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Rapid analysis of covalently and non-covalently fluorophore-labeled proteins using ultra-thin-layer sodium dodecylsulfate gel electrophoresis

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Abstract

Gel electrophoresis is one of the most frequently used tools for the separation of complex biopolymer mixtures. In recent years, there has been considerable activity in the separation and characterization of protein molecules by sodium dodecylsulfate (SDS) gel electrophoresis with particular interest in using this technique to separate on the basis of size and to estimate molecular mass and protein purity. Although the method is informative, it is cumbersome, time consuming and lacks automation. In this paper we report an automated, high-performance SDS gel electrophoresis system that is based on electric-field-mediated separation of SDS–protein complexes using an ultra-thin-layer platform. The integrated fiber optic bundle-based scanning laser-induced fluorescence detection technology readily provided high sensitivity, real-time detection of the migrating solute molecules. Rapid separations of covalently and non-covalently labeled proteins were demonstrated in the molecular mass range 14 000 to 205 000 in less than 9 and 16 min, respectively. Excellent quantitation and lane-to-lane migration time reproducibility were found for all the solute components using the multilane separation platform. The limit of detection was found to be 1.5–3 ng/band for both labeling methods, with excellent linearity over a six times serial double-dilution range. Molecular mass calibration plots were compared for both covalently and non-covalently labeled proteins. A linear relationship was found between the molecular mass and electrophoretic mobility in the case of covalently labeled samples, while a non-linear relationship was revealed for the non-covalently labeled samples.

Keywords: Fluorescence detection; Detection, electrophoresis; Derivatization, electrophoresis; Sodium dodecylsulfate gel electrophoresis; Ultra-thin-layer sodium dodecylsulfate gel electrophoresis; Proteins

1. Introduction

Electrophoresis in polyacrylamide or other gels containing ionic detergents, such as sodium dodecylsulfate (SDS), has proven to be a powerful tool for size separation of protein molecules, estimation of their molecular mass and assessment of their purity [1]. In the presence of thiol reducing agents the disulfide bridges are cleaved and the detergent (SDS) dissociates proteins into their constituent subunits and binds to the polypeptide chains in a way that similar charge-to-mass ratios of the resulting complexes are obtained [2]. In gel electrophoresis,

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the polymer network structure creates a sieving media so that separation can be performed based on the size of the solute molecules [3]. In sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins, a linear relationship is observed when the logarithm of the molecular mass of standard polypeptide chains is plotted against their electrophoretic mobilities [4]. The method is reliable and reproducible in the molecular mass range of 10 000 to several hundreds of thousands, generally within 5–10% of those obtained by mass spectrometry (MS). Prior to sample application to the separation gel, the proteins are denatured by heat treatment (100°C/5 min) in the presence of β-mercaptoethanol or dithiothreitol. Rod or slab gels of a variety of crosslinked polyacrylamides have conventionally been used as sieving matrices to separate the SDS–polypeptide complexes according to their size [5].

In the past several years gel electrophoresis has been successfully utilized in capillary dimensions, mainly for size separation and purity assessment of synthetic oligonucleotide probes and primers [6] and polymerase chain reaction (PCR) products [7]. Early attempts to apply capillary gel electrophoresis for protein separations by SDS–PAGE involved highly concentrated crosslinked polyacrylamide as sieving medium [8] using UV detection. Later, lower concentration crosslinked polyacrylamide [9] and linear (non-crosslinked) polyacrylamide was also employed in capillaries as sieving material [10]. Also, non-polyacrylamide-type-low-viscosity polymer solutions such as dextrans or polyethylene oxides with excellent UV transparency at 214 nm were utilized to obtain size separations [11]. Recently, covalent fluorophore labeling by various high-sensitivity fluorescent dyes was introduced to enhance detectability of the proteins separated by SDS capillary gel electrophoresis. Such pre-separation covalent fluorophore labeling was reported earlier by Craig et al. [12] and more recently by Hunt and Nasabeh [13], who used 5-TAMRA.SE as a very sensitive approach for monitoring the consistency of biotechnology products, requiring several hours of derivatization time. A novel approach using non-covalent fluorophore labeling was demonstrated a short while ago in capillary dimensions [14,15].

Ultra-thin-layer gel electrophoresis was introduced in the late 1990s as an efficient separation technique for the analysis of nucleic acids [16–19]. Its applicability was also demonstrated in automated ultra-thin-layer SDS gel electrophoresis of proteins [20], which is a unique combination of SDS–PAGE (high-throughput multilane format) and capillary SDS gel electrophoresis (high-performance separations). In this paper we report the rapid analysis of covalently and non-covalently labeled proteins with molecular masses ranging from 14 000 to 205 000 in less than 16 min in capillary dimensions using a multilane separation format. Lane-to-lane migration time and quantitation reproducibility, as well as the calibration plots for molecular mass determination, were examined with both covalently and non-covalently labeled samples.

2. Materials and methods

2.1. Instrumentation

The automated ultra-thin-layer (190 μm) gel electrophoresis system was equipped with real-time laser-induced fluorescence detection [21]. This particular setup consisted of a fiber optic bundle-based scanning illumination/detection system, using a 532 nm frequency doubled green Nd–YAG laser excitation source and avalanche photodiode detection (APD) with a 585±25 nm wide band interference filter. A lens set scanned across the multilane separation platform by means of a high-speed translation stage and collected the emitted fluorescent light [22]. The analog signal from APD was digitized in a micro-controller and acquired by a personal computer. Integrated fluoresence values of the separated peaks were obtained using the Gel-Pro Analyzer software package form Media Cybernetics (Silver Spring, MD, USA).

The-ultra-thin layer separation platform was a 100 mm×75 mm×0.19 mm float glass cartridge with built-in 15 ml plastic buffer reservoirs at both ends. The inside surfaces of the cassettes were coated by linear polyacrylamide [23] in order to minimize electroosmotic flow (EOF) at the separation pH of

8.4. Effective separation lengths of 1.5 and 3 cm (measured from the loading well to the scanning detection zone) were used, unless specified otherwise. Submicroliter sample volumes (0.2–0.5 μl) were injected into pre-formed loading wells (2.5× 4×0.19 mm).

2.2. Chemicals

In all the experiments, 1% low electroendosmosis (EEO; $-m_r = 0.06$) Amresco's Agarose-III (Solon, OH, USA) and 2% linear polyacrylamide (M_r 700 000–1 000 000) (Polysciences, Warrington, PA, USA) were dissolved in 50 m*M* Tris, 50 m*M* TAPS [*N* - tris(hydroxymethyl)methyl - 4 - aminobutanesulfonic acid] buffer (pH 8.4), containing 0.05% SDS (referred to as TTS). Tris, TAPS, SDS and mercaptoethanol were obtained from ICN (Costa Mesa, CA, USA), all of electrophoresis grade. The covalently labeled protein molecular mass marker set (fluorescein-5-isothiocyanate, FITC) containing trypsin inhibitor (M_r 20 100), carbonic anhydrase (M_r 29 000), alcohol dehydrogenase (M_r 39 000), bovine serum albumin (M_r 66 000), β-galactosidase (M_r 116 000) and myosin (M_r 205 000) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.25 ml double-deionized water in a final total protein concentration of 1 mg/ml. The Bromophenol Blue, which interfered with the fluorescent signal of the FITC label on the proteins, was removed from the samples by using TTS buffer equilibrated Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA).

The non-covalent fluorescent staining dye Sypro Red (SR) was purchased from Molecular Probes (Eugene, OR, USA) in 5000× concentration. Standard proteins α-lactalbumin (M_r 14 200), carbonic anhydrase (M_r 29 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), phosphorylase B (M_r 94 700) and β-galactosidase (M_r 116 000) were obtained from Sigma and dissolved in a final concentration of 0.5 mg/ml in 50 m*M* Tris, 50 m*M* TAPS, 0.05% SDS and 10% sucrose-containing buffer, and boiled in a water bath for 5 min after the addition of 2.5% 2-mercaptoethanol. The samples were then cooled to room temperature and were non-covalently labeled by complexation with the fluorescence staining dye Sypro Red ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 625$ nm), which was added to the samples in a final concentration of 1× immediately prior to loading.

2.3. Procedures

The appropriate amount of agarose powder was

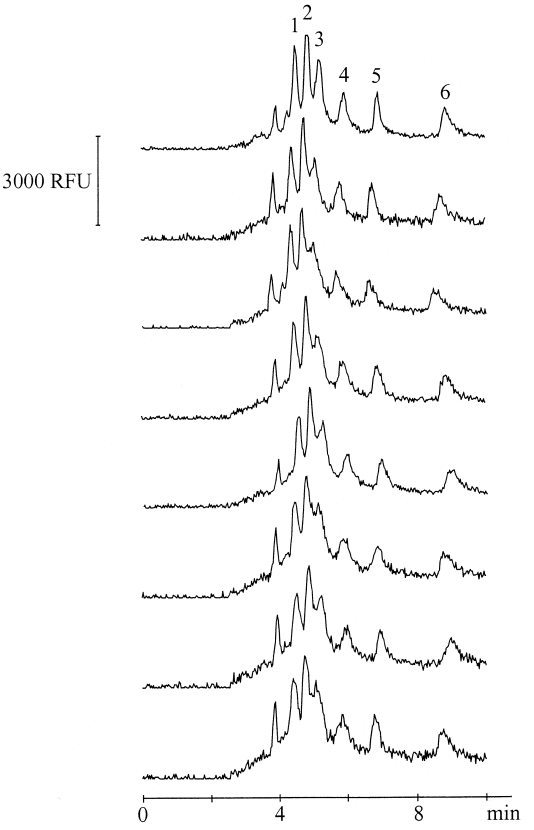

Fig. 1. Ultra-thin-layer SDS gel electrophoresis separation of covalently (FITC) labeled protein molecular mass markers using laser-induced fluorescence scanning detection on a multilane platform. Peaks: 1=trypsin inhibitor, M_r 20 100; 2=carbonic anhydrase, M_r 29 000; 3=alcohol dehydrogenase, M_r 39 000; 4=bovine serum albumin, M_r 66 000; 5=β-galactosidase, M_r 116 000; 6=myosin, M_r 205 000. Separation conditions: gel, 1% agarose, 2% LPA (M_r 700 000–1 000 000) in 50 m*M* Tris, 50 m*M* TAPS, 0.05% SDS (pH 8.4); separation buffer, 50 m*M* Tris, 50 m*M* TAPS, 0.05% SDS (pH 8.4); separation voltage, 900 V (14–16 mA); effective separation length, 1.5 cm; gel thickness, 190 μm; temperature, 25°C; sample loading, 0.5 μl into 2.5×4× 0.19 mm injection wells.

suspended in TTS buffer, boiled repetitively in a microwave oven until clear. Linear polyacrylamide (LPA, M_r 700 000–1 000 000) was added to the melted agarose in 2% final concentration. The composite matrix was then kept at 60°C for 10 min before use. The preheated separation cassettes (45–50°C) were filled with the melted composite separation matrix and after several minutes of cooling/solidification the gel-filled cassette was ready for use. After each run, the used gels were replaced in the ultra-thin-layer separation cassette by simply pumping through fresh, melted composite matrix. An aluminum heat sink was employed to hold the ultra-thin-layer separation cartridge in the horizontal position and to dissipate extra Joule heat. The temperature of the heat sink was regulated by a thermostated air bath with a precision of ±1°C. The actual separation temperature was measured at the middle of the heat sink by a thermocouple.

3. Results and discussion

3.1. Separation of covalently labeled proteins

Fig. 1 shows simultaneous (multilane) separation of the six-protein test mixture containing trypsin inhibitor, M_r 20 100 (1); carbonic anhydrase, M_r 29 000 (2); alcohol dehydrogenase, M_r 39 000 (3); bovine serum albumin, M_r 66 000 (4); β-galactosidase, M_r 116 000 (5) and myosin, M_r 205 000 (6) using automated ultra-thin-layer SDS gel electrophoresis. All proteins in the mixture were covalently labeled by FITC [24]. The effective separation distance of the ultra-thin-layer platform was 1.5 cm (from the injection point to the detection zone) and all the proteins were detected within 8.5 min. Excellent quantitation and lane-to-lane migration time reproducibility was obtained, as depicted in Table 1 (covalent columns). The average RSDs for migration time and quantitation were 0.52 and 6.06%, respectively. The limit of detection (LOD) was determined by injecting a series of double dilutions (96, 48, 24, 12, 6 and 3 ng/band) onto the multilane separation platform and the resulting detection signal (integrated fluoresence) was plotted (Fig. 2). As can be seen, good detection linearity ($r^2 = 0.9995$) was also obtained. The detection limit was found to be 3 ng/band for the FITC-labeled standard proteins.

3.2. Separation of non-covalently labeled proteins

Fig. 3 depicts the multilane separation of the five-protein test mixture of α-lactalbumin, M_r 14 200 (1); carbonic anhydrase, M_r 29 000 (2); ovalbumin, M_r 44 000 (3); bovine serum albumin, M_r 66 000 (4) and β-galactosidase, M_r 116 000 (5) on automated ultra-thin-layer SDS gel electrophoresis. All proteins were labeled by the non-covalent staining dye Sypro Red (1× concentration in the sample) immediately prior to the injection process [25]. In this instance the effective separation length of the multilane platform was 3.0 cm which is reflected in the longer

Table 1
Lane-to-lane migration time and quantitation reproducibility (RSD) of covalently and non-covalently labeled standard proteins, analyzed in Figs. 1 and 3 (t_M, migration time)

Protein	M_r	$RSD_{covalent}$ (%)		$RSD_{non\text{-}covalent}$ (%)	
		t_M	Integrated fluoresence	t_M	Integrated fluoresence
α-Lactalbumin	14 200			0.75674	5.857
Trypsin inhibitor	20 100	0.356	10.6174		
Carbonic anhydrase	29 000	0.526	3.6774	0.61293	4.570
Alcohol dehydrogenase	39 000	0.732	5.6227		
Ovalbumin	45 000			0.74053	6.370
Bovine serum albumin	66 000	0.521	4.6891	0.5952	5.871
β-Galactosidase	116 000	0.561	6.5410	0.6806	9.077
Myosin	205 000	0.427	5.2083		
Average		0.520	6.0593	0.6772	6.349

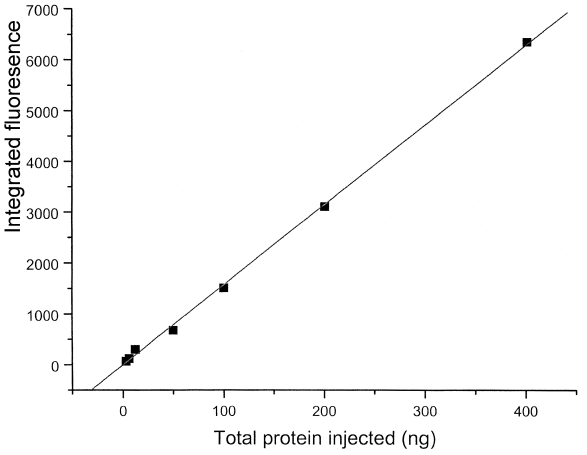

Fig. 2. Detection linearity of serially diluted covalently (FITC) labeled protein molecular mass markers. Injected amounts: 96, 48, 24, 12, 6 and 3 ng/band.

migration times of up to 16 min. Baseline separation was obtained for all components. The lane-to-lane reproducibility of the migration times of the separated proteins and their quantitation is listed in Table 1 (non-covalent columns) which shows an average migration time RSD = 0.68% and quantitation RSD = 6.35%. Similar to the covalently (FITC) labeled proteins, good detection linearity (Fig. 4, $r^2 = 0.9976$) was observed by plotting the detection signal as a function of the injected amount (48, 24, 12, 6, 3 and 1.5 ng/band). The limit of detection was 1.5 ng/band, comparable to the sensitivity level of the commonly used silver staining technique [26].

3.3. Molecular mass calibration plots

Fig. 5 compares the separations of the two differently labeled protein standard mixtures and the molecular mass evaluation of phosphorylase B using ultra-thin-layer SDS gel electrophoresis. Fig. 5A depicts the separation of the non-covalently (SR) stained five-protein test mixture of α-lactalbumin (ALA), carbonic anhydrase (CBA), ovalbumin (OVA), bovine serum albumin (BSA) and β-galactosidase (BGA). The trace in Fig. 5B corresponds the molecular mass analysis of the non-covalently (SR) labeled phosphorylase B. Fig. 5C shows the separation of the covalently (FITC) labeled five-protein test mixture of tripsin inhibitor (TRI), carbonic

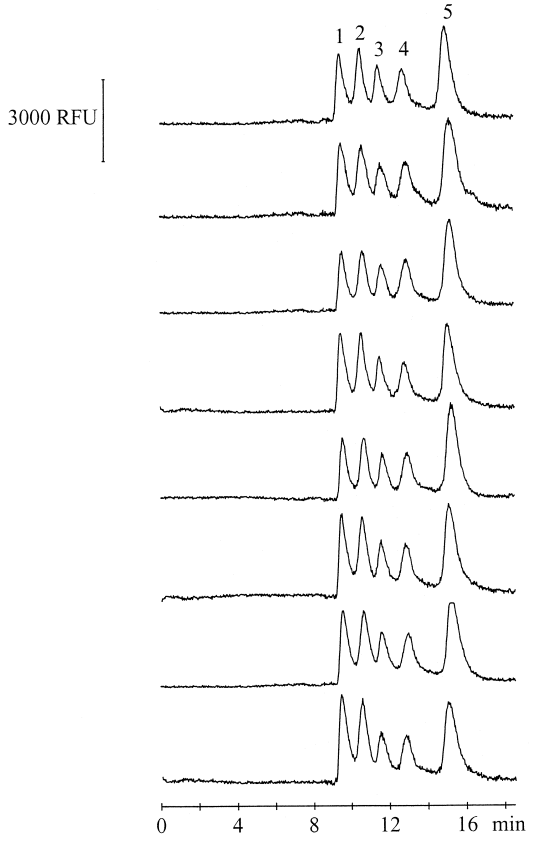

Fig. 3. Separation of non-covalently labeled protein molecular mass markers by multilane ultra-thin-layer SDS gel electrophoresis using the laser-induced fluorescence scanning detection system. Peaks: 1 = α-lactalbumin, M_r 14 200; 2 = carbonic anhydrase, M_r 29 000; 3 = ovalbumin, M_r 44 000; 4 = bovine serum albumin, M_r 66 000; 5 = β-galactosidase, M_r 116 000. Separation conditions: gel, 1% agarose, 2% linear polyacrylamide (LPA, M_r 700 000–1 000 000) in 50 m*M* Tris, 50 m*M* TAPS, 0.05% SDS (pH 8.4); separation buffer, 50 m*M* Tris, 50 m*M* TAPS, 0.05% SDS (pH 8.4); separation voltage, 420 V; current, 5 mA; gel thickness, 190 μm; effective separation length, 3.0 cm; temperature, 25°C; sample loading, 0.2 μl into 2.5×4×0.19 mm injection wells. Sample buffer contained 0.05% SDS and 1× Sypro Red.

anhydrase (CBA), alcohol dehydrogenase (ADH), bovine serum albumin (BSA) and β-galactosidase (BGA). Note that non-covalent labeling of the sample and standard proteins took place immediately prior to the loading process. Detection of the migrating SDS–protein complexes was accomplished in real time during the electrophoresis separation process. The actual separation distance in this experi-

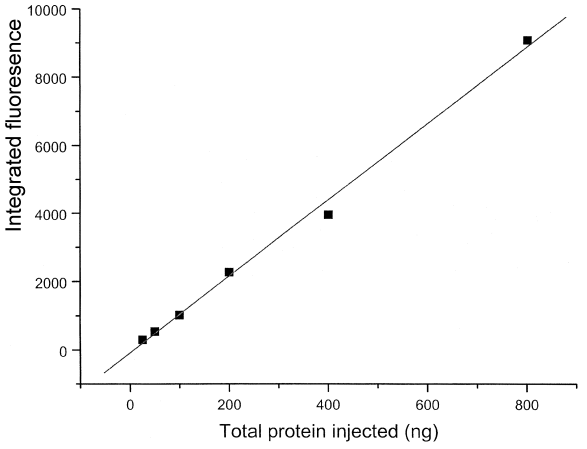

Fig. 4. Detection linearity of serially diluted non-covalently (SR) labeled protein molecular mass markers. Injected amounts: 48, 24, 12, 6, 3 and 1.5 ng/band.

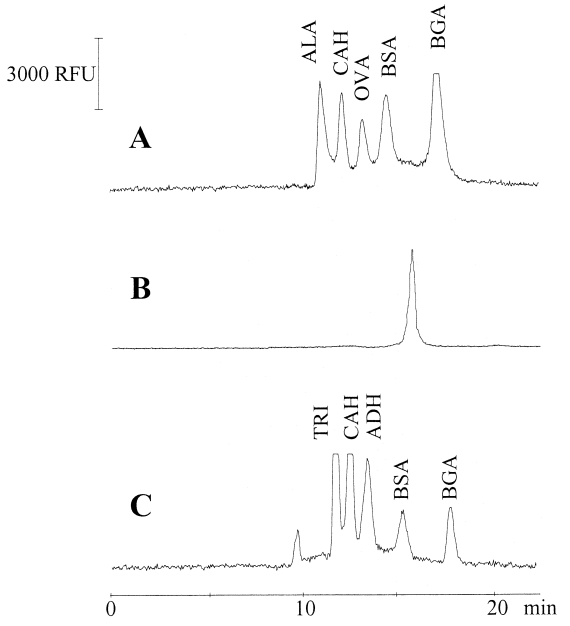

Fig. 5. Molecular mass analysis of phosphorylase B (B) and the separation of the non-covalently labeled protein markers (A; ALA, α-lactalbumin; CBA, carbonic anhydrase; OVA, ovalbumin; BSA, bovine serum albumin; BGA, β-galactosidase) and the covalently labeled protein markers (C; TRI, trypsin inhibitor; CAH, carbonic anhydrase; ADH, alcohol dehydrogenase; BSA, bovine serum albumin; BGA, β-galactosidase). Effective separation length, 3.5 cm; other conditions were the same as in Fig. 3; sample buffer for the non-covalently labeled samples contained 0.05% SDS and 1× Sypro Red.

ment was 3.5 cm, in order to obtain high resolution and a rapid analysis time.

Standard curves for molecular mass estimation were constructed by plotting the logarithmic molecular masses of the corresponding proteins in the standard test mixtures against their electrophoretic mobilities (Fig. 6). For non-covalent staining, the best fit was obtained by applying a second-order polynomial function, exhibiting an extremely high confidence level for this relationship ($r^2 = 0.9999$, solid line). The slight curvature of the calibration plot of the non-covalently stained proteins is probably caused by dynamic complexation with the negatively charged staining dye, which causes a slight increase in the overall charge of the resulting complex and in its electrophoretic velocity. This is in contrast to the linear relationship found between the logarithmic molecular mass and electrophoretic mobility for the covalently labeled proteins ($r^2 = 0.9995$, dashed line). We estimated the molecular mass of phosphorylase B (Fig. 5B) to be 97.250 based on the calibration plot derived from the non-covalently (SR) labeled protein standards (solid line). This value represents a 0.15% error compared to the literature value of M_r 97 400 [27]. Based on the calibration plot derived from the covalently (FITC) labeled protein standards (dashed line) the molecular mass of the same protein was estimated as 89 750, in this instance, representing a 7.8% error compared to the literature value.

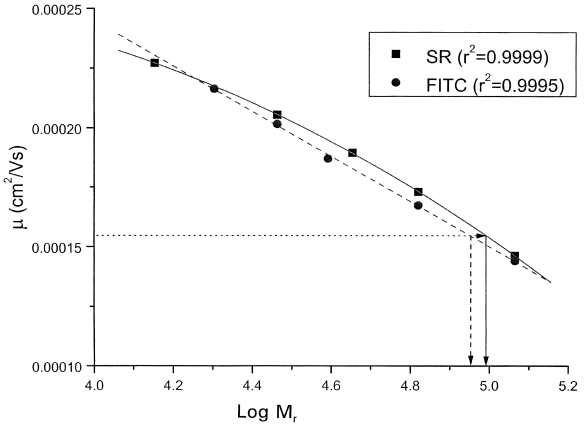

Fig. 6. Standard curves of electrophoretic mobility vs. logarithmic molecular mass (derived from the data of Fig. 5A and C) for molecular mass estimation using covalently (- - -) and non-covalently (———) labeled protein standards.

4. Conclusion

In this paper we have demonstrated the rapid analysis of covalently (FITC) and non-covalently (SR) labeled proteins in ultra-thin-layer format using a multilane separation platform and laser-induced scanning fluorescence detection. Fast separation of all standard proteins in the test mixtures was demonstrated in the molecular mass range 14 000 to 205 000. The lane-to-lane migration time reproducibilities were found to be RSD = 0.52% for the covalently and RSD = 0.68% for the non-covalently labeled protein standards. Eight samples were analyzed in parallel on the multilane separation platform, but it is important to note that the system is capable of analyzing up to 32 individual lanes simultaneously. The LOD was found to be in the low nanogram regime, 1.5 and 3 ng/band for the non-covalently and covalently labeled proteins, respectively. Excellent detection linearity was exhibited over the examined six-times serial double-dilution range for both the covalently and non-covalently labeled samples. Note that, due to its speed and simplicity, the instant, non-covalent fluorophore labeling of SDS–protein complexes reported in this paper can open up new horizons in the emerging field of proteomics. Molecular mass calibration plots were compared for the differently labeled protein standards. A linear relationship was found between the molecular mass and electrophoretic mobility in the analysis of the covalently labeled samples and a second-order polynomial relationship was observed using non-covalent fluorophore labeling. This latter, non-linear relationship was probably caused by the charge-to-mass ratio shift due to the complexation with the negatively charged dye molecules. Molecular mass estimation with higher accuracy (0.15%) was obtained when the non-covalent labeling method was used for both the standard and sample proteins.

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