Chapter 2

Discrimination of Glycoproteins from Unglycosylated Proteins in Capillary Electrophoresis: Two-Color LIF Detection Coupled with Post-column Derivatization

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Abstract

Glycosylation is one of the most important posttranslational modifications (PTMs) which lead to the functionalization of proteins. Here, we describe one method for discriminating glycosylated proteins from unglycosylated ones in their mixture sample by capillary electrophoretic separation and two-color laser-induced fluorescence detection coupled with post-column derivatization. Two lasers emitting at 450 and 532 nm permit the detection of amino groups of proteins derivatized by naphthalene-2,3-dicarboxaldehyde and a fluorescently labeled lectin, tetramethylrhodamine-labeled concanavalin A (Rh-Con A), respectively. When a protein mixture react with Rh-Con A, the glycoproteins bound with Rh-Con A exhibit signals at the same migration time in two electropherograms obtained by 450- and 532-nm lasers whereas unbound proteins show a signal only in the electropherogram of the 450-nm laser. So, when one protein is glycosylated it is detected at the same migration time in the electropherograms obtained by two lasers.

Key words Capillary electrophoresis, Glycoprotein, Postcolumn derivatization, Two-color laser-induced fluorescence

1 Introduction

Proteins play many roles in biological systems such as enzymatic reactions, immunological reactions, and maintenance of structure in cell membranes and organs. Proteins are synthesized in biological cells through translation of genes, and then are subject to post-translational modifications (PTMs) such as glycosylation, phosphorylation, and methylation, which lead to the functionalization of proteins in biological systems. Among these PTMs, glycosylation of proteins has key roles in cellular recognition, protein folding, and protein trafficking [1] so that discrimination of glycoproteins from unglycosylated proteins is an important issue in the study of biological systems.
Several analytical methods have been developed for glycoproteins, glycans, and glycoforms based on chromatographic or electrophoretic separations coupled with mass spectrometry (MS) [2–5]. However, intact proteins are, in general, digested by enzymes since they are too large to be measured directly by MS. Conversely, capillary electrophoresis (CE) has several advantages in protein analysis because of its high resolution of large proteins, rapid separation, and low consumption of samples. Therefore, CE showed excellent performance in the analyses of glycans [6], glycoforms [7], and glycoproteins [8].

In protein analyses, laser-induced fluorescence (LIF) is a high sensitive method that permits the detection of single molecules [9] and the analysis of single cells [10]. However, one of the difficulties in coupling LIF with CE is the need to label proteins with a fluorescent dye before separation. Conversely, post-column [11] and on-column [12] derivatizations are advantageous since native proteins can be injected directly into a capillary with no labeling. Previously, we employed the post-column derivatization for proteins separated by capillary sieving electrophoresis, which permitted direct injection of denatured proteins and their LIF detection [13, 14].

We also developed a novel LIF method to discriminate glycosylated proteins in a protein mixture which contained both glycosylated and unglycosylated proteins [15]. The method consists of CE separation followed by LIF detection with two visible lasers coupled with post-column derivatization. A glycoprotein, thyroglobulin, was clearly discriminated from bovine serum albumin which is not glycosylated using a lectin probe, concanavalin A labeled with tetramethylrhodamine (Rh-Con A).

2 Materials

Prepare all solutions using analytical grade reagents and ultrapure water (see Note 1). Store all protein solutions and labeling reagents in a refrigerator at 4 °C. Store all buffer solutions at room temperature. Filter all solutions with 0.2-μm membrane filter before the use (see Note 2).

Use a CE system equipped with a laser-induced fluorescence (LIF) detector and a post-column reactor using sheath flow, in which a separation capillary was inserted into a large bore capillary to react the analytes with the labeling reagent at the outlet of the separation capillary (Fig. 1) [16]. Regulate the collection of the fluorescence signals and the switching of the beam stoppers synchronously by a LabView program (National Instruments, CA, USA) using a personal computer equipped with an A/D converter (NI 9215, National Instruments, CA, USA).
1. Running buffer for capillary electrophoresis: 50 mM sodium borate buffer. Weigh 4.767 g of sodium tetraborate decahydrate (Borax, MW = 381.37) in a 200 mL beaker. Add about 100 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 250-mL volumetric flask and make up to 250 mL with water.

2. Derivatization solution: 100 mM sodium borate buffer. Weigh 3.814 g of sodium tetraborate decahydrate (Borax, MW = 381.37) in a 100 mL beaker. Add about 50 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 100-mL volumetric flask and make up to 100 mL with water.

3. 5 mM Naphthalene-2,3-dicarboxaldehyde (NDA) solution: 5 mM NDA. Weigh 0.0184 g of NDA in a 20 mL vial and add 20 mL of methanol to dissolve the powder (see Note 3).

Fig. 1 Photos of the capillary holder (upper) and the tip of the tapered capillary inserted into the large bore capillary (lower)
4. 80 mM 2-Mercaptoethanol (2-ME) solution: 80 mM 2-ME. Weigh 0.125 g of 2-ME in a 20 mL vial and add 20 mL of methanol (see Note 4). Store the solutions of NDA and 2-ME in a refrigerator.

5. Derivatization solution. Mix 7 mL of 100 mM sodium borate buffer, 2 mL of 5 mM NDA, and 1 mL of 80 mM 2-ME, resulting in a solution containing 1 mM NDA, 8 mM 2-ME, 70 mM borate, and 30% methanol.

6. Protein solutions (see Note 5): 4.9 × 10^{-5} M Thyroglobulin solution. Weigh 0.0032 g of thyroglobulin in a 1-mL centrifugal tube and add 100 μL of the running buffer. 4.9 × 10^{-4} M Albumin solution. Weigh 0.0032 g of albumin in a 1-mL centrifugal tube and add 100 μL of the running buffer (50 mM borate) (see Note 6).

7. Metal solutions: 20 mM MnCl₂ solution. Weigh 0.0792 g of Manganese (II) chloride tetrahydrate in a 10 mL beaker. Add about 10 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 20-mL volumetric flask and make up to 20 mL with water. 20 mM CaCl₂ solution. Weigh 0.0588 g of calcium chloride dihydrate in a 10-mL beaker. Add about 10 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 20-mL volumetric flask and make up to 20 mL with water.

8. Rh-Con A: Obtain rhodamine concanavalin A from Vector Laboratories (catalog No.: RL-1002, CA, USA), store in a refrigerator at 4 °C, and use without any pretreatment. The commercially available Rh-Con A solution contains 25 mg of protein at the concentration of 5.0 mg/mL as active conjugate, 10 mM HEPES buffer (pH 7.5), 0.15 M NaCl, 0.1 mM Ca²⁺, 0.01 mM Mn²⁺, and 0.08% sodium azide (see Note 7).

9. 2.5 × 10^{-5} M sodium fluorescein: Weigh 0.004 g of sodium fluorescein in a 50 mL beaker. Add about 30 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 100-mL volumetric flask and make up to 100 mL with water to prepare a stock solution of 0.1 mM sodium fluorescein. Take 2.5 mL of 0.1 mM sodium fluorescein into a 10-mL volumetric flask and make up to 10 mL with water.

2.2 Sample Preparation

1. Sample of thyroglobulin: Take 10 μL of 4.9 × 10^{-5} M thyroglobulin, 25 μL of 2.5 × 10^{-5} M sodium fluorescein (internal standard), and 65 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

2. Sample of Rh-Con A: Take 10 μL of Rh-Con A, 2.5 μL of 20 mM MnCl₂ solution, 2.5 μL of 20 mM CaCl₂ solution (see Note 8), 25 μL of 2.5 × 10^{-5} M sodium fluorescein, and 60 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.
3. Sample of thyroglobulin containing Rh-Con A: Take 10 μL of 4.9 × 10^{-5} M thyroglobulin, 10 μL of Rh-Con A, 2.5 μL of 20 mM MnCl₂ solution, 2.5 μL of 20 mM CaCl₂ solution, 25 μL of 2.5 × 10^{-5} M sodium fluorescein (internal standard), and 50 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

4. Sample of albumin: Take 10 μL of 4.9 × 10^{-4} M albumin (see Note 9), 25 μL of 2.5 × 10^{-5} M sodium fluorescein (internal standard), and 65 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

5. Sample of albumin containing Rh-Con A: Take 10 μL of 4.9 × 10^{-4} M albumin, 10 μL of Rh-Con A, 2.5 μL of 20 mM MnCl₂ solution, 2.5 μL of 20 mM CaCl₂ solution, 25 μL of 2.5 × 10^{-5} M sodium fluorescein, and 50 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

6. Mixture sample of thyroglobulin and albumin containing Rh-Con A: Take 10 μL of 4.9 × 10^{-5} M thyroglobulin, 10 μL of 4.9 × 10^{-4} M albumin, 10 μL of Rh-Con A, 2.5 μL of 20 mM MnCl₂, 2.5 μL of 20 mM CaCl₂, 25 μL of 2.5 × 10^{-5} M sodium fluorescein, and 40 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

2.3 Post-column Reactor

1. Fill a tapered capillary (50 μm i.d., 360 μm o.d., and 40 cm length, PicoTip EMITTER, TT360-50-50-CE-5, coating; P200P, NEW OBJECTIVE, MA, USA) with 0.1 M NaOH by a 100-μL microsyringe connected with the inlet of the capillary using a piece of polytetrafluoroethylene tube and activate the inner surface of the capillary overnight (see Note 10).

2. Connect the tapered capillary with a tee connector fixed on a home-made holder (Fig. 1).

3. Connect a large bore capillary (530 μm i.d., 660 μm o.d., and 13 cm length, GL Science, Tokyo, Japan) with the tee connector so as to insert the tip of the tapered capillary into the large bore capillary (Fig. 1) (see Note 11).

2.4 LIF Detector

1. Align two lasers emitting at 450 nm (20 mW Z20M18H-F-450-pe, Z-LASER, Germany) and 532 nm (40 mW, Z40M18B-F-532-pz, Z-LASER, Germany), a dichroic filter (XF2077, Omega Optical, Inc., VT, USA), aluminum mirrors, a plane-convex lens (synthesized quartz, φ = 10 mm, focal distance=50 mm) for focusing the laser beams, and a detection box consisting of a photomultiplier tube (Model R3896, Hamamatsu, Shizuoka, Japan), a pinhole (φ = 0.5 mm), and a microscope objective (Olympus, LWD LSPlan 50×/N. A., 0.60) for collecting the fluorescence signal (Fig. 2).

2. Overlap the two laser beams by adjusting the dichroic filter, mirror 1, and mirror 2 (see Note 12) and locate the two beam
3. Place a long-pass filter (HQ470LP, Chroma Technology Corporation, VT, USA), and notch filters (NF01-532U-25, Semrock, NY, USA and 532 nm Rugate Notch Filter, Edmund Optics Inc., NJ, USA) (Fig. 3) between the microscope objective and the photomultiplier tube in the detection box.

stoppers (F116-1, SURUGA SEIKI, Shizuoka, Japan) controlled by a shutter controller (F77-7, SURUGA SEIKI, Shizuoka, Japan) between the lasers and the dichroic filter (Fig. 2).
4. Adjust the capillary so as that the center of the tapered capillary overlaps with the image of the pinhole placed in front of the photomultiplier tube in the detection box (Fig. 4a).

5. Focus the laser beams onto the tapered capillary, and then move the laser beams so as to overlap with the pinhole (Fig. 4b) by moving the plane-convex lens.

6. Move the capillary to adjust the distance between the tip of the capillary and the laser beams to 750 μm (Fig. 4c) (see Note 13).

7. Observe change in the fluorescent signal by injecting a fluorophore solution such as fluorescein and rhodamine B with a microsyringe to confirm whether alignment of the detection system is correct or not.

Fig. 4 Alignment of the capillary and the laser beams in the post-column fluorescence detection
3 Methods

1. Condition the separation capillary (tapered capillary) by rinsing with 0.1 M NaOH for 5 min, deionized water for 5 min, and the running buffer for 5 min, sequentially.

2. Condition the reaction capillary (large bore capillary) by rinsing with water and the running buffer sequentially.

3. Fill the separation capillary and the reaction capillary with the running buffer and the derivatization solution, respectively (see Note 14).

4. Inject a sample solution (one of them described in Subheading 2.2) hydrodynamically for 10 s into the capillary from the sample vial raised 10 cm above the outlet vial (see Note 15).

5. Apply a constant potential of +10 kV to the inlet side of the separation capillary by a high-voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan), and flow the derivatization solution into the reaction capillary at a flow rate of 0.2 μl/min during the separation (see Note 16).

6. Save the data as a text file, and then open the text using a spreadsheet software such as Excel to show two electropherograms (Fig. 5 (the results for (A) thyroglobulin, (B) Rh-Con A, and (C) their mixture), Fig. 6 (the results for (A) albumin, (B) Rh-Con A, and (C) their mixture), Fig. 7 (the results for a mixture of thyroglobulin, albumin, and Rh-Con A) (see Note 17).

7. Fill the separation capillary with 0.1 M NaOH to keep the surface of the capillary activated after the experiments.

8. Flush the reaction capillary with 0.1 M NaOH and fill it with water after the experiment (see Note 18).

4 Notes

1. Filter pure water before the use for preparation of solutions. A disposable syringe filter was usually employed for rapid filtration.

2. The filtration helps removing particulate matters in solutions since they appear as spike noises in the electropherogram due to scattering of laser light.

3. Store the NDA solution in a refrigerator within 1 week and prepare a fresh solution every week.

4. Wear gloves and weigh in a fume hood for the preparation of the solution since 2-ME is toxic and volatile.
Fig. 5 Electropherograms of thyroglobulin, Rh-Con A, and their mixture. (a) 4.9 μM thyroglobulin (sample 1), (b) 4.9 μM Rh-Con A (sample 2), (c) a mixture of 4.9 μM thyroglobulin and 4.9 μM Rh-Con A (sample 3). (1) thyroglobulin, (2) fluorescein, (3) Rh-ConA, (4) complex between thyroglobulin and Rh-Con A. Blue lines and green lines are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons.
Fig. 6 Electropherograms of albumin, Rh-Con A and a mixture of albumin and Rh-Con A. (a) 49 μM albumin (sample 4), (b) 4.9 μM Rh-Con A (sample 2), (c) 49 μM albumin with 4.9 μM Rh-Con A (sample 5). (1) Albumin, (2) fluorescein, (3) Rh-Con A. Blue lines and green lines are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons.
5. Employ molecular masses of 660 and 66 kDa for thyroglobulin and albumin, respectively, to calculate the concentrations of the protein solutions.

6. Prepare fresh samples when the peaks of proteins are distorted.

7. The concentration of the active concanavalin A was calculated to be $4.9 \times 10^{-5}$ M, using molecular mass of 102,000 for concanavalin A. The number of conjugated fluorophore to one concanavalin A molecule given by the manufacture was 3.5, that is, the product was a mixture of multiply labeled Rh-Con A molecules.

8. The concentrations of CaCl$_2$ and MnCl$_2$ must be kept at 0.5 mM in order to obtain a sharp peak for Rh-Con A.

9. Note that the concentration of albumin is 10 times higher than that of thyroglobulin due to the poor sensitivity of albumin-NDA derivative.

10. Suppress adsorption of negatively charged proteins on the capillary surface by activation. The surface of a fused-silica capillary was charged negatively by flushing 0.1 M NaOH.

11. Insert the tapered capillary into the large bore capillary carefully, connect the large bore capillary with the tee connector, and then fix the large bore capillary on the holder so as not to damage the tip of the tapered capillary.

12. Adjust the height of two laser beams at 12 cm which is the same height as that of the detector, adjust the angles of the laser beams to be parallel to the plain face of a laboratory table by moving the lasers, and then overlap the laser beams using the dichroic filter and mirrors.

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**Fig. 7** Electropherogram of a mixture of thyroglobulin, albumin, and Rh-Con A (sample 6). (1) Complex between thyroglobulin and Rh-Con A, (2) albumin, (3) fluorescein. Blue lines and green lines are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons.
13. Fix the capillary holder on a stage equipped with three-dimensional micrometer heads. Adjust the distance between the laser beam and the capillary tip by the micrometer head for Z-axis.

14. Bubbles must be completely removed from the tapered capillary and the large bore capillary. To remove bubbles, both the buffer solution and the derivatization solution were flushed fast.

15. Keep the length between the liquid levels of the outlet reservoir and the sample reservoir 10 cm.

16. Monitor the electric current to confirm the application of an electric field. The electric current is, in general, constant at 5 μA under the present conditions.

17. Check alignment of the laser, the capillary, and the pin-hole when no signal is obtained. The reason of no signal is, in most cases, attributed to wrong alignment of the detection system. Another reason is due to no injection of the sample.

18. Keep the separation capillary activated after the experiment.

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References


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