary. Fluorescence from the optical region was collected by a 20× microscope objective lens (Edmund) set at right angles to the laser beam and isolated with the use of a 520 nm interference filter (Edmund) and a OG515 longpass cutoff filter (Edmund). These filters were fastened directly onto the outside of the 6 in.×3.5 in.×10 in. metal box (1 in.=2.54 cm) which housed the photomultiplier. A spatial mask was also applied to the face of the housing to minimize stray light. Signals from the photomultiplier (Type R1527, Hamamatsu, Bridgewater, NJ, USA), which was held at −950 V, were then passed on to an RC circuit (300 ms time constant) for current to voltage conversion and smoothing. Data collected via UV absorbance detection utilized a Spectra 100 (ThermoSeparation Products, Fremont, CA, USA) detector.

Analog absorption or fluorescence data were collected via chart recorder or on a 20 bit data acquisition system (ChromPerfect for Windows, DT2804 board, Justice Innovations, Mountain View, CA, USA) installed on a 486DX2 computer.

2.2. Reagents

Acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), SDS, 2-mercaptoethanol, FITC (fluorescein isothiocyanate)-labeled bovine serum albumin, NBD-F, NBD-Cl, DTAF (5-([4,6-dichlorotriazin-2-yl]amino)fluorescein) and all proteins were purchased from Sigma (St. Louis, MO, USA). Tris was purchased from Fisher (Fair Lawn, NJ, USA). CHES [2-(cyclohexylamino)ethanesulfonic acid] and potassium chloride were purchased from Aldrich (Milwaukee, WI, USA). Sodium borate was purchased from Mallinckrodt Chemical (St. Louis, MO, USA). All reagents were of reagent grade or better and were used as received. ProSort SDS Protein analysis reagent was purchased from Applied Biosystems (Foster City, CA, USA) and used as directed.

2.3. Solutions and procedures

2.3.1. Preparation of non-cross-linked polyacrylamide gels

Linear, non-cross-linked-polyacrylamide gels with an acrylamide concentration of 6% were polymerized, in situ, essentially as described by Wu and Regnier [19] except that our gels were prepared in a solution of 0.1 *M* Tris–CHES at a pH of 8.7. Briefly, 15 ml of 12% acrylamide stock solution and 15 ml of 0.1 *M* Tris–CHES pH 8.7 were pipetted into a 125 ml filtering flask. The solution was swirled, stoppered and degassed via a water aspirator for 30 min. After degassing, the solution was divided into two 15 ml portions and placed into 20 ml glass buffer vials. To each vial, 300 μl of 10% ammonium persulfate, 60 μl of 10% TEMED and 150 μl of 10% SDS were added. Each vial was swirled to further initiate the polymerization reaction. Without delay, a small amount of this solution was sucked into a 3 ml syringe which was then used to fill the separation capillary. The capillary was flushed with three to four column volumes of the polymerizing gel solution. The ends of the capillary were then placed into the 20 ml buffer vials containing the polymerizing gel. The gel was allowed to polymerize in situ for 2 h before the application of high voltage. High voltage was initially applied at a field near 0 V and was then slowly increased until a voltage of 13.8 kV (230 V/cm) was attained. Before any injections were performed, the voltage was applied for 10 min in order to get a stable current. Protein samples were injected into the capillary from the grounded end by electrokinetic injection. The polyacrylamide gel solutions in the buffer vials were replaced every seven to ten days. The gels that were polymerized within the capillary had an average lifetime of a few hours to two days. Non-functional gels were removed from

the capillary using a polyimide ferrule adapter (Valco, Houston, TX, USA) and nitrogen gas.

2.3.2. Preparation of non-cross-linked entangled polymer gels

As an alternative to in situ polymerization, a commercial preparation of a entangled polymer gel formulation was utilized. The fused-silica columns used were conditioned prior to initial use by flushing manually with deionized water for 10 min, 0.1 *M* NaOH for 10 min and ProSort gel for 60 min. The separation column was flushed manually with ProSort gel for 5 min between injections.

2.3.3. Preparation of NBD-F protein derivatives

In a 13 mm×100 mm test tube, 300–700 μl of an individual protein stock solution (1.0–1.4 mg/ml in 50 m*M* sodium borate–100 m*M* KCl pH 7.9) were mixed with 300–600 μl of NBD-F stock solution (0.2 mg/ml in HPLC-grade acetonitrile). The mixture was heated for 5 min in a 50°C water bath. A 1 ml volume of protein sample buffer was added to denature the protein. Protein sample buffer consisted of 0.1 *M* Tris at a pH of 8.1, 5% (v/v) 2-mercaptoethanol and 2.5% (w/v) SDS. The mixture was heated for 1 min in a boiling water bath. The resulting individual NBD–protein conjugates were at final concentrations of 0.167–0.389 mg/ml. The solutions were transferred to individual 4 ml glass vials and stored in a refrigerator. All NBD stock solutions were prepared fresh and used immediately.

All proteins were initially labeled individually. Mixtures of the NBD-F protein conjugates were prepared by diluting specified amounts of the desired individual NBD-F protein conjugate solutions with protein sample buffer.

2.3.4. Preparation of NBD-Cl protein derivatives

In a 2 ml glass vial, 140 μl of an individual protein stock solution (10 mg/ml in 50 m*M* sodium borate, 100 m*M* potassium chloride, pH 7.9) were mixed with 140 ml of three other individual protein stock solutions to generate a mixture of trypsin inhibitor, ovalbumin, bovine serum albumin (BSA) and conalbumin. To this mixture, 40 μl of NBD-Cl stock solution (50 mg/ml in HPLC-grade acetonitrile) were added. A series of such mixtures were then allowed to react at varying temperatures for 10 min. O ixture was reacted at each of the following temperatures: 22, 35, 45, 60, 75°C. Each vial was placed in a water bath at the desired temperature for 10 min. A 600 μl volume of a solution composed of 5% (v/v) 2-mercaptoethanol and 5% SDS was then added to each vial. The resulting mixtures were heated for 15 min in a boiling water bath. The resulting individual NBD-Cl labeled protein conjugates were at final concentrations of 1.16 mg/ml. Solutions were stored in a refrigerator. All NBD stock solutions were prepared fresh and used immediately.

The labeling of solutions containing only BSA was carried out in the manner above with the following exceptions. A 390 μl volume of protein stock solution (10 mg/ml in 50 m*M* borate, 100 m*M* KCl, pH 7.9) was mixed with 110 μl of 25 mg/ml NBD-Cl prepared in HPLC-grade acetonitrile. After the desired reaction interval at the desired temperature, 500 μl of a solution composed of 5% (v/v) 2-mercaptoethanol and 5% SDS was added to the vial. The resulting mixture was then heated for 15 min in a boiling water bath. Solutions were refrigerated for storage.

2.3.5. Preparation of unlabeled proteins

Unlabeled control protein solutions were prepared for comparison with labeled proteins. Unlabeled protein solutions were prepared by mixing 390 μl of a protein stock solution (composed of 5 mg/ml of an individual protein) with 110 μl of HPLC-grade acetonitrile. After the desired heating interval at the desired temperature, 500 μl of a solution composed of 5% (v/v) 2-mercaptoethanol and 5% SDS was added to the vial. The resulting mixture was then heated for 15 min in a boiling water bath. Mixtures of proteins were generated from these solutions. Solutions were refrigerated for storage.

2.3.6. Preparation of labeled FITC-BSA

Stock FITC-BSA (0.1 mg/ml BSA) was prepared by dissolving 1 mg of the solid in 10 ml of 0.1 *M* Tris–CHES pH 8.7. In a 13 mm×100 mm test tube, 1 ml of the FITC-BSA (Sigma) stock solution was reacted with 1 ml of the protein sample buffer. The mixture was swirled and then heated for 1 min in a boiling water bath. This 50 μg/ml solution was then

transferred to a 4 ml glass vial and was subsequently placed in the refrigerator for storage.

2.3.7. Estimation of injection volumes

Injection volumes for the electrokinetic injections utilized here were estimated through the use of the following relationship:

$$V_{inj} = (T_{inj}/T_{mig})(E_{inj}/E_{mig})(V_{cap})$$

Here, V_{inj} represents the volume injected while V_{cap} represents the volume of the capillary from the injection point to the detector. T_{inj} and T_{mig} represent the injection and migration times while E_{inj} and E_{mig} represent injection field strength and migration field strength.

3. Results and discussion

3.1. Choice of labeling reagent

The enhancement of detection limits in SDS-CGE separations of proteins through the use of LIF detection is an important analytical goal. One of the critical aspects of developing the tools and techniques required to achieve this goal is the selection of the optimum fluorescent labeling agent. The ideal labeling reagent would be fluorogenic, possess an excitation maximum which was accessible via a commonly available laser source, not alter the separation properties of the analytes, provide excellent detection limits and react rapidly under mild conditions. We focused on investigating labeling reagents which could be excited via the 488 nm line of an air cooled argon-ion laser due to their high power and low cost. We also concentrated on the use of well known amine labeling reagents, although fluorescent labeling of protein thiols has also been reported [20]. We utilized four amine labeling reagents for these studies: DTAF, FITC, NBD-Cl and NBD-F. Due to the superior spectroscopic qualities of FITC and DTAF, we initially investigated their use as precolumn labeling reagents in SDS-CGE. However, we were unable to generate useful electropherograms from DTAF and FITC labeled analytes due to the large excess of unlabeled reagent remaining in the sample mixture after our labeling reaction (data not shown). The alternative reagents, NBD-F and NBD-Cl, would seem to possess several of the qualities of our ideal labeling reagent. For instance, they are fluorogenic and have spectroscopic qualities compatible with sensitive laser-based analyses [21,22], although they cannot provide the detection limits achieved with FITC labeled analytes. In addition, these reagents have been shown to react rapidly with amine functionalities under mild conditions, even being used in postcolumn reactors [23]. For these reasons, our remaining studies focused upon these species.

3.2. Optimization of labeling reaction and gel preparation

One of the most attractive possibilities of the use of prelabeling in SDS-CGE is the opportunity to dramatically improve the detection limits available with no loss, ideally, in separation performance as measured by efficiency. Accomplishing this feat requires the covalent labeling of the protein without a significant alteration in the net separation properties of the protein–SDS complex. Two divergent strategies for achieving this goal seemed plausible. The approach most likely to succeed would be to label the proteins only minimally. Alternatively, one might attempt to utilize a highly reactive labeling reagent to fully label all potential sites on the protein. NBD-F is substantially more reactive than NBD-Cl and other alternative amine labeling reagents, such as FITC or DTAF, which typically require hours of reaction time. Fig. 2 shows a typical electropherogram generated from a SDS-CGE separation of NBD-F labeled proteins. As described in Section 2, the labeling process consisted of short heating of the reagent and protein prior to addition of SDS or 2-mercaptoethanol. The proteins utilized are commonly employed as molecular mass standards for SDS-CGE separations. Analyses such as these clearly demonstrate that the use of LIF detection in SDS-CGE is feasible. However, a number of operational parameters require optimization. For instance, one can see that the lower-molecular-mass proteins in the electropherogram have substantially greater efficiencies than the higher-molecular-mass proteins. This could potentially be due to the labeling process, the gel formulation or the specific proteins involved.

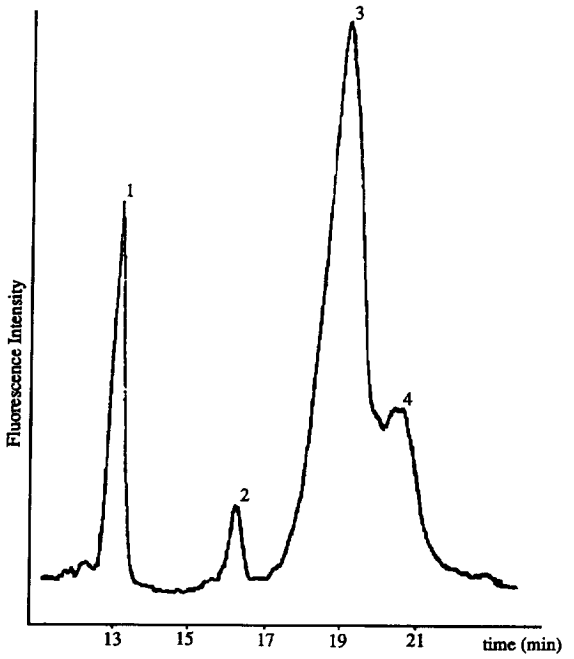

Fig. 2. Electropherogram of NBD-F labeled proteins using 6% T non-cross-linked acrylamide gel and LIF detection. Proteins: (1) soybean trypsin inhibitor; (2) ovalbumin; (3) bovine serum albumin (BSA); (4) conalbumin. Conditions: uncoated 75 μm I.D. capillary, 40 cm separation length, 60 cm total length. Gel prepared in situ as described in text. 10 s electrokinetic injection at migration voltage. Migration voltage of 230 V/cm. Laser output: 3 mW at 488 nm.

While the non-cross-linked acrylamide gel formulation we utilized provided separations based on protein molecular mass, we believed that a more easily reproducible entangled polymer formulation would provide advantages for these studies. A commercially available non-cross-linked entangled polymer formulation was used for all further studies.

To ascertain how efficiency was affected by the extent of the labeling reaction, we studied the effects of a range of labeling conditions upon analyte efficiency. We initially investigated the effects of reaction conditions upon the efficiency of BSA, one of the proteins exhibiting poor separation efficiencies in the NBD-F reactions. Our initial studies involved determining the reaction conditions (minimum reaction temperature for a short, constant reaction time) required for maximum separation efficiency. We varied our reaction temperature while keeping the reaction interval constant at 10 min. The electropherograms in Fig. 3 represent typical results from injections of the reaction mixtures at various temperatures. We can draw several conclusions from the electropherograms shown in Fig. 3. First, we see that BSA is labeled by NBD-Cl even with a room temperature incubation over a ten minute interval. The observed peak at 10.7 min is entirely due to absorption of the NBD moiety as control electropherograms of unlabeled BSA gave no detectable peak (data not shown). The broad peak observed eluting prior to BSA is an, as yet unidentified, contaminant of our solutions. We also see that the extent of the labeling process (as measured by analyte peak height) increases by roughly a factor of 3 for the reaction at 35°C relative to the reaction at 22°C. The reaction carried out at 45°C shows roughly a 4-fold increase in labeling relative to the room temperature reaction. With incubation temperatures above 45°C, analyte peak heights actually decrease slightly. This is due to the fact that as reaction temperature increases, a gradual loss in separation efficiency is observed. This preliminary study indicates that, at least for BSA, reaction conditions optimized for efficiency and analytical signal would involve reaction of NBD-Cl with BSA at temperatures of 22–45°C. We undertook more extensive studies in order to generate optimal incubation conditions for a wider range of proteins.

To more fully understand how labelling conditions altered our separation efficiencies, we must compare efficiencies of labeled and unlabeled species directly. We can accomplish this by utilizing absorbance detection at 215 nm for our electropherograms. Absorption detection at this wavelength monitors labeled and unlabeled species simultaneously due to absorption of the peptide backbone. Typical electropherograms of a labeled and unlabeled four component mixtures are shown in Fig. 4. In Fig. 4, the electropherograms for the 35 and 60°C reactions have been omitted for clarity. Efficiency data for all the electropherograms is summarized in Table 1 ($n = 1$ for all trials).

Several conclusions can be drawn from the electropherograms shown in Fig. 4 and the data shown in Table 1. First, we can see that the mild incubation conditions which provided optimum separation efficiency for BSA also provide optimum efficiencies for the other three proteins in our mixture. The loss

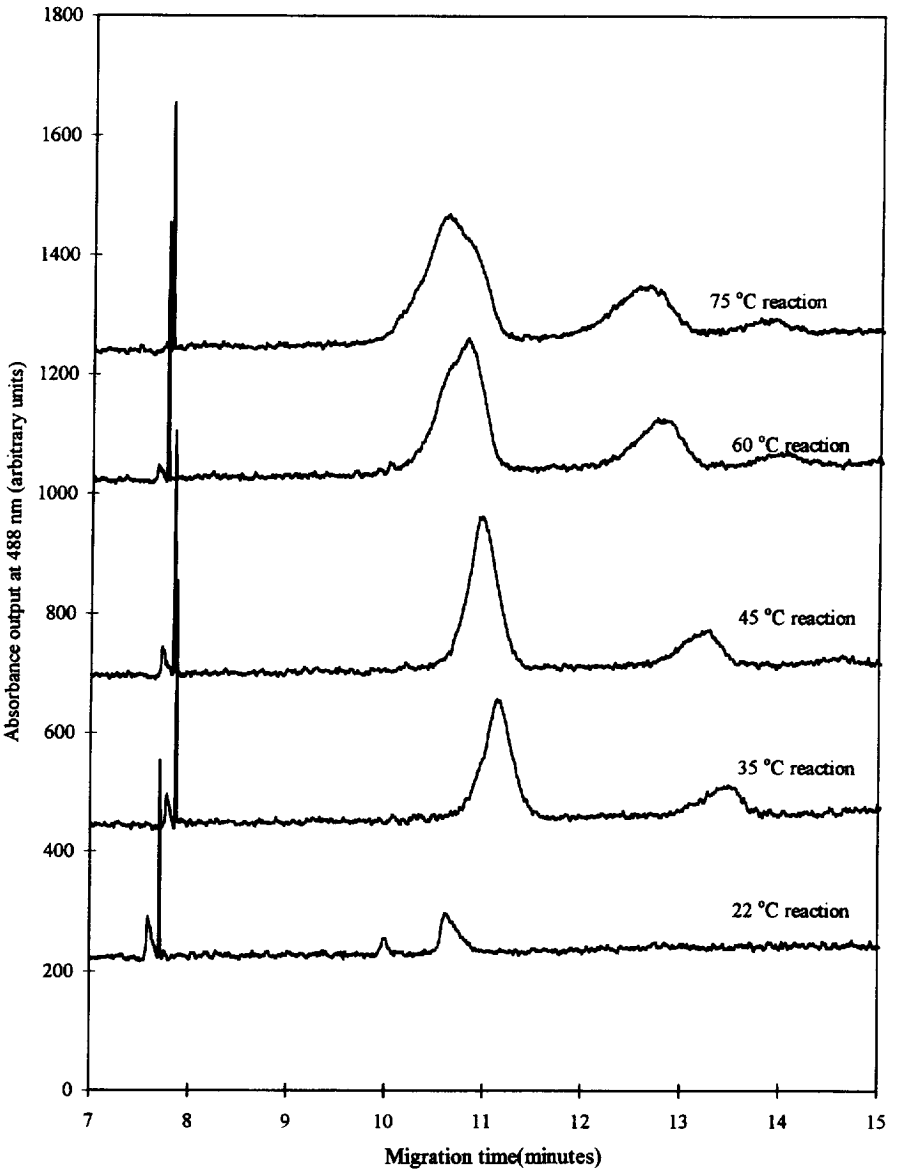

Fig. 3. Electropherograms of NBD-Cl labeled BSA labeled at various temperatures for 10 min. Uncoated 50 μm separation capillary of 52 cm total length, 30 cm separation length using ProSort non-cross-linked gel. Absorption detection at 488 nm. Electrokinetic injection of 20 s at 119 V/cm. Migration field strength of 287 V/cm.

of separation efficiency observed for the 35°C data (summarized in Table 1) relative to the 22 and 45°C data is believed to be due to the imperfect nature of that individual analysis rather than the incubation temperature. This is supported by the gradual, monotonic trends we observed in the labeling of BSA in Fig. 3. We can also see that efficiencies of labeled proteins at 22 and 45°C are essentially equivalent to the mean efficiency of five electropherograms of unlabeled proteins. Essentially, no loss in efficiency is observed due to the labeling process at these low incubation temperatures. More vigorous reaction temperatures again result in measurable loss in efficiency. In the electropherogram corresponding to

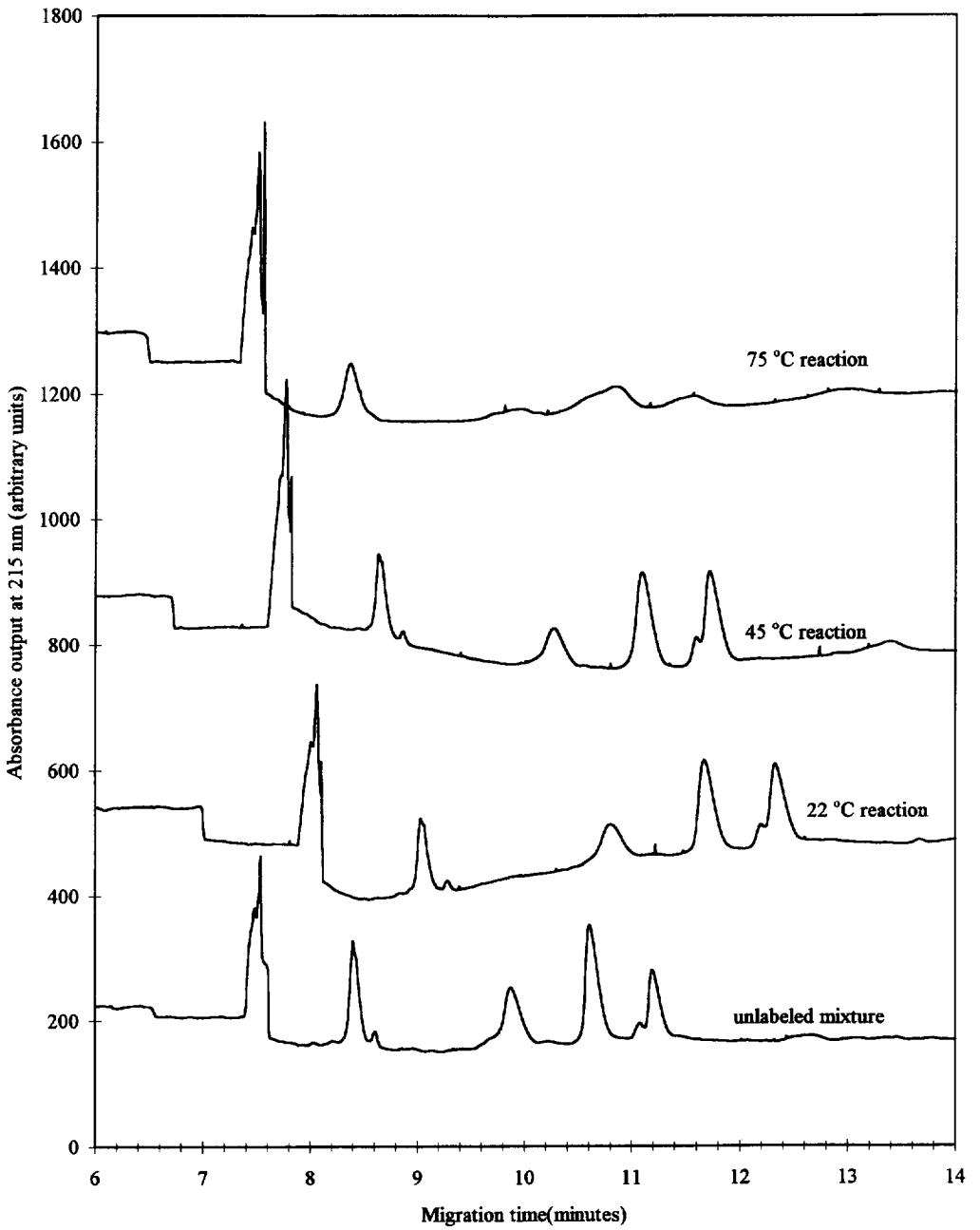

Fig. 4. Electropherograms of unlabeled and NBD-Cl labeled proteins labeled at various temperatures for 10 min. Absorption detection at 215 nm. Elution order: unidentified low-molecular-mass compound; soybean trypsin inhibitor; ovalbumin; bovine serum albumin (BSA); conalbumin. Other conditions as in Fig. 3.

an incubation temperature of 75°C, for instance, we see a very dramatic loss of efficiency for the labeled proteins relative to unlabeled species separated under nearly identical conditions. The cause of this temperature dependence is not clear at this time. One possibility is that the presence of larger quantities of

Table 1
Numbers of theoretical plates for unlabeled and NBD-Cl labeled proteins for a series of labeling temperatures

Protein	Trypsin Inhibitor	Ovalbumin	Serum Albumin	Conalbumin
N values for unlabeled proteins				
mean ($n=5$)	38 200	12 100	23 600	31 100
S.D.	8 000	2 750	7 140	13 600
N values for labeled proteins				
Reaction temperature (°C)				
22	35 900	11 100	28 300	32 000
35	27 600	7 160	15 500	13 300
45	31 600	14 100	22 700	32 400
60	23 000	9 890	13 200	11 600
75	12 100	6 320	2 650	7 300

NBD moiety extending from the surface of the protein tend to disrupt optimal SDS binding.

Comparison of the efficiencies obtained for proteins labeled with NBD-Cl at temperatures ranging from 22–45°C with unlabeled proteins under nearly identical conditions suggests that no further optimization of labelling conditions is required to maximize efficiency. A typical electropherogram generated with these optimized conditions is shown in Fig. 5. A dramatic improvement relative to the less refined electropherogram shown in Fig. 2 is seen. This is partially due to the optimized reaction temperatures and conditions as well as the use of more predictable gel matrices.

3.3. Detection limits

The primary motivation for this investigation was to provide improved detection limits for proteins in CGE relative to those available for current systems which utilize UV–Vis absorbance detection. Fig. 6 shows the peak observed for NBD-F labeled BSA at an injected concentration of 250 ng/ml after separation via SDS-CGE and detection via LIF. The detection limit of 25 ng/ml which results compares quite favorably to the best detection limits reported for UV absorbance detection in CGE (500 ng/ml) [24]. An improvement of roughly 20-fold is observed. When compared to typical concentration detection limits for Coomassie Blue staining (2.5 ug/ml) [25], an improvement of 100-fold is seen. In addition, the concentration detection limits observed here are quite competitive with those obtained with the much more complex silver staining process (10 ng/ml) [26,27]. We calculated mass detection limits for BSA by estimating our injection volume as 17 nl (see Section 2). This injection volume correlates to a mass detection limit of $7 \cdot 10^{-18}$ mol or 0.5 pg of protein. It is important to note that these detection limits describe the ability of the instrumentation to monitor low concentrations of a fluorescent species. Labeling of species at ultratrace levels is often difficult and reaction kinetics may limit detectability. It is also important to note that the labeling procedures utilized here may be influenced by levels of analyte present in the sample as well as levels of matrix species present. Until a full investigation of the quantitative nature of the labelling process is complete, one must assume the procedure to be qualitative in nature.

Detection limits obtained for NBD-F labeled species should be equally applicable for NBD-Cl labeled species as the two reagents vary only in their reactivity and produce spectroscopically identical products. The poor efficiency of the BSA peak demonstrated in Fig. 6 can be rectified by utilization of reaction conditions optimized for efficiency as shown in Fig. 4. These milder reaction conditions should result in no loss in detection capabilities as demonstrated in Fig. 3. The milder reaction conditions provide equivalent peak height with a substantial decrease in peak broadening.

In order to see if the detection limits we have obtained here represent an optimum, we investigated several potential sources of improvement in detection capability. First, NBD derivatives are known to be highly sensitive to environmental influences and the prospect of significant quenching in the aqueous environment of the gel is possible. By making injections of commercially available preparations of BSA labeled with FITC, we were able to obtain detection limits for the "ideal" fluorophore. Injections of the FITC labeled BSA provided detection limits of 10 ng/ml in our 6% non-cross-linked acrylamide gel (data not shown). Comparison of this detection limit with the detection limits obtained from NBD labeled proteins imply that the spectro-

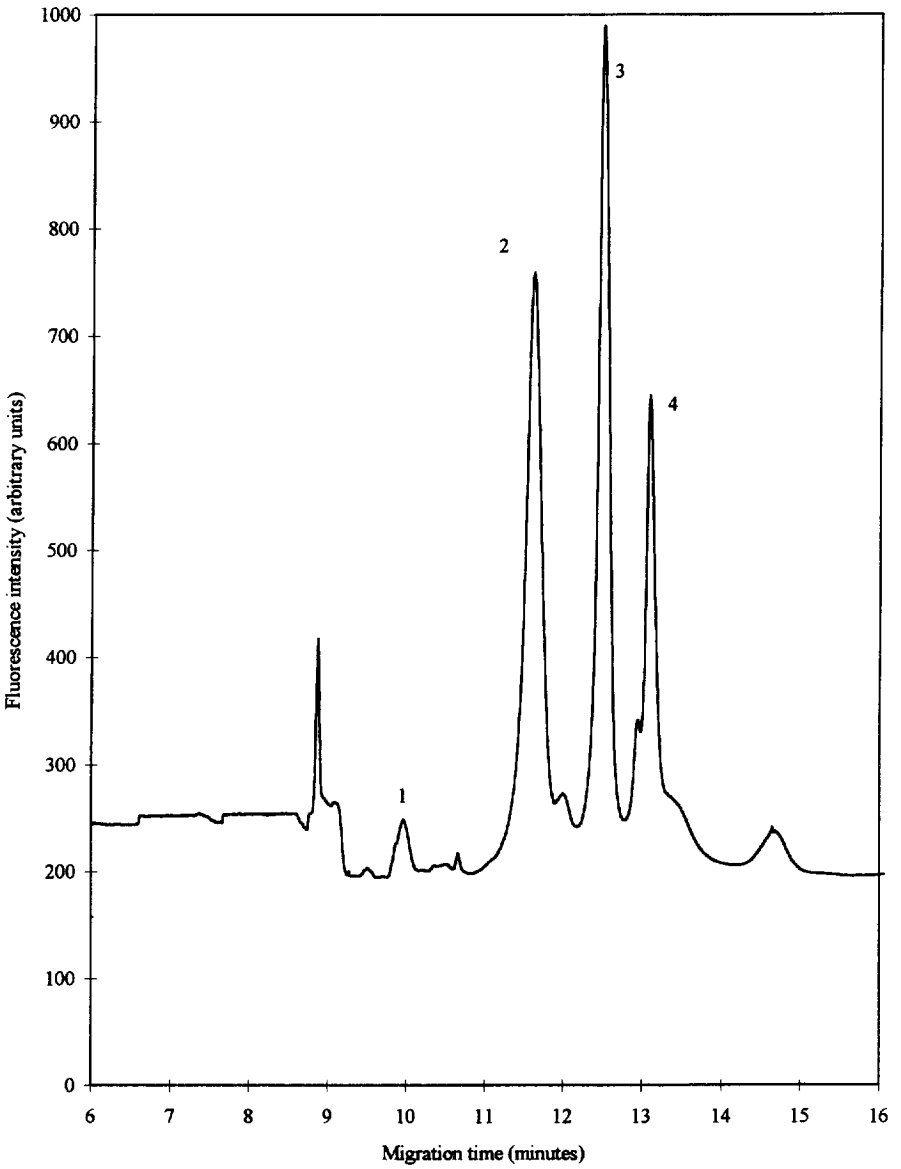

Fig. 5. Electropherogram of NBD-Cl labeled protein mixture detected via laser induced fluorescence detection. Labeling temperature: 35°C. Laser output: 3 mW at 488 nm. Other conditions as in Fig. 4.

scopic properties of the NBD label are favorable in the gel environment. NBD conjugates possess a lower molar absorptivity and quantum yield than FITC conjugates and are expected to provide somewhat slightly inferior detection limits. It is possible that the micellar environment of the gel matrix minimizes the quenching observed with NBD derivatives in aqueous environments. Further comparison of the detection limits obtained for the FITC labeled BSA with literature sources imply that further gains in detection limits can be achieved. Similar systems have achieved detection of $3 \cdot 10^{-12}$ M fluorescein in capillary electrophoresis systems [28]. We believe that our concentration detection limits ($4 \cdot 10^{-10}$ M) do not match these levels primarily due to the presence of enhanced background noise levels from

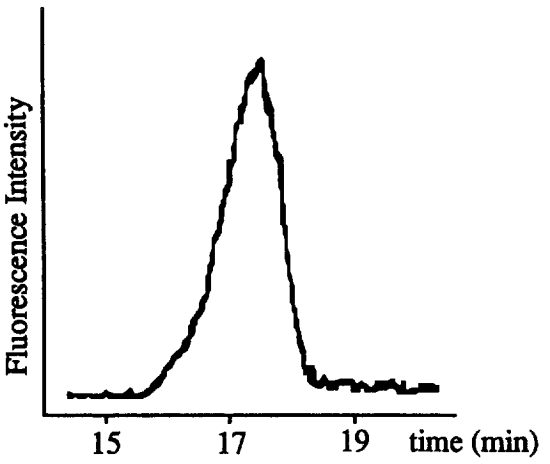

Fig. 6. Electropherogram of NBD-F labeled BSA detected via LIF detection. Laser output: 15 mW at 488 nm. A 10 s electrokinetic injection at migration voltage (230 V/cm) was used. Injected concentration of BSA: 250 ng/ml. Other conditions as in Fig. 2.

the gel matrix. With further attention to minimization of stray light from the entangled polymer matrix, attainment of these levels may be possible.

Previous groups have reported the use of pre-column labeling in conventional slab gel systems. Comparable systems reported have utilized scanning fluorometric detection in slab gels with conventional UV illumination [9] and photographic detection in slab gels after argon-laser-based excitation [11]. The concentration and mass detection limits obtained for these representative systems are 40–16 ng/ml, respectively, and 1–0.4 ng of detected protein, respectively. The work we describe provides, in general, comparable concentration detection limits relative to these systems and mass detection limits which are improved by roughly a factor of one thousand.

3.4. Estimation of protein molecular mass

An important distinction between a zone electrophoresis and a SDS-CGE separation is that the gel electrophoresis mode provides an additional dimension of qualitative information (i.e. estimation of protein molecular mass) which is not readily available using zone electrophoresis. In order to retain this advantage, our prelabeling procedure must not degrade the ability of the gel system to accurately provide molecular mass estimates of separated proteins. To investigate the effects of our labeling procedures on the estimation of analyte molecular mass, we initially found the linear relationship existing between the logarithm of protein molecular mass and protein relative migration for a series of four electropherograms of unlabeled proteins. Data was collected via UV absorption at 215 nm and our relationship was as follows: $y = -2.617x + 6.633$. The logarithm of the protein molecular mass represented the y-axis while the protein relative migration represented the x-axis. The low-molecular-mass peak seen at 7–8 min in Fig. 4 was used as the reference mobility marker. We then collected relative migration data for a series of protein mixtures labeled at a variety of temperatures between 22 and 60°C. At each temperature, at least one electropherogram was collected via absorption detection (215 nm) and one electropherogram via LIF detection. Relative migration data was generated. For the fluorescence data, the peak associated with NBD-OH (observed at 8.8 min in Fig. 5) was used as our reference mobility marker. Using the linear relationship derived exclusively from the unlabeled proteins, we can predict molecular masses for the labeled proteins using their relative migration data. The molecular mass predicted for the labeled proteins can then be compared to the literature value for the unlabeled protein and a percent error calculated. The resulting data is summarized in Table 2.

Several conclusions can be drawn from the data contained in Table 2. First, we see that by using data obtained exclusively from the unlabeled proteins we can accurately obtain the true molecular mass of the labeled species simply by measuring their relative migration. The mean percent error for all trials over all proteins was 4.5%. Conventional SDS-PAGE slab gel systems are reported to provide molecular mass estimates accurate to within 3000 u [29]. An absolute error of 3000 u represents % error values ranging from 15% to 4% for proteins with molecular masses between 20 100 and 77 000. Our overall % error values of 4.5% compare very favorably with these figures. Thus, it would appear that the labeling process has not influenced our ability to accurately estimate the molecular mass of separated species.

Several additional factors should be noted. The data for the unlabeled and labeled species was collected several days apart on entirely different

Table 2
Accuracy of molecular mass estimates of labeled proteins. Molecular masses are predicted using calibration data from unlabeled proteins. Literature values of molecular mass [31] are: STI, 20 100; OVA, 45 000; BSA, 66 000; CON, 77 000

Reaction Temp	Relative migration data			Predicted molecular mass			Mean % error
22°C	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
STI	0.892	0.880	0.874	19 891	21 382	22 169	5.21
OVA	0.746	0.772	0.776	47 942	40 990	40 014	−4.48
BSA	0.690	0.714	0.716	67 185	58 137	57 442	−7.69
CON	0.654	0.660	0.680	83 461	80 497	71 358	1.87
35°C	Trial 1	Trial 2		Trial 1	Trial 2		
STI	0.890	0.905		20 131	18 391		−4.17
OVA	0.764	0.766		43 0.14	42 499		−4.99
BSA	0.710	0.701		59 557	62 876		−7.25
CON	0.677	0.663		72 659	79 0.54		−1.48
45°C	Trial 1	Trial 2		Trial 1	Trial 2		
STI	0.900	0.889		18 954	20 253		−2.47
OVA	0.756	0.760		45 139	44 064		−0.87
BSA	0.700	0.709		63 256	60 061		−6.58
CON	0.662	0.674		79 533	74 029		−0.28
60°C	Trial 1	Trial 2		Trial 1	Trial 2		
STI	0.901	0.899		18 886	19 069		−5.59
OVA	0.767	0.760		42 244	44 064		−4.10
BSA	0.718	0.707		56 822	60 643		−11.0
CON	0.682	0.667		70 503	77 172		−4.11

electrophoresis systems. Even better agreement may be possible with the use of characterized, labeled standards run just before a sample electropherogram is obtained on the laser-based system. We must also note that the protein providing the least accurate molecular mass estimate is that of BSA. BSA is known to possess an anomalous degree of SDS-binding (0.9 g/g protein vs. 1.4 g/g protein [30]). Thus, this atypical protein provides overly pessimistic data regarding the accuracy of the molecular mass estimation process. Without considering BSA, a mean % error of 3.3% over all trials and proteins is obtained.

4. Conclusions

The use of laser-induced fluorescence detection in SDS-CGE separations of proteins can provide a substantial improvement in detection capability relative to conventional slab gel systems and CGE systems utilizing UV absorption detection. No significant sacrifice in separation efficiency or in accuracy of molecular mass estimates is required. Further studies investigating the quantitative aspects of the technique and the ability of the technique to separate and identify analytes in more complex matrices will be required to fully develop the technique's potential.

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