BioChromatography

Time-Resolved Immunofluorometric Detection of Antigens Separated by High-Performance Liquid Chromatography and Coated to Polystyrene

Eleftherios P. Diamandis and Theodore K. Christopoulos
Toronto Western Hospital

ABSTRACT

We use high-performance liquid chromatography with fraction collection to separate an antigen of interest. The antigen is then immobilized on polystyrene microtiter wells and detected with a specific antibody, followed by a second biotinylated antibody and streptavidin labeled with a fluorescent europium chelate. Fluorescence can be quantified with the use of time-resolved fluorescence. Antigen detectability down to 2–3 × 10^{-17} mol was achieved. This method could be used as an alternative to Western blot in certain applications.

INTRODUCTION

The Western blot technique, introduced in 1979 by Towbin et al. (6) and reviewed in 1984 (5), still enjoys widespread use in biotechnology applications. The method involves electrophoretic separation of proteins in polyacrylamide gels and subsequent transfer to nitrocellulose by electroelution. The proteins are then detected by use of specific antibodies. An alternative to Western blotting would be the separation of proteins by other means (e.g., liquid chromatography) and immunological detection of proteins in the eluate fractions by using either competitive or noncompetitive immunoassay. This method has been used frequently, but is more demanding than a Western blot because (a) in noncompetitive immunoassays, two antibodies against the antigen of interest are needed and (b) in competitive immunoassays, labeled antigen or highly purified solid-phase antigen are needed to set up the assay.

Table 1. Detection Limits, Range of Linear Response and Slopes of Calibration Curves for Antigens Coated on Polystyrene Microtiter Wells

Antigen	Detection Limit[a]		Linear Regression Analysis[b]	
	pg/well	Moles × 10^{-17}/well	Linear Range Moles × 10^{-17}/well	Slope
Mouse IgG	4	2.5	13–3200	28
α-Fetoprotein	1.7	2.5	74–4736	14
Carcinoembryonic antigen	2.7	1.4	20–640	117
Ferritin	13	2.8	17–4400	22

[a]Detection limit is defined as the amount of antigen distinguishable from zero with 99% confidence.

[b]Calibration curves were the plots of log (fluorescence) vs. f[log (antigen amount)]. Fluorescence was in arbitrary units and antigen amount was in mol × 10^{-17}/well.

We have examined the possibility of using solid-phase coating of antigen present in chromatographically separated fractions as an alternative to Western blot. The coated antigen was detected by using a monoclonal antibody specific for the antigen, followed by a biotinylated goat anti-mouse antibody (GAMIg) and streptavidin labeled with the fluorescent europium chelate of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (2,4). The final product formed on the solid phase (polystyrene microtiter wells) is solid-phase-antigen-antibody (mouse)-GAMIg-biotin-streptavidin-BCPDA-Eu^{3+}. This complex can be measured on the dry solid phase by using time-resolved fluorometry (1).

MATERIALS AND METHODS

We coated white opaque polystyrene microtiter wells (Dynatech Labs, Alexandria, VA) with varying amounts of antigens. Coating (50 µl/well) of antigens dissolved in a 50-mM Tris buffer, pH 7.40, was performed for 3 h with mechanical shaking at room temperature (RT). After washing the wells once with the wash solution (this and other solutions not described are as published in Reference 3), we added the

primary mouse antibody (500 ng/100 µl/well) and incubated it for 2 h, as described above. We then washed once before adding the biotinylated GAMIg (100 ng/100 µl/well) for 1 h. After another wash, we added the streptavidin tracer and incubated it for 30 min, followed by washing 4×. The wells were then dried with a stream of forced air. The fluorescence was quantified on a Model 615 time-resolved fluorometer from CyberFluor, Toronto, Canada. The diluent for the primary antibody and GAMIg was a 50-mM Tris buffer, pH 7.40, containing 3% (wt/vol) of bovine serum albumin (BSA). Primary antibodies used were from Medix Biochemica, Kauniainen, Finland (carcinoembryonic antigen, CEA, Code No. 5914; α-fetoprotein, AFP, Code No. 5108) and OEM Concepts, Toms River, NJ (Ferritin, Code No. A027). The GAMIg was from Atlantic Antibodies, Scarborough, ME, and was biotinylated as previously described (3). Antigens were from Scripps Laboratories, San Diego, CA. All glassware, tubes and pipet tips were blocked with a 1% BSA solution and washed thoroughly with water before use.

RESULTS AND DISCUSSION

The detection limits of the antigens coated to the wells with the above method and the slopes of the calibration curves in the near-linear response range are shown in Table 1. In Figure 1, a calibration curve obtained by coating varying amounts of mouse IgG is shown. In this case, primary antibody is not needed for detection. The curves for all other antigens tested had a similar shape and leveled off at 220 000 arbitrary fluorescence units, although the slopes of the near-linear region are different (Table 1). The leveling off of fluorescence indicates the point of saturation of the solid phase with antigen which is around 6 ng/well.

Individual antigens were injected into a high-performance gel filtration liquid chromatography column (Superose 6, Pharmacia LKB Biotechnology, Piscataway, NJ) eluted with a 50-mM Tris buffer; pH 7.20, containing 9 g/l NaCl. The flow rate was 0.5 ml/min, and individual fractions were collected (0.5 ml fraction size). Fraction aliquots (50 µl/well, triplicate) were pipetted into polystyrene microtiter wells and coated and stained with antibodies and streptavidin, as described above. An example of the results obtained is shown in Figure 2 for AFP (8 ng of antigen injected). When the column was calibrated with molecular weight markers under identical conditions (markers from Bio-Rad Labs [Canada] Ltd., Mississauga, Ontario), AFP was detected at the point which corresponds to a molecular weight of 78 000 (theoretical 69 000). The proposed method has the following advantages: very low detection limits (pg vs. ng for typical Western blots) (5); no need for electrotransfer; ability to study and simultaneously obtain pure fractions of antigen of interest; and potential for quantification by using calibration curves as shown in Figure 1.

The disadvantages are inferior resolution and potential for misleading results if other proteins co-elute at the same fraction at much higher concentrations than the antigen of interest. This problem is not due to antibody cross-reactivity, but rather to saturation of the solid phase with the co-eluting protein. If this happens, the coating of antigen of interest may be prevented or severely reduced. When there is some knowledge of the problem at hand, this method could be a useful alternative to Western blotting.

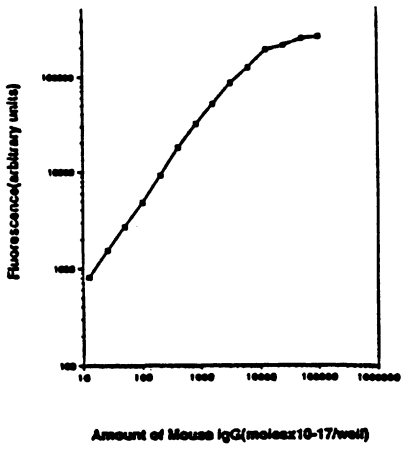

Figure 1. Relationship between fluorescence and amount of mouse IgG coated onto polystyrene microtiter wells. A near-linear relationship exists up to 3200×10^{-17} mol of IgG/well. Similar curves were obtained for the other antigens listed in Table 1.

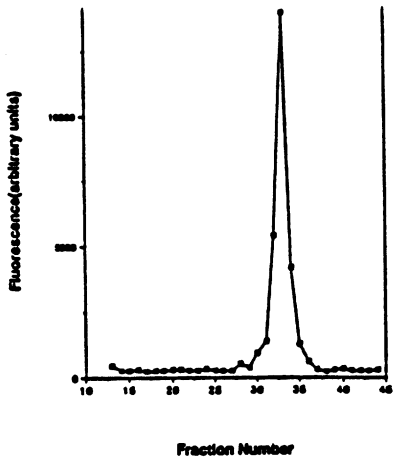

Figure 2. Elution pattern of AFP from the column, when detected with the proposed method. About 8 ng of antigen were injected and 50 µl were coated from the 500-µl fraction. The calculated mol wt of AFP from the elution fraction was ca. 78 000 (theoretical 69 000).

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Address correspondence to:
E.P. Diamandis
Department of Clinical Biochemistry
Toronto Western Hospital
399 Bathurst Street
Toronto, Ontario M5T 2S8, Canada