Analytical Biochemistry 534 (2017) 19–23

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Quantitation of CRM197 using imaged capillary isoelectric focusing with fluorescence detection and capillary Western

John W. Loughney, Sha Ha, Richard R. Rustandi*

Biologics and Vaccines, Merck Research Laboratories, Merck and Co., Inc., Kenilworth, NJ, USA

ARTICLE INFO

Article history:
Received 27 March 2017
Received in revised form
31 May 2017
Accepted 26 June 2017
Available online 27 June 2017

Keywords:
Maurice
Imaged capillary IEF
Fluorescent detection
CRM197
Wes
Western blot

ABSTRACT

Maurice is a new instrument that can perform imaged capillary isoelectric focusing (icIEF). The standard detection for icIEF is UV absorbance at 280 nm, which limits its application to high protein concentration samples and non-complex samples. Here we describe an icIEF instrument with fluorescence detection. We demonstrate the advantage of using either icIEF with fluorescence detection or quantitative Western Blot to measure diphtheria toxin mutant CRM197 protein titer in crude cell lysates and purified samples. These two techniques have great potentials to become standard methods to analyze protein titers in crude cell lysate or other complex samples types.

Introduction

Imaged capillary isoelectric focusing (icIEF) is an established method used to analyze charge heterogeneity of biological molecules such as proteins, virus, and cells [1–4]. This technique has been described in detail by Wu et al. [5,6]. This icIEF technique has robust separation power and excellent linearity, and can be used to measure protein concentration in complex samples. Previously we demonstrated that the concentration of diphtheria toxin mutant CRM197 protein can be measured during the purification process [7].

Automated capillary Western Blot is a relatively new technique that has been previously described for various qualitative and quantitative based applications [8–10]. This new technique is superior to the traditional Western Blot because the detection has a wide linear range and offers standard curve-based quantitation. In the capillary Western Blot method, the analysis is performed under a denatured condition by adding SDS and heating the sample, making it a good method to analyze protein concentration in complex samples such as cell lysates. We have demonstrated the advantage of this quantitative Western to analyze protein in cell lysate samples of a fermentation process [11].

CRM197 protein is used as a common carrier in most polysaccharide conjugate-based vaccines [12]. Four assays for CRM197 have been established using icIEF technique, namely, identity by its pI, charge heterogeneity, stability, and protein concentration measurement [7]. All four icIEF assays have been performed using either iCE280 or iCE3 instrumentations with a standard 280 nm UV absorbance detection. Using UV absorbance, CRM197 protein concentration can be measured for fermentation and purification samples. However, for crude cell lysate samples, it is impossible to measure CRM197 using icIEF with UV detection alone. Here we describe the advantages of using either icIEF with fluorescence detection or quantitative capillary Western to measure CRM197 protein in cell lysates and downstream purification samples.

Materials and methods

Chemicals and reagents

Methylcellulose (MC) containing solutions, icIEF fluorescence calibration, system suitability standards, pI marker 4.65, and the icIEF cartridge were obtained from ProteinSimple (Santa Clara, USA). Pharmalyte ampholytes (pH 3–10 and pH 4–6.5) were purchased from GE Healthcare (Uppsala, Sweden). Glycerol and pI marker 7.60 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), molecular weight (MW) fluorescent

* Corresponding author. 770 Sumneytown Pike, P. O. Box 4, WP42A-30, West Point, PA 19486, USA.
E-mail address: richard_rustandi@merck.com (R.R. Rustandi).

http://dx.doi.org/10.1016/j.ab.2017.06.013
0003-2697/

standards, luminol-S, hydrogen peroxide, biotinylated molecular weight markers, Streptavidin HRP conjugated, secondary goat-anti mouse antibody, 10x sample buffer, antibody diluent 2, wash buffer, 10–230 kDa Wes plate, and capillaries were purchased from ProteinSimple (Santa Clara, CA). Pierceable adhesive film was purchased from 4titude (UK). The primary anti-CRM197 antibody and CRM197 were generated in house as previously described [7].

icIEF sample preparation

The icIEF sample preparation has been previously described [7]. Briefly, an ampholyte solution was prepared by combining 2 parts of the ampholytes pH 4–6.5 with 1 part of the ampholytes pH 3–10. The sample was prepared by combining 70 μL of 1% methylcellulose, 9 μL of ampholyte solution, 40 μL of glycerol, 1 μL of each pI marker 4.65 and 7.60 with various volumes of CRM197 to make standard curves and various amount of water to obtain a final volume of 200 μL for each sample. The standard curve ranges from 15 to 250 μg/mL.

The samples were centrifuged at 5000 x g for 5 min before 120 μL of each sample was transferred to the 96-well plate. Lastly the plate was covered with pierceable film, before it was placed into the Maurice instrument.

Maurice icIEF instrument

The Maurice is a new instrument from ProteinSimple that can perform CE SDS and icIEF. The icIEF part is similar to other iCE280 or iCE3 instruments, except the capillary is now in a “plug and run” cartridge format. In addition, Maurice is the only commercially available icIEF instrument that has the added fluorescent emission detection at 330 nm and 280 nm excitation in combination to the standard UV absorbance at 280 nm. The IEF separation cartridge has a 50 mm long, 100 μm ID x 200 μm OD silica capillary coated with fluorocarbon (ProteinSimple, Santa Clara, CA, USA). The catholyte consists of 0.1 M NaOH in 0.1% MC and the anolyte is 0.08 M phosphoric acid in 0.1% MC. All other needed reagents (e.g., system suitability standard, fluorescence calibration standard, 0.5% methylcellulose) were prepared according to vendor's recommendations. The samples were pre-focused for 1 min at 1500 V followed by a focusing for 8 min at 3000 V. Samples were imaged using both the absorbance and fluorescent detectors. Fluorescent exposure times of 3sec, 5sec, 10sec, and 20sec were collected. All data analyses were performed with the 3sec fluorescent exposure time, unless specified otherwise.

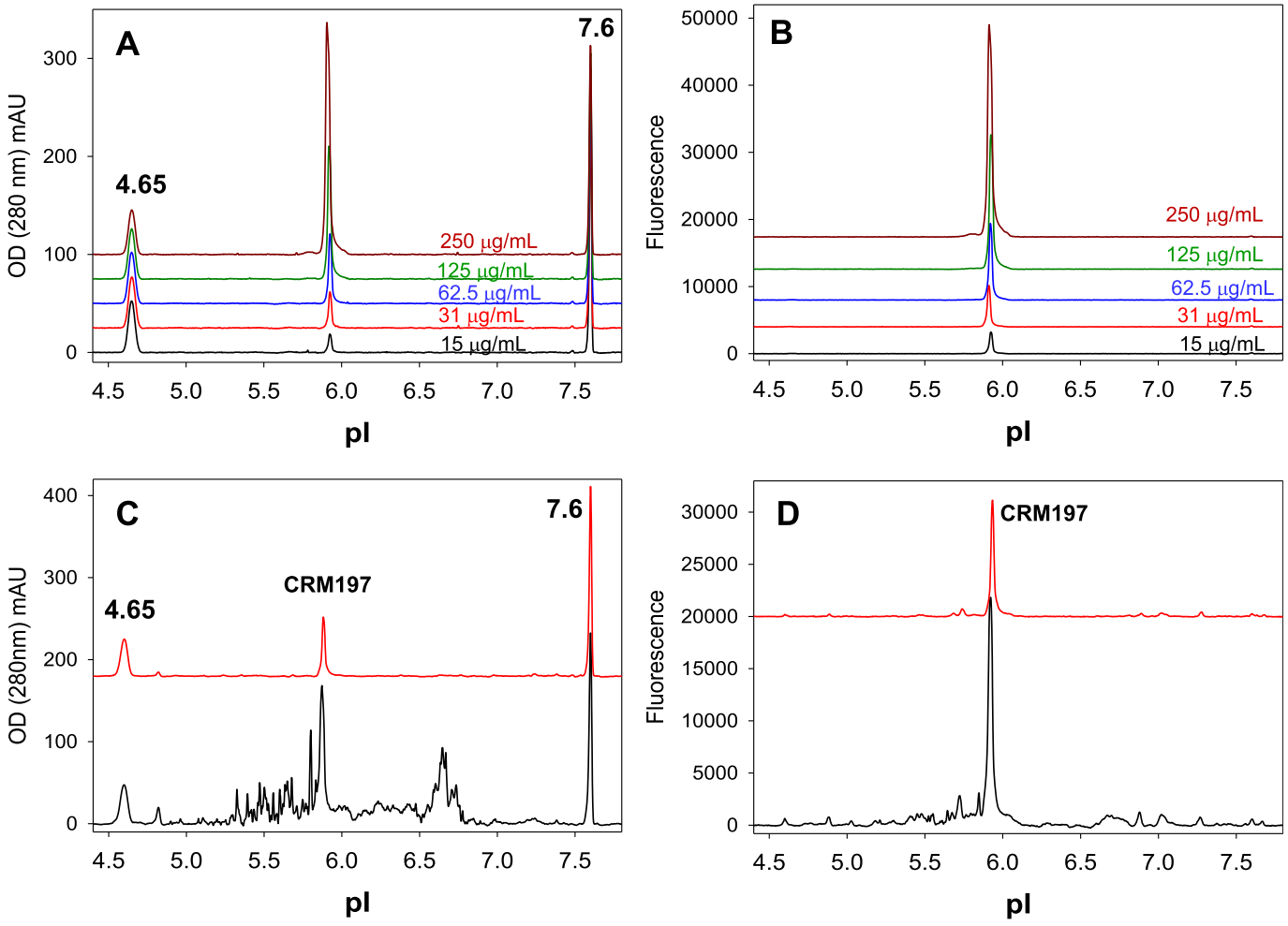

Fig. 1. Electropherogram of icIEF traces from CRM197 standards (15–250 μg/mL) performed in icIEF with (A) UV at 280 detection and (B) fluorescence detection (280 nm excitation and 330 nm emission). The pI markers of 4.65 and 7.6 were only for absorbance since both do not give fluorescence. Both provide good linearity with $r^2 \geq 0.998$. Comparison of icIEF electropherograms of process intermediate samples detected in UV (C) and fluorescence (D) for CRM197 during purification steps.