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Short communication

Labeling effects on the isoelectric point of green fluorescent protein

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Abstract

We studied the effects of fluorescent labeling on the isoelectric points (p*I* values) of proteins using capillary isoelectric focusing with laser-induced fluorescence detection (cIEF–LIF). Specifically, we labeled green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* with the fluorogenic dye 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). cIEF–LIF was used to monitor the native fluorescence of GFP and showed p*I* changes in GFP's FQ-labeled products. Multiple labeling of GFP with FQ produced a series of products with p*I* values shifted towards a low pH. We verified cIEF–LIF results with traditional slab gel IEF. Our cIEF–LIF technique can routinely detect 10^{-11} *M* of FQ-labeled protein, whereas traditional slab gel IEF with silver stain detection gives detection limits of 10^{-7} *M* in the same samples.

Keywords: Isoelectric focusing; Fluorescence detection; Detection, electrophoresis; Proteins; Green fluorescent protein; Furoylquinolinecarboxaldehyde

1. Introduction

Traditionally isoelectric focusing (IEF) has been performed on slab gels, which consist of polyacrylamide-immobilized pH gradients. However, with the advent of capillary electrophoresis (CE), the use of CE to perform IEF (cIEF) was introduced [1]. cIEF seeks to improve on slab gel IEF with regard to laborious staining procedures that result in poor sensitivity [2] and the small pore size of the gel matrix, which in slab gel IEF prevents macromolecules from attaining their isoelectric point (p*I*) [3]. cIEF also brings with it all the advantages of CE, including small sample volumes, effective Joule heat dissipation, and real-time data acquisition.

cIEF is usually performed with UV detection [1,4–6]; however there are sensitivity limits to this approach. Fluorescent labeling of proteins and laser-induced fluoresence (LIF) detection are used in CE to improve detection limits [7]. Typically CE–LIF detection limits of proteins are of the order of 10^{-12} to 10^{-13} *M* [8]. Because cIEF is a concentrating technique, cIEF–LIF detection limits should be superior to those of CE–LIF by at least an order of magnitude. Few reports of cIEF–LIF exist; most reports rely on native fluorescence [9,10] or, in a few cases, use of a fluorescent dye to tag the molecules of interest [11,12].

Concerns exist surrounding the use of a fluorescent dye to tag a protein for cIEF–LIF. First of all, it has been shown that there are heterogeneous labeling products when peptides and proteins are tagged with fluorescent dyes [13,14,7]. Another concern is that the p*I* value of the protein will be changed when it is labeled with a fluorescent tag. It has been noted that upon conjugation with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate, rabbit IgG experiences a p*I* decrease [15,16]. However, this

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decrease in p*I* may simply be due to the negative charge of fluorescein.

We chose green fluoresent protein (GFP) as the model protein in a study of whether or not conjugation of a protein and a fluorogenic dye changes the p*I* value of the protein. GFP from the jellyfish *Aequorea victoria* is a common gene marker which can be expressed in both eukaryotic and prokaryotic systems [17]. Another significant advantage of GFP is that it does not require any additional cofactors from the jellyfish to fluoresce [17]. GFP's native fluorescence allowed us to monitor, through IEF, any changes in the p*I* value of GFP on labeling with the fluorogenic dye 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). We used slab gel IEF to confirm our cIEF–LIF results.

2. Experimental

2.1. Materials

Fused-silica capillary (50 μm I.D.×140 μm O.D.) was obtained from PolyMicro Technologies (Phoenix, AZ, USA). Phastgel IEF 4–6.5 IEF slab gels were acquired from Pharmacia (Quebec, Canada). Recombinant GFP was purchased from Clontech (Palo Alto, CA, USA). Bio-Lyte 4/6 ampholytes and ammonium persulfate were from BioRad (Hercules, CA, USA) and N,N,N′,N′-tetramethylethylenediamine was purchased from Gibco (Grand Island, NY, USA). Sodium hydroxide was obtained from Caledon (Georgetown, Canada) and phosphoric acid was acquired from Fisher (Fair Lawn, NJ, USA). Disodium tetraborate and sodium carbonate were from BDH (Toronto, Canada). Vinylmagnesiumbromide, tetrahydrofuran, and potassium cyanide were obtained from Aldrich (Milwaukee, WI, USA). FQ was acquired from Molecular Probes (Eugene, OR, USA). Formaldehyde (37% photographic grade) and glutaraldehyde were from Sigma (St. Louis, MO, USA). Silver nitrate was from ACP (Montreal, Canada). Trichloroacetic acid (TCA) and glacial acetic acid (HAc) were obtained from Anachemia (Montreal, Canada). Ethanol (EtOH) was obtained from Commercial Alcohols (Winnipeg, Canada). Acryloylaminopropanol (AAP) was graciously provided by Professor P.G. Righetti.

2.2. Sample preparation

FQ labeling of GFP for slab gel IEF was done in the following manner: 9 μl of $3.72 \cdot 10^{-5}$ *M* GFP, 1 μl of 25 m*M* KCN (in 10 m*M* borate), and 100 nmol of dry FQ were reacted for 1 min at room temperature. The reaction mixture was diluted 100× with 10 m*M* borate (pH 9.2) to slow the reaction. This $3.35 \cdot 10^{-7}$ *M* FQ-labeled GFP mixture was loaded onto an application comb and then onto the gel. For cIEF–LIF, FQ labeling of GFP was done as follows: 1 μl of $3.72 \cdot 10^{-5}$ *M* GFP, 9 μl of 25 m*M* KCN (in 10 m*M* borate), and 100 nmol of dry FQ were reacted for 1 min at room temperature. The reaction mixture was then diluted 100× with 10 m*M* borate (pH 9.2) to slow the reaction. A sample was made of $3.72 \cdot 10^{-9}$ *M* FQ-labeled GFP and 2% Bio-Lyte 4/6 ampholytes in water. This sample was put into a syringe and loaded into the capillary.

2.3. Slab gel isoelectric focusing

A Pharmacia LKB Phastsystem was used for slab gel IEF. The slab gels had a 4–6.5 pH gradient. A Pharmacia low p*I* isoelectric focusing calibration kit (pH 2.5–6.5) was used for standard purposes. The IEF program is shown in Table 1.

After IEF, the gels were developed using a slightly modified version of the silver stain protocol supplied by Pharmacia. The development protocol is shown in Table 2. After development, the gels were air-dried.

2.4. Capillary isoelectric focusing with laser-induced fluorescence detection and anodic mobilization

The laboratory-made single-capillary instrument with sheath flow cuvette used for cIEF–LIF is

Table 1
Pharmacia LKB Phastsystem IEF program

Step	Voltage (V)	Current (mA)	Power (W)	Temperature (°C)	Volthours
1	2000	2.0	3.5	15	75
2	200	2.0	3.5	15	15
3	2000	5.0	3.5	15	410

Table 2
IEF silver stain development protocol. Note that all solutions were v/v percentages except for silver nitrate, which was w/v percent. Also note that the developer was made of 0.03% (v/v) 37% formaldehyde in 2.5% (v/v) Na_2CO_3

Step	Solution	Time (min)	Temperature (°C)
1	20% TCA	5	20
2	50% EtOH, 10% HAc	2	50
3	10% EtOH, 5% HAc	2	50
4	10% EtOH, 5% HAc	4	50
5	8.3% glutaraldehyde	6	50
6	10% EtOH, 5% HAc	3	50
7	10% EtOH, 5% HAc	5	50
8	ddH_2O	2	50
9	ddH_2O	2	50
10	0.5% $AgNO_3$	10	40
11	ddH_2O	0.5	30
12	ddH_2O	0.5	30
13	Developer	0.5	27
14	Developer	4.5	27
15	5% HAc	5	50

ddH_2O, distilled deionized water.

described elsewhere [18]. A manual Hamilton T-valve (Chromatographic Specialties, Brockville, Canada) was added to the instrument's sheath flow line for mobilization purposes. A blue argon ion laser (3.5 mW, λ=488 nm) (Uniphase, San Jose, CA, USA) was used for excitation. Fluorescence was filtered through a 515DF20 bandpass filter (Omega Optical, Brattleboro, VT, USA) and was detected with an R1477 photomultiplier tube (Hamamatsu, Middlesex, NJ, USA).

IEF was performed in a 35 cm×50 μm I.D.×140 μm O.D. fused-silica capillary. The capillary was Grignard coated with polyAAP as described elsewhere [19]. Focusing was performed for 30 min using a reversed electric field polarity of 350 V/cm. The running buffer (catholyte) was 40 m*M* NaOH and the sheath flow buffer (anolyte) was 20 m*M* H_3PO_4. To mobilize the sample past the detector, the sheath flow buffer was switched from 20 m*M* H_3PO_4 to 40 m*M* NaOH while the electric field was kept at −350 V/cm.

3. Results

Slab gel determination of the p*I* of GFP produced three bands (see Fig. 1). The predominant band was at p*I* 5.00±0.04 (n=5 gels). The second and third bands flanked the major band at p*I* values of 4.88±0.05 and 5.19±0.04.

Slab gel results show that GFP's p*I* is significantly changed upon reaction with the fluorogenic dye FQ. The p*I* of GFP is decreased when it is labeled with FQ and multiple labeling products are produced from the reaction. Specifically, the slab gel results show that a series of labeling products are created which have p*I* values in the range of 4.6–4.9. These products are not distinguishable from one another and appear as smears on the IEF gels. These are the concentrations of the labeling products which were greater than 10^{-7} *M*; lower concentration proteins were not visible on the silver stained IEF slab gels.

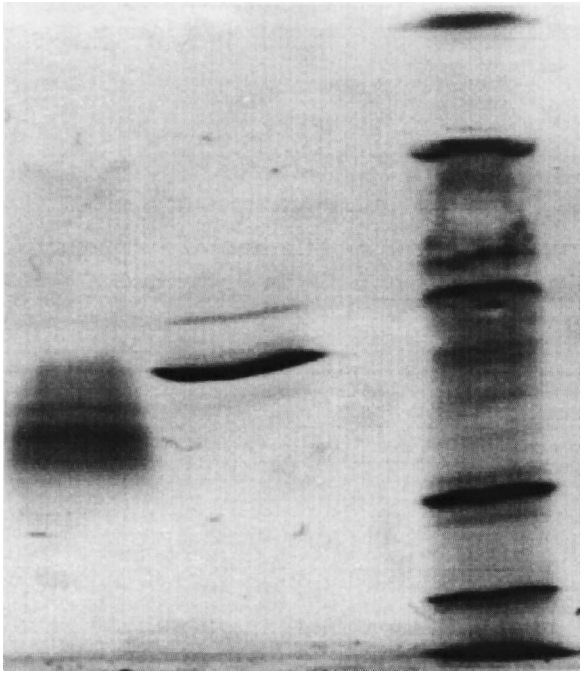

Fig. 1. Slab gel IEF: multiple labeling of GFP with FQ. IEF was performed using a Pharmacia LKB Phastsystem. Slab gels were 4–6.5 pH gradient (pH 6.5 is at the top). A silver staining protocol was used to stain and detect the sample. From left to right, the samples are as follows: FQ-labeled GFP ($3.35 \cdot 10^{-7}$ *M*), GFP ($3.72 \cdot 10^{-7}$ *M*), and Pharmacia low p*I* isoelectric focusing calibration kit standards. The visible standards and their p*I* values are: human carbonic anhydrase (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), glucose oxidase (4.15), and methyl red (3.75).

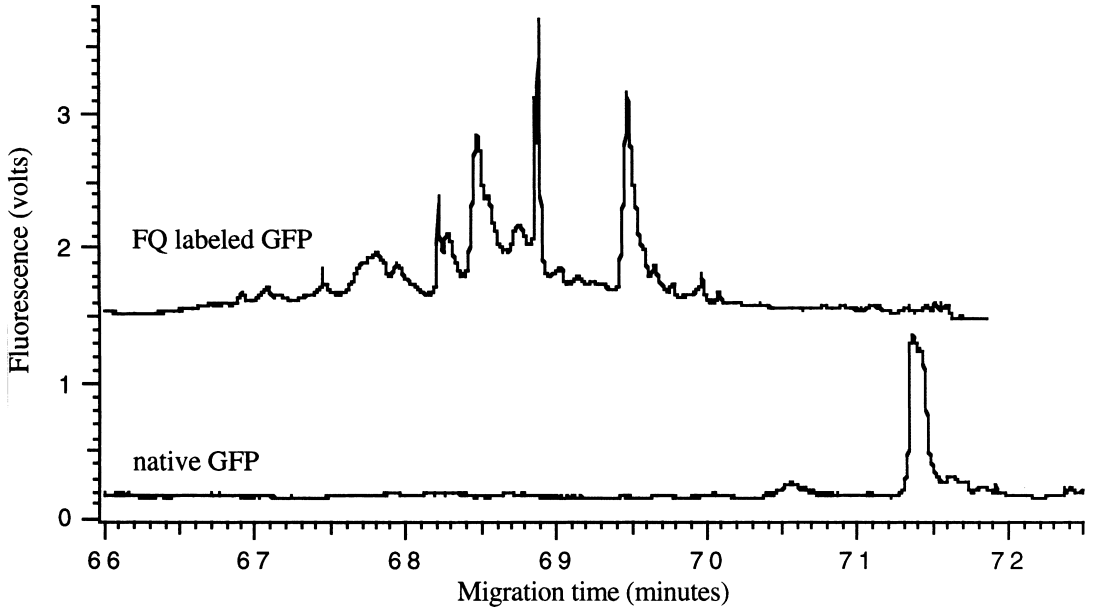

Fig. 2. cIEF: Multiple labeling of GFP with FQ. Separation was in a polyAAP Grignard coated capillary of dimensions 35 cm×140 μm×50 μm at a reversed electric field of 350 V/cm. Catholyte was 40 m*M* NaOH, anolyte was 20 m*M* H_3PO_4, and mobilization was at 30 min by changing the anolyte to 40 m*M* NaOH. Detection was by LIF, excitation at 488 nm and emission at 515 nm. Native GFP is 10^{-12} *M* GFP and 2% Bio-Lyte 4/6 ampholytes in water. FQ-labeled GFP is $3.72 \cdot 10^{-9}$ *M* FQ-labeled GFP and 2% Bio-Lyte 4/6 ampholytes in water.

One peak is seen in cIEF of GFP (Fig. 2). However its p*I* value was not determined using fluorescent p*I* markers because none are commercially available. The cIEF–LIF instrument easily detected peaks produced by 10^{-13} *M* GFP. A few broad peaks also flank the predominant peak.

The cIEF results of the FQ-labeled GFP show the presence of many peaks with various p*I* values. Many more labeling products are seen with LIF detection than with silver staining of slab gels because LIF is much more sensitive. Again, the exact p*I* values of the labeling products were not determined because fluorescent p*I* markers are not available at present.

4. Discussion

FQ is a neutral molecule that reacts with the ε-amine of a lysine group. It can be rationalized that the loss of positively charged groups during the labeling step will produce a decrease in the p*I* value of a protein labeled with FQ, i.e. the protein will become more acidic upon labeling with FQ. The shift in p*I* due to this labeling is shown by gel IEF. A similar decrease in p*I* has been noted when rabbit IgG is labeled with the negatively charged dye fluorescein isothiocyanate [15,16]. Again the argument can be used that when negative charges are added to a protein, its p*I* will become more acidic.

We have already reported that labeling of GFP with FQ generates at least six components when analyzed by capillary zone electrophoresis [7]. Similarly, the reaction of FQ with successive lysine residues will titrate the positive charge on the protein, shifting the p*I* to acidic values.

5. Conclusions

We demonstrate here the effects on the p*I* value of GFP when labeled with the fluorogenic dye, FQ. We determined the p*I* of GFP to be 5.00 by slab gel IEF with silver staining, with minor bands at 4.88 and 5.19 flanking this predominant band. GFP's multiple labeling products also have different acidically

shifted p*I* values in the range 4.6–4.9. As a more sensitive alternative, we used cIEF–LIF to detect p*I* changes of GFP. cIEF–LIF took advantage of GFP's native fluorescence. cIEF–LIF could routinely detect 10^{-12} *M* GFP whereas slab-gel IEF (with silver staining) was not able to detect less than 10^{-7} *M* GFP.

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