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Reaction of fluorogenic reagents with proteins III. Spectroscopic and electrophoretic behavior of proteins labeled with Chromeo P503

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Abstract

The spectroscopic and electrophoretic properties of proteins labeled with Chromeo P503 were investigated. Its photobleaching characteristics were determined by continually infusing Chromeo P503-labeled α-lactalbumin into a sheath-flow cuvette and monitored fluorescence as a function of laser power. The labeled protein is relatively photo-labile with an optimum excitation power of about 2 mW. The unreacted reagent is weakly fluorescent but present at much higher concentration than the labeled protein. The unreacted reagent undergoes photobleaching at a laser power more than an order of magnitude higher than the labeled protein. One-dimensional capillary electrophoresis analysis of Chromeo P503-labeled α-lactalbumin produced concentration detection limits (3σ) of 12 pM and mass detection limits of 0.7 zmol, but with modest theoretical plate counts of 17 000. The reagent was employed for the two-dimensional capillary electrophoresis analysis of a homogenate prepared from a Barrett's esophagus cell line; the separation quality is similar to that produced by 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), a more commonly used reagent.

Keywords

Chromeo P503; fluorogenic reagents; protein labeling; capillary electrophoresis; laser-induced fluorescence

1. Introduction

In the previous two papers in this journal, we reported the reaction kinetics of proteins with the fluorogenic reagents Chromeo P465 and P503, and some electrophoretic and spectroscopic properties of proteins labeled with Chromeo P465 [1–2]. That reagent generated an unacceptable background signal. In this manuscript we investigate the related reagent Chromeo P503, which has much better electrophoretic properties. This reagent appears to be identical to Py1, a reagent introduced by Wolfbeis [3].

This group and others have employed the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) for analysis of complex protein samples [4–12]. That reagent is non-

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fluorescent until it reacts with a primary amine, it can be excited with the 473 nm solid-state laser, and produces yoctomole detection limits when used to label proteins. Any new reagent used for ultrasensitive protein analysis must compete with the performance of FQ.

2. Experimental

2.1 Chemicals and reagents

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions were made with distilled deionized water (Barnstead Nanopure, Boston, MA, USA) and vacuum filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA). 3-(2-furoyl)quinoline-2-carboxaldeyde (FQ) was purchased from Molecular Probes (Eugene, OR, USA). Chromeo P503 was a kind gift from Professor Otto Wolfbeis of the University of Regensburg; additional material was purchased from Chromeo (Regensburg, Germany). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The dynamic coating reagent UltraTrol LN was purchased from Target Discovery (Palo Alto, CA, USA).

2.2 CP18821 cell line tissue culture

An immortalized cell line of premalignant epithelial esophageal cells (CP18821) was a kind gift from Peter Rabinovitch of the Department of Pathology, University of Washington. Cells were cultured with keratinocyte-serum free medium supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (rEGF) (Invitrogen, Carlsbad, CA, USA). After a brief exposure to 0.25% trypsin, cells were collected in phosphate-buffered saline (PBS) and rinsed eight times in PBS to remove the media from the cell suspension. Cells were lysed in 1% sodium dodecyl sulfate (SDS) and aliquots were stored at −80 °C.

2.3 Fluorescent labeling

Dry aliquots containing 100 nmol of FQ or of Chromeo P503 were stored in the dark at −20° C until use.

Solutions of α-lactalbumin (90 μM) and of an 8 standard protein ladder (α-lactalbumin bovine milk (14.2 kDa), trypsin inhibitor soybean (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase *E. coli* (116 kDa), and myosin rabbit muscle (205 kDa)) (Sigma) were prepared in 1% SDS and stored at −80°C until use. All samples were denatured at 95°C for 5 min before labeling.

To label with FQ, 5 μL of denatured sample was mixed with 5 μL of 5 mM KCN in a tube with 100 nmol of lyophilized FQ. This mixture was heated at 65°C for 5 min. FQ-labeled α-lactalbumin was diluted with 100 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES), 100 mM Tris, 15 mM SDS to a final concentration of 0.45 nM. FQ-labeled protein ladder and cellular homogenate was diluted with 90 μL of 100 mM CHES, 100 mM Tris, 15 mM SDS. Samples were stored on ice until analysis.

To label with P503, 100 nmol of lyophilized Chromeo P503 was dissolved in 100 μL methanol. 10 μL of denatured sample and 10 μL of dissolved Chromeo P503 were added to 10 mM borate and heated at 50°C for 10 min. Chromeo P503 -labeled α-lactalbumin was diluted with 100 mM CHES, 100 mM Tris, 15 mM SDS to a final concentration of 0.25 nM. Chromeo P503-labeled protein size standards and cellular homogenate were diluted with 400 μL of 100 mM CHES, 100 mM Tris, 15 mM SDS. Samples were stored on ice until analysis.

2.3 One-dimensional CE

Separation was performed in a 25-cm long, 30-μm I.D. fused silica capillary. Between runs, the capillary was rinsed for 5 min at 5 psi with 0.5 M NaOH, water, UltraTrol_LN and separation buffer. Separation was performed at -17 kV. Sample was injected for 1 s at -1 kV. Separation was performed by capillary sieving electrophoresis (CSE) in a 100 mM CHES, 100 mM Tris, 3.5 mM SDS, 5% Dextran (500 kDa) buffer.

2.4 Two-dimensional CE

We have previously described the instrumentation for two-dimensional capillary electrophoresis (2D-CE) [12–14]. In brief, two separation capillaries are connected at a Plexiglas interface. Analytes are separated in the first-dimension according to their size by CSE, and in the second dimension by micellar electrokinetic capillary chromatography (MECC). In this paper, both fused-silica capillaries were 23 cm long, 31 μm inner diameter, 147 μm outer diameter and were coated with the UltraTrol LN dynamic coating.

Between runs, separation capillaries and the interface were rinsed for 5 minutes each at 5 psi with 0.5 M NaOH, water, Ultratrol_LN and separation buffer. The first dimension separation buffer contained 100 mM CHES, 100 mM Tris, 3.5 mM SDS, 5% Dextran (500 kDa). The second dimension separation and sheath buffers contained 100 mM CHES, 100 mM Tris, 15 mM SDS.

Using a multipurpose injection block [15], sample was electrokinetically injected by the application of 5 kV across the first capillary for 2 s. To perform capillary sieving electrophoresis, a field strength of 740 V/cm was applied across the first capillary. After 100 seconds, the fastest moving components had migrated near the end of the first capillary. Analytes were then transferred sequentially across the interface into the second capillary by the application of 10 kV for 1 s. Sample was separated in the second dimension by applying 17 kV to both electrodes for 13 s; by applying the same potential to the two electrodes, 0 kV net potential was applied across the first capillary, holding its contents stationary. The second capillary experienced a field strength of 740 V/cm to drive a MECC separation. Potentials were applied in negative polarity such that the anode was at the detector. These transfer and MECC separation cycles were repeated approximately 250 times until all components from the first dimension had been analyzed.

Analytes were detected using a postcolumn sheath flow cuvette [12,16–17]. Fluorescence was excited by a 473 nm solid-state diode laser (Lasermate Group, Ponoma, CA, USA), collected with an M-PLAN 60x, 0.7 NA microscope objective (Universe Kogaku, Oyster Bay, NY USA), and filtered with a 580LP long-pass filter (Omega Optical, Brattleboro, VT, USA). Laser intensity was adjusted with a linear polarizer placed in the beam path. Light was detected by an avalanche photodiode single-photon counting module (EG&G Canada, Vaudreuil, Canada). Voltage programming and fluorescence measurement were controlled by LabView software. Data were processed with Matlab. The signal was corrected to account for the dead-time response of the photodetector [18].

3. Results and discussion

3.1 Photobleaching

We first report the photobleaching characteristics of Chromeo P503-labeled α-lactalbumin. In these experiments, we continually infused the labeled protein, the reagent blank, and the buffer blank into our sheath-flow cuvette detector, and measured the signal as a function of laser power; the data are summarized in Figure 1 and Figure 2.

Photobleaching removes fluorescent compounds from the sample, decreasing the fluorescence signal. Photobleaching can be modeled by analogy to three-level optical saturation

$$I(P)=\frac{I_{\max}P}{P_{\text{PhotoBleach}}+P}$$

where I is the observed fluorescence intensity, P is laser power, I_{max} is the asymptotic signal observed at very high laser powers, and $P_{PhotoBleach}$ is the laser power required to reach half the photobleaching limit.

The background generated by the buffer system was weak and showed a slight deviation from linearity across the laser power range available; a non-linear regression analysis yielded $P_{PhotoBleach}$ = 80 mW. This behavior suggests that the buffer is contaminated at the femtomolar level with fluorescent compounds, such as fluorescein, which is ubiquitous in the environment at these very low levels.

We continually infused the derivatizing reagent to characterize its saturation properties. The reagent blank was roughly twice that generated by the buffer blank; although the unreacted dye has low molar absorptivity at 473 nm, it is present at high concentration and generates sufficient fluorescence to be detectable in our system. We subtracted the signal from the buffer blank and plotted the saturation behavior of the reagent itself in figure 1; $P_{PhotoBleach}$ = 70 mW, which is similar to that of the buffer blank.

Finally, we continually infused a dilute solution of Py-labeled α-lactalbumin. The background signal due to unreacted reagent was subtracted from the data before plotting, Figure 1. $P_{PhotoBleach}$ = 2 mW, which is roughly half the value observed for FQ-labeled proteins.

3.2 Photobleaching

Detection limit is defined as that concentration of protein that generates a signal three times larger than the noise in the background. For continual infusion experiments, the background signal is generated by unreacted reagent. Figure 2 presents a signal-to-noise plot as a function of laser power for Chromeo P503-labeled α-lactalbumin. The fluorescence signal was generated from continual infusion of the protein, corrected for the background from unreacted derivatization reagent. The noise is the standard deviation of the signal generated by infusing the reagent blank; we treated this data with a median filter and Gaussian filter to reduce the effects of light scatter from particulates in solution. The signal-to-noise plot maximizes at about 5 mW, slightly higher than the photobleaching parameter. The maximum signal-to-noise ratio was about 1,500, which corresponds to a 3σ detection limit of 20 pM Chromeo P503-labeled α-lactalbumin that is continuously infused.

3.3 Capillary sieving analysis of labeled protein

To compare the electrophoretic performance of FQ and Chromeo P503, α-lactalbumin was labeled with each dye and then diluted to 0.45 nM and 0.25 nM respectively. An aliquot of labeled protein was injected and characterized by CSE (Figure 3). The data consists of a peak generated by labeled protein peak and several smaller peaks, presumably generated by impurities in the protein sample. The data were treated with a 5-point median filter to eliminate noise spikes and then smoothed by convolution with a 0.8-s wide Gaussian function. Signal was defined as peak height above the baseline while noise was estimated as the standard deviation of the baseline between 4 and 7 minutes. The 3σ limit of detection for FQ-labeled protein was 0.9 zmol; the 3σ limit of detection for Chromeo P503-labeled protein was 0.7 zmol. Separation efficiencies for the smoothed electropherograms of the labeled protein were ~15 000 plates.

It is interesting that the Chromeo P503 and FQ-labeled proteins have essentially the same mobility in this buffer. The FQ-labeled products are neutral while the Chromeo P503 products are cationic, which should result in a large difference in their free-solution mobility. However, the separations are performed with a buffer that contains the anionic surfactant SDS, which ion-pairs with cationic groups. In the case of FQ, the ion-pair helps reduce band broadening due to incomplete labeling of lysine residues. In the case of Chromeo P503, the ion pair is generated with both the labeled and unlabeled lysine residues. In both cases, lysine residues are neutralized, eliminating differences in mobility due to differences in charge state of the labeled products.

3.4 Two-dimensional capillary electrophoresis

Chromeo P503 labeled proteins behave well in two-dimensional capillary electrophoresis. We first separated a mixture of standard proteins (Figure 4). The spots are quite narrow in the sieving dimension but rather broad in the MECC dimension; such behavior is commonly observed with standard proteins. Curiously, spots 3 and 7 are not aligned along the MECC dimension but instead fall on a diagonal. This behavior suggests that the proteins are not pure —higher molecular weight components tend to have longer migration time in the MECC dimension. The highest molecular weight proteins in our standards kit (β-galactosidase *E. coli* and myosin rabbit muscle) were not detected, presumably because they had either degraded during storage or because they generated very broad peaks that faded into the background.

We then analyzed a BE cellular homogenate, which is a complex sample that contains amino acids, biogenic amines, peptides, and proteins. Figure 5 presents the data generated when the sample was labeled with Chromeo P503 and Figure 6 presents the data generated when the same sample was labeled with FQ. Both electropherograms show high efficiency separation in the CSE dimension but relatively poor separation efficiency in the MECC dimension.

4. Conclusions

Proteins labeled with FQ and Chromeo P503 have similar electrophoretic properties and detection limits. Chromeo P503 reacts with proteins more quickly than FQ [1], but the unreacted reagent produces a modest fluorescence signal. Chromeo P503 appears to be a useful replacement for FQ in many protein analyses.

Acknowledgements

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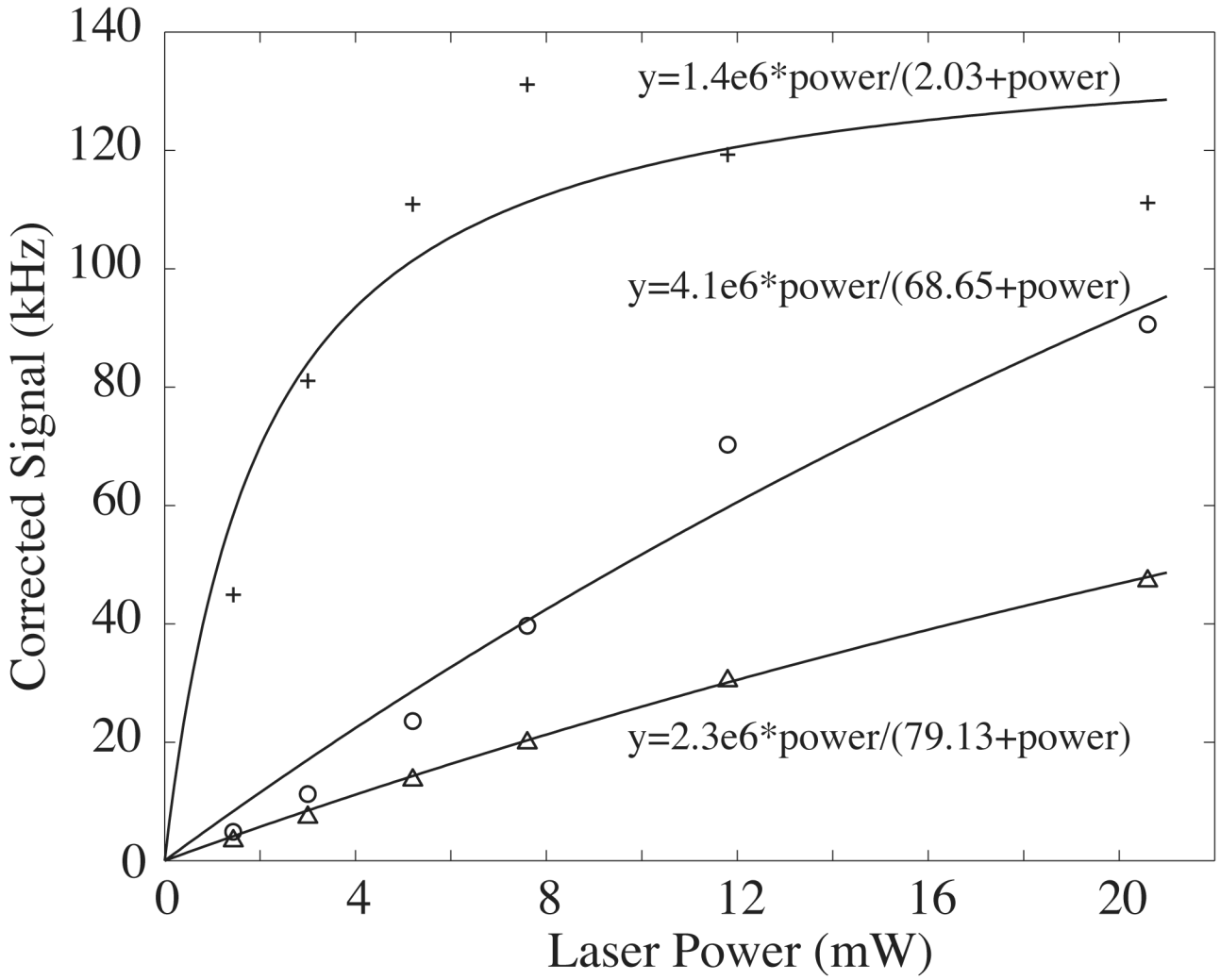

Figure 1.
Photobleaching curves for buffer (triangles), Chromeo P503 reagent blank (circles), and 9 nM Py-503 labeled α-lactalbumin (plus signs). The smooth curves are the least squares fit to three-level optical saturation curve, where power is laser power in mW.

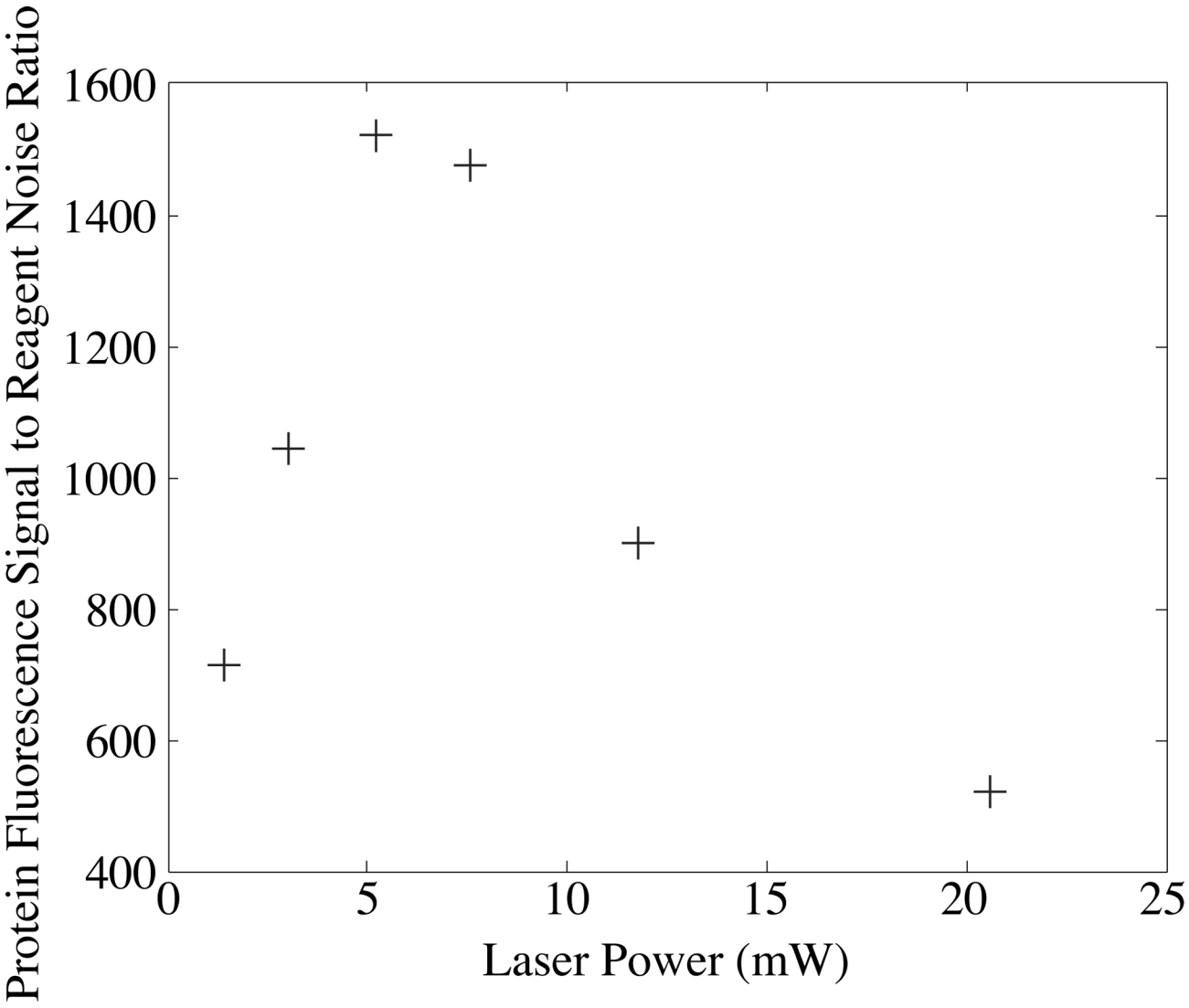

Figure 2.

Signal-to-noise ratio optimization versus laser power for a 9 nM solution of Py-503 labeled α-lactalbumin. Signal is the fluorescence intensity above the reagent blank, and noise is the standard deviation of the reagent blank. Data are first treated with a 5-point median filter to remove noise and then convoluted with a 0.5-s wide Gaussian filter.

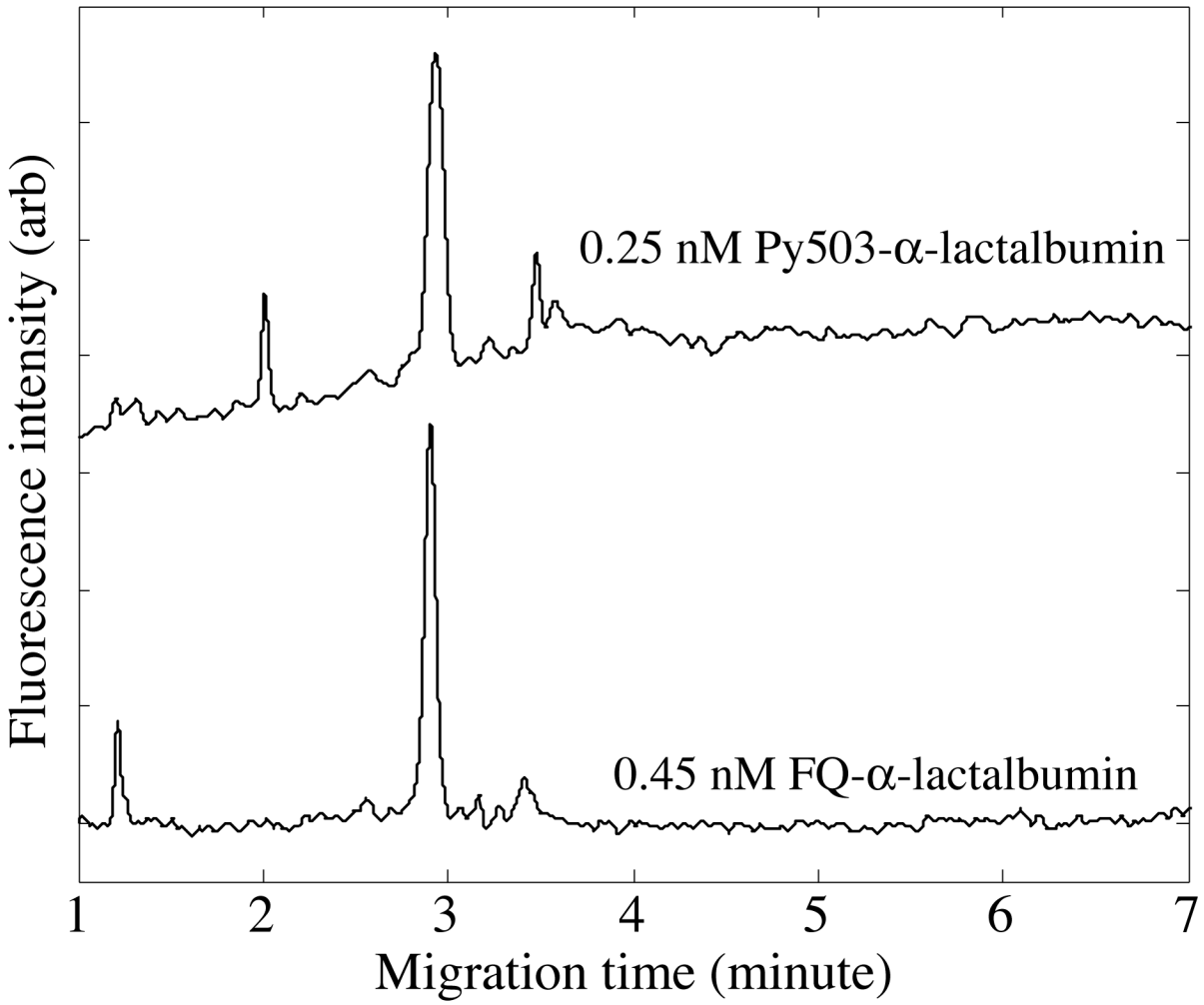

Figure 3.
CSE analysis of solutions of 0.25 nM Chromeo P503 α-lactalbumin and 0.45 nM FQ α-lactalbumin. Samples were injected for 1 s at 1 kV and separated at 17 kV. Detection limits are ~0.9 zmol injected onto the capillary for both compounds.

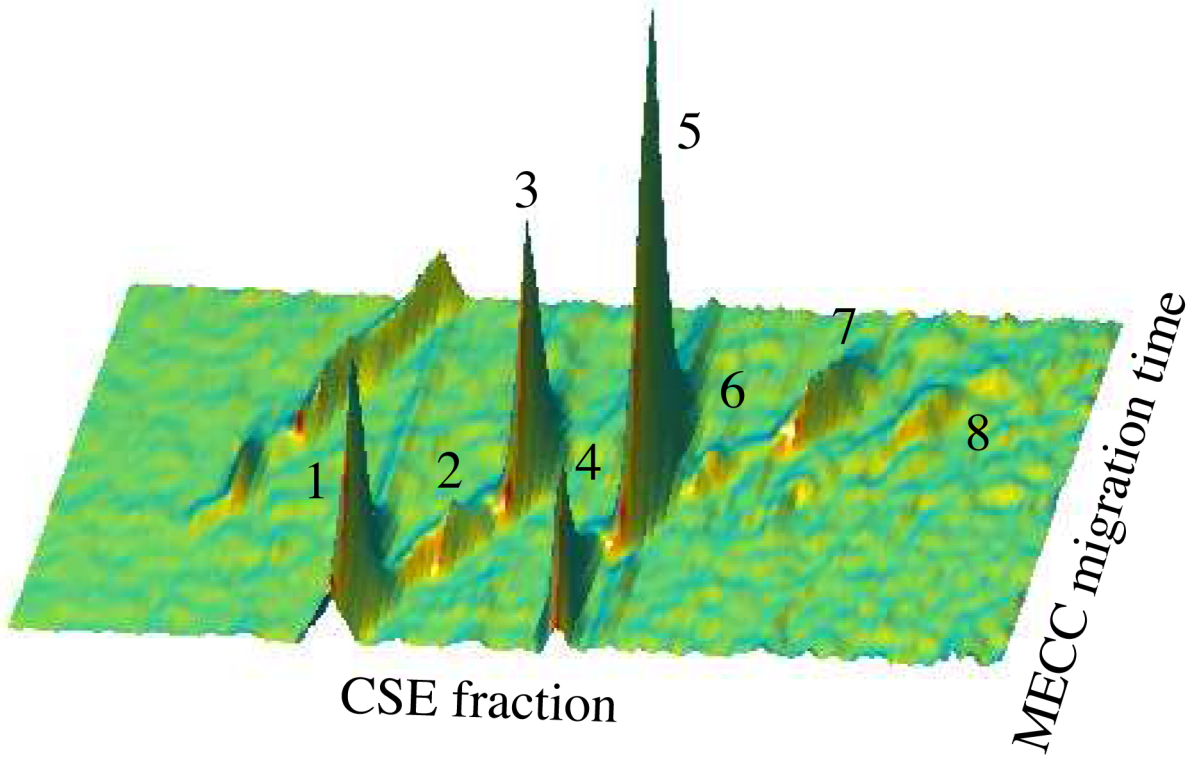

Figure 4.

Two-dimensional electropherogram of standard proteins. Peaks: 1: α-lactalbumin bovine milk (14.2 kDa), 3: trypsin inhibitor soybean (20.1 kDa), 4: carbonic anhydrase (29 kDa), 5: ovalbumin (45 kDa), 7: bovine serum albumin (66 kDa), 8: phosphorylase b (97.4 kDa). Peaks 2 and 6 were impurities. β-Galactosidase *E. coli* and myosin rabbit musclewere included in the standard but were not detected.

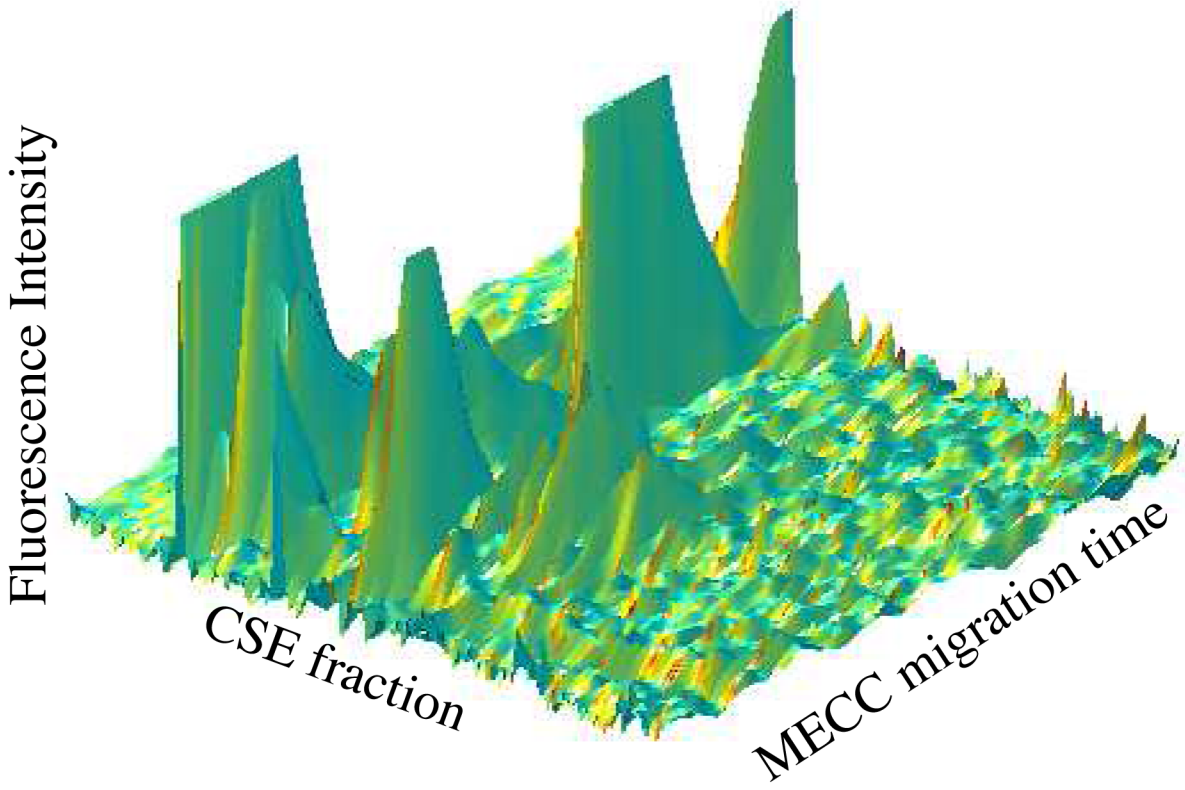

Figure 5.
Two-dimensional capillary electrophoresis analysis of a homogenate prepared from a Barrett's esophagus cell line and labeled with Chromeo P503. The higher amplitude components were truncated to allow visualization of lower amplitude components.

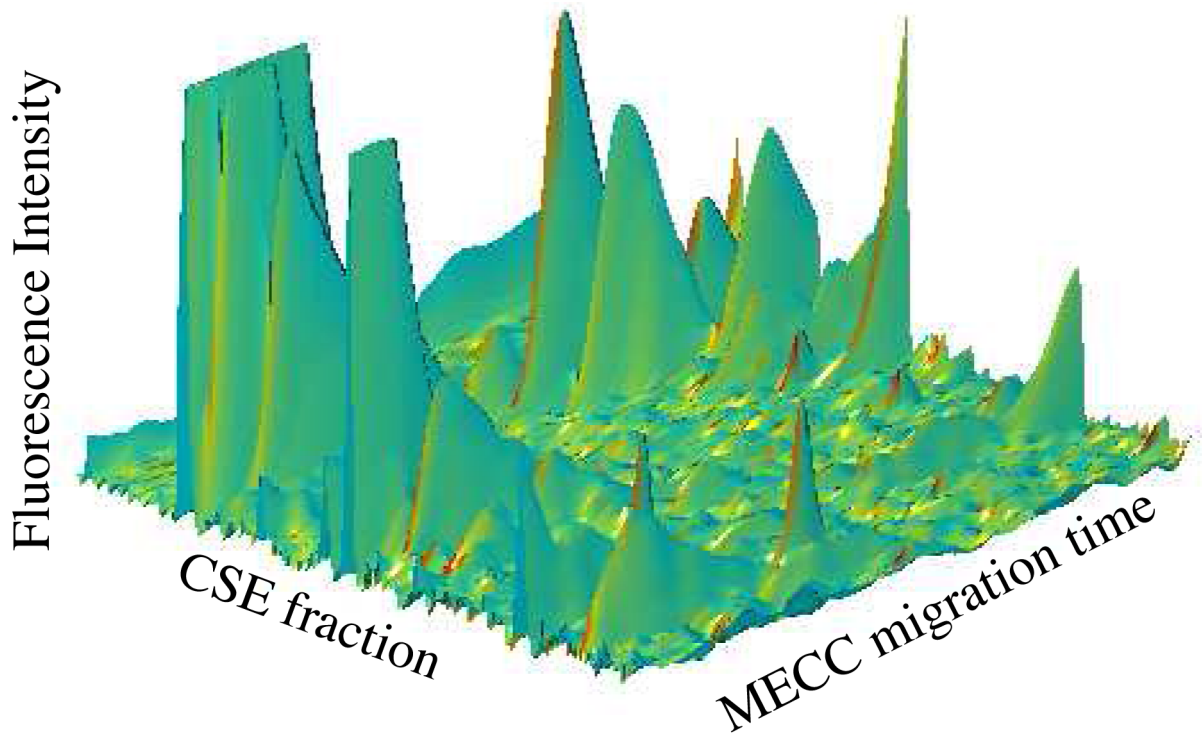

Figure 6.
Two-dimensional capillary electrophoresis analysis of a homogenate prepared from a Barrett's esophagus cell line and labeled with FQ. The higher amplitude components were truncated to allow visualization of lower amplitude components.