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Characterization of charge-modified and fluorescein-labeled antibody by capillary electrophoresis using laser-induced fluorescence
Application to immunoassay of low level immunoglobulin A

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Abstract

An affinity-purified and fluorescein-labeled antibody was chemically modified to alter its electrophoretic mobility. The functional activity of the resulting modified and fluor labeled antibody appeared was preserved. Immunoassay is carried out by the addition of an appropriate amount of the labeled antibody to a sample containing the antigen. Separation of the antigen-bound and free labeled antibody was performed by capillary electrophoresis with laser-induced fluorescence detection. Both free and antigen-bound labeled antibody species may be analyzed simultaneously. Quantitation of the immunoassay is achieved with an on-line data analysis.

1. Introduction

Immunoassay is based on the specificity of the reaction between an antigen and its corresponding antibody. Quantitation of antigen requires the ability to detect and discriminate by some means, between the antigen–antibody complex and either the free antibody or free antigen. The discrimination may be based on a measurable change in physical property [1] or a biochemical property [2] as a result of the immuno reaction, or by the actual physical separation [3] of the antigen–antibody complex from the free species. Analysis of immunoglobulins in biological fluids is traditionally achieved by radial immunodiffusion-based precipitation on an agar plate [1] or by immunonephelometric or immunoturbidimetric analysis [2,3]. The sensitivities of immunonephelometric or immunoturbidimetric assay are on the order of 10^{-6} to 10^{-7} M for most of the proteins. For high-sensitivity immunoassay of proteins at 10^{-9} M or below, a solid phase-based sandwich immunoassay is a standard method. Immunoreaction is performed with two antibodies, one of the antibodies is immobilized on the solid phase, while the other is labeled for detection purpose [4].

Capillary electrophoresis (CE) is one of the most powerful tools for separating ionic species, particularly for charged biomolecules such as peptides, proteins and DNA [5–14]. In open-tube CE, the separation is based on the differences in electrophoretic mobility, which depends upon the charge-to-mass ratio. The method is rapid, applicable to small samples and very well suited to automation with real-time data analysis.

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We propose a direct immunoassay using CE. To measure the free and bound species in the presence of numerous potentially interfering substances, a uniquely detectable label on the immunochemical reactants, antibody for example, is required. Such label should provide good sensitivity in order to detect species at a relatively low concentration [15,16]. Furthermore, for many protein antigens, the antigen–antibody complex may not be readily separable from the free antibody. In these cases it is necessary also to provide means to facilitate the separation.

To achieve an effective separation of the labeled antibody from the antigen–antibody complex, the electrophoretic mobility of the antibody must be modulated by chemical modification with a well defined charge-bearing organic molecule. Succinic anhydride appears to be one of the most effective and convenient choices for charge modification of proteins. For each ϵ-NH2 of the lysine residues in proteins, succinylation results in a net gain of two negative charge at neutral buffer pH. A proper control of the succinylation should allow a significant change of its electrophoretic mobility without sacrificing its functional activity. For use in CE, fluorescent labels and laser-induced fluorescence (LIF) detection are a preferred combination. We report here the synthesis of such a charge-modifying antibody and its functional utility for the immunoassay of IgA.

2. Experimental

2.1. Materials

A fluorescein-labeled affinity pure $F(ab')_2$ fragment isolated from goat antiserum to human IgA was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Succinic anhydride (Gold Label) was derived from Aldrich (Milwaukee, WI, USA).

2.2. Charge modification of antibody

The fluorescein-labeled $F(ab')_2$ was dialyzed against 0.1 *M* sodium hydrogencarbonate at pH 8.2. The visible spectrum of the resulting solution was measured and the concentration of fluorescein in the labeled product was 10^{-5} *M*, based on the absorption of fluorescein at 495 nm ($\epsilon = 75\,000\ M^{-1}\ cm^{-1}$). A portion of the above solution, containing 2 nmol fluorescein-labeled $F(ab')_2$ in 200 μl of 0.1 *M* hydrogencarbonate buffer, pH 8.2 was mixed with 20 μl of succinic anhydride (40 mg/ml in acetonitrile) at room temperature. A portion of the reaction mixture was diluted to 20 mm phosphate, 75 mm sodium chloride, pH 7.0 (phosphate-buffered saline, PBS) and monitored by CE–LIF. The reaction reached its end-point essentially in an hour, but can be terminated readily by the addition of 1.0 *M* Tris base. The resulting product was chromatographed on a Sephadex G-25 column (25 cm × 1 cm) eluted with PBS. The molar concentration of the resulting succinyl fluorescein 5-isothiocyanate (FITC)-$F(ab')_2$ was determined from its visible spectrum.

2.3. Immunoassay protocols

Human serum-based IgG, IgA and IgM calibrators were obtained from Beckman Instruments (Brea, CA, USA). The fluorescein-labeled antibody and serum samples or calibrator were diluted in PBS containing 2 mg/ml bovine serum albumin (BSA). Appropriately diluted samples or calibrator of 10 μl each was added to 100 μl of the labeled antibody containing fluorescein-5-carboxylic acid as the internal reference. The resulting mixture was analyzed on an automated CE system equipped with a LIF detectorn as will be described later.

2.4. CE procedures

A P/ACE TM 2100 equipped with LIF detector by Beckman Instruments (Fullerton, CA, USA) was used with P/ACE system software controlled by an IBM PS/2 Model 55 SX computer. Post-run data analysis was performed on System Gold software by Beckman Instruments (Fullerton, CA, USA). The capillary column, typically 27 cm (20.5 cm to detector window) ×

20 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) was assembled in the P/ACE cartridge format with an ellipsoidal mirror to collect fluorescence. A 5.0-mW argon-ion laser emitting at 488 nm was purchased from Beckman Instruments (Fullerton, CA, USA). The fluorescence signal was collected through a narrow-band filter of 520 nm ± 9 nm (Oriel, Straford, CT, USA) while the laser beam was rejected by a notch filter at 488 nm (Barr Associates, Westford, MA, USA). During the electrophoresis run, the capillary was maintained at ambient temperature (usually 23°C) with a circulating coolant surrounding the capillary. Samples were introduced by low-pressure injection 0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 20 s. Electrophoresis was performed in a borate buffer at pH 10.2 with a voltage gradient of 800 V/cm and current of about 13.5 μA. Between runs, the capillary was sequentially washed with sodium hydroxide and water, followed by reconditioning with the running buffer.

3. Results and discussion

The fluorescein-labeled $F(ab')_2$ migrates at similar position as that of the immunoglobulins under the same electrophoretic condition (Fig. 1, curve A). Succinylation of FITC-$F(ab')_2$ antibody results in substantial differences in electrophoretic mobility from that of the FITC-$F(ab')_2$ shown in Fig. 1 (curve B). The use of succinic anhydride provides an example of the charge-based modification of the antibody for immunoassay. The extent of succinylation on the FITC-$F(ab')_2$ can be monitored by CE–LIF and may be terminated by quenching with Tris base. Most importantly, the modification is controlled by the functional requirement to alter the mobility change of the antibody relative to the mobility of the antigen for immunoassay procedure in the CE–LIF system.

Functional analysis of the charge-modified FITC-$F(ab')_2$ is evident from Fig. 2. Curve A is the electropherogram of the antibody alone at

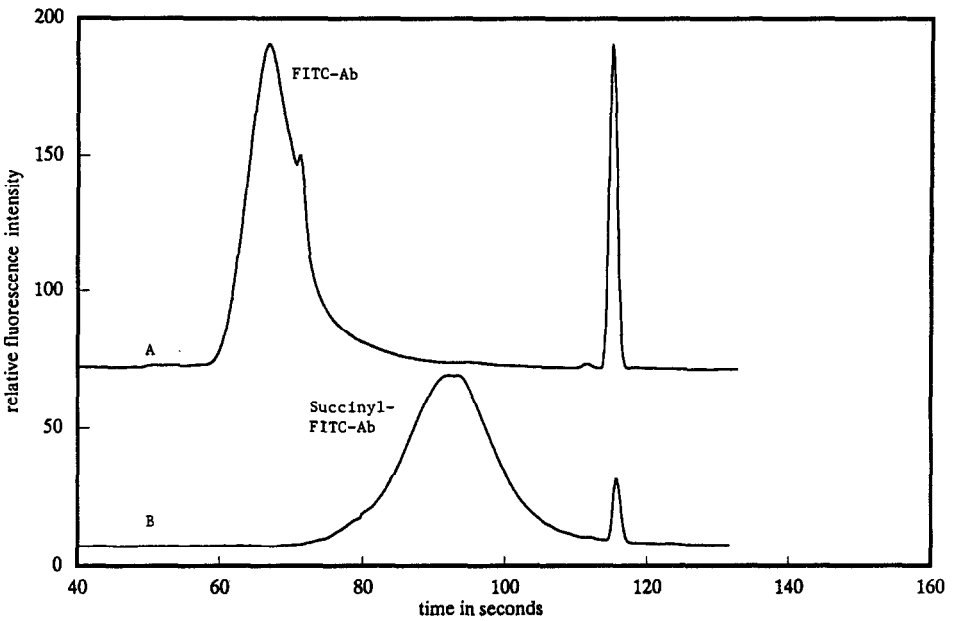

Fig. 1. A: Electropherogram of FITC-antibody to human IgA at 10^{-7} *M*. B: Electropherogram of succinyl FITC-antibody to human IgA at $5 \cdot 10^{-8}$ *M*. Conditions: 25 cm × 20 μm capillary; 80 m*M* borate buffer, pH 10.0; applied potential, 20 kV/13.5 μA; excitation wavelength, 488 nm, emission wavelength, 510 nm ± 10 nm.

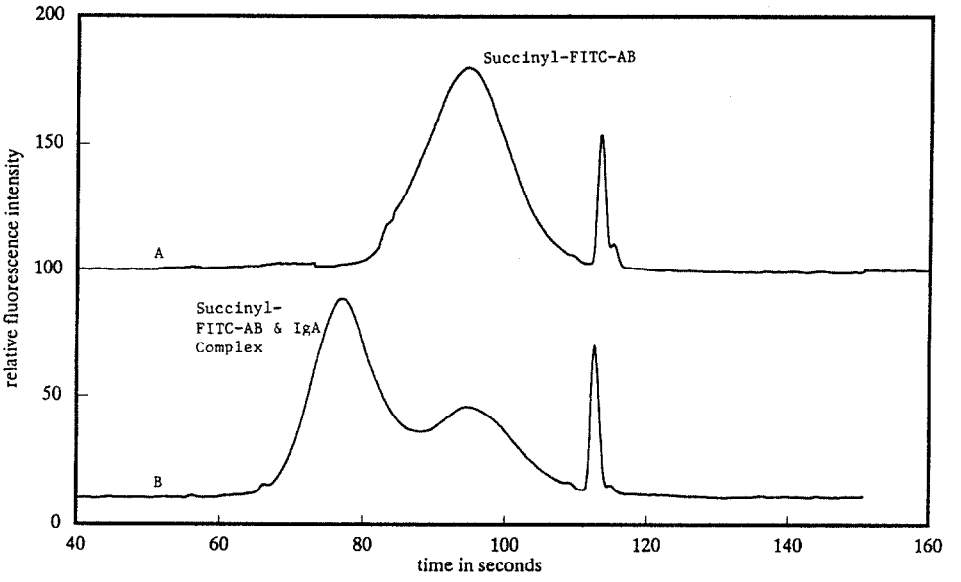

Fig. 2. A: Electropherogram of succinyl FITC-antibody to human IgA at $5 \cdot 10^{-8}$ *M* (100 μl). B: Electropherogram of reaction mixture of IgA (3 μl of 1 to 10 dilution of human serum with 1.3 mg/ml IgA) with succinyl FITC-antibody to human IgA at $5 \cdot 10^{-8}$ *M* (100 μl). Conditions as in Fig. 1.

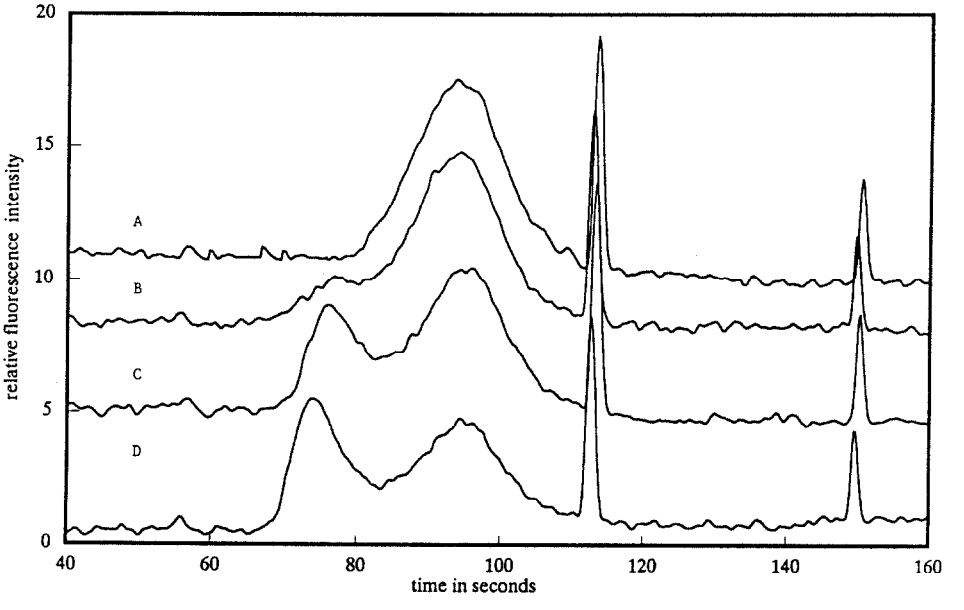

Fig. 3. A: Electropherogram of succinyl FITC-antibody to human IgA at $5 \cdot 10^{-9}$ *M* (100 μl) (no IgA). B: Electropherogram of reaction mixture of IgA (10 μl of of human serum calibrator diluted to 0.1 μg/ml IgA) with succinyl FITC-antibody to human IgA at $5 \cdot 10^{-9}$ *M* (100 μl). C: Electropherogram of reaction mixture of IgA (10 μl of human serum calibrator diluted to 0.5 μg/ml IgA) with succinyl FITC-antibody to human IgA at $5 \cdot 10^{-9}$ *M* (100 μl). D: Electropherogram of reaction mixture of IgA (10 μl of of human serum calibrator diluted to 1.0 μg/ml IgA) with succinyl FITC-antibody to human IgA at $5 \cdot 10^{-9}$ *M* (100 μl). Conditions as in Fig. 1.

$5 \cdot 10^{-8}$ M, while curve B exhibits a reaction mixture of 3 μl diluted human serum (1 to 10) with 100 μl of succinyl FITC-antibody ($5 \cdot 10^{-8}$ M). The final IgA concentration of the reaction mixture is 0.4 μg/ml. As the amount of IgA in the sample increases, the amount of the complex also increases indicating the quantitative relationship by the present technique (data shown in Fig. 3). Normal serum exhibits fluorescence background that is associated with the albumin fraction. At the present serum sample dilution, 1 to 334, the amount of fluorescence in the assay is negligible.

Addition of IgA calibrators at 0.1, 0.5 and 1.0 μg/ml to the succinyl FITC-F(ab')$_2$ showed a progressive increment of the complex formation between IgA and the succinyl FITC-F(ab')$_2$ (Fig. 3, curves B, C and D), which suggests that the formation of complex between succinyl FITC-F(ab')$_2$ and IgA is directly proportional to IgA concentration. The present method provides detection sensitivity of 0.1 μg/ml IgA, equivalent to $6.6 \cdot 10^{-10}$ M.

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