Immunofixation Following Electrophoresis or Isoelectric Focusing for Identification and Phenotyping of Proteins

A. MYRON JOHNSON, M.D.

Department of Pediatrics,
University of North Carolina School of Medicine,
Chapel Hill, NC 27514

ABSTRACT

Immunofixation following electrophoresis or isoelectric focusing has proven to be a powerful tool for the phenotyping of plasma proteins, the identification of proteins such as M-components and the study of conversion or breakdown products of proteins such as C3. One of the major advantages is the ability to compare, side by side, the mobilities of relatively low-concentration proteins. The technique is relatively simple, can be used with most types of gels and can be enhanced when necessary by the use of enzyme- or radiolabeled antibodies. The cost is competitive with other immunochemical procedures which are more tedious but which give less information.

Introduction

Wilson19 and Afonso1 first proposed the direct precipitation of proteins in gels following electrophoresis. This “direct immunoelectrophoresis” involved a standard electrophoresis in agar on microscope slides, with antiserum pipeted over the gel rather than placed in troughs, as is done in routine immunoelectrophoresis. Precipitin patterns were thus enhanced, but resolution was poor compared to immunoelectrophoresis.

Alper and Johnson3 utilized antigen fixation by precipitation with monospecific antiserum following high-resolution agarose gel electrophoresis for the identification of genetic variants of human ceruloplasmin and group-specific component and of conversion products of the third component of complement, C3. Subsequent studies by these and other authors have confirmed the usefulness of this method, called “immunofixation electrophoresis,” for the study of these proteins4,11 as well as α1-antitrypsin,10,15,17 orosomucoid,12 immunoglobulins (normal and M-components),18 properdin factor B,2 transferrin and others.16 In the cases of orosomucoid and α1-antitrypsin, acidic gels (each circa pH 5) of starch or agarose have also been used.12,15,17

More recently, immunofixation has been used following isoelectric focusing in agarose-acrylamide10 and acrylamide5 gels. In addition, Arnaud and coworkers have suggested the use of a cellulose acetate overlay method which results in an “immunofixation print,” or “immunoprint.”5
Principle

Specific antibodies applied to the surface of a gel following electrophoresis or isoelectric focusing diffuse into the gel and form immune complexes with the respective antigen at the site to which it has migrated. The antigen-antibody complexes are too large and insoluble to wash out of the gel's pore structure as long as slight to moderate antibody excess is present. The remaining unprecipitated proteins are washed out of the gel, leaving only the desired immunoprecipitates. These may then be stained with protein stains or reacted with second antibodies labeled with enzymes, such as horseradish peroxidase, or radioisotopes, washed and identified appropriately.

Reagents and Standards

High titer, specific antisera to the proteins of interest may be raised or purchased commercially. Several antisera, raised in goats* or rabbits†, have been found to have adequate titers for the usual procedures. For use following isoelectric focusing in acrylamide gels, the IgG fraction of rabbit antibodies has been found by the author to work best.

Samples of serum or other fluids must be diluted so that the protein in question has a concentration of approximately 0.1 to 0.5 g per liter (10 to 50 mg per dl), depending upon the protein and the titer of the antiserum. Previous reports may be used as starting points for serum and antiserum dilutions, particularly reference 16.

For lower concentration antigens, the antibodies, or second antibodies directed against the first, may be labeled with an enhancing agent. The use of horseradish peroxidase, coupled by the two-step method of Boorsma and Kalsbeek,§ with 3-amino-9-ethylcarbazole¶ as substrate is recommended by the author.

For distinguishing and identifying genetic variants of proteins, samples with known phenotypes must be available for comparison with unknown test samples. For conversion products, as with C3, intact and converted protein should be run beside test samples.

For routine staining, saturated Amido Black or 0.1 percent Coomassie Brilliant Blue R250 in methanol:acetic acid:water, 9:2:9, is recommended. The same solvent mixture is used for destaining. Coomassie Blue G250 is not as sensitive as R250, since it stains only the proteins at the gel surface and must be destained very cautiously to avoid the loss of all color.

No special apparatus is required for the immunofixation and subsequent processing. Almost any system for high-resolution electrophoresis or isoelectric focusing may be used for the preliminary separation.

Procedure

Samples of serum or other fluids are diluted as discussed previously and are then subjected to separation in agarose, starch, or acrylamide gels. Immediately following the separation procedure, the gel, which is on a mylar, plastic or glass support, is laid on a flat surface.

High-titer monospecific antiserum is applied to the gel to cover the area where the protein of interest is known to migrate. Markers such as bromophenol blue-albumin or hemoglobin A may be used during separation to aid in the location. Application of antiserum may be made in either of two ways: (1) one or two drops of antiserum per square centimeter are applied and spread over the desired area with a glass rod or fingertip or (2) a cellulose acetate strip is saturated with antiserum, drained and applied to the surface of the gel, being careful to avoid air

* Atlantic Antibodies, Westbrook, ME 04092; Technicon Instruments Corporation, Tarrytown, NY 10591.
† DAKO-immunoglobulins a/s, 22 Guldborgvej, DK-2000 Copenhagen F, Denmark; Accurate Chemical and Scientific Corporation, 28 Tec Street, Hicksville, NY 11801.
bubbles. If strips with different antisera are used on the same gel, the strips must not touch each other. With either technique, a small volume of high-titer antiserum will give better precipitates than a larger volume of dilute antiserum. The second method is more likely to result in antigen excess than the first; therefore, samples generally should be diluted two to ten times more.

The gel is then covered with a tray, preferably with a moist paper towel taped to the inside of the top, and incubated at room temperature for 30 to 120 minutes. Longer times and warmer temperatures may result in excessive drying unless the chamber is quite moist. The time required for immunoprecipitation depends upon the type and thickness of gel. Agarose gels one to 1.5 mm thick require 30 to 60 minutes, whereas sieving gels require up to two hours.

Following diffusion and precipitation, the cellulose acetate strip (if used) is removed. If an immunoprint is desired, the strip may be placed in saline overnight, with the gel-contact surface upwards, exposed to the saline. Fingerprints on the surface must be avoided. The strip is then rinsed with water and stained by brief immersion in Coomassie Brilliant Blue R250. Ten percent glycerine should be added to the destaining solution to avoid subsequent cracking. The strip may then be blotted dry and preserved.

Meanwhile, the agarose gel is covered with a wet sheet of Whatman No. 1 filter paper, several absorbent paper towels, a plate of glass or thick plastic and a two to three kg weight. The gel should be pressed for at least ten minutes. In the case of starch or acrylamide gels, the surface may be rinsed gently with saline, but pressure on the surface should be avoided.

The unwanted proteins are then removed by soaking the gel in physiological saline, preferably with mild agitation as with a slow orbital shaker. If whole or partially fractionated antiserum is used, agarose gels will clear in 16 to 24 hours, whereas sieving gels take 48 to 72 hours.

**Figure 1.** Immunofixation patterns of group-specific component, or Gc-globulin, phenotypes in amniotic fluid (top) and serum. The initial separation was by agarose gel electrophoresis at pH 8.6.
with two or three changes in saline. With purified IgG fractions, background clearing is faster and more complete. The saline is removed by soaking the gel in tap water for at least 30 minutes. The gel is then processed and stained by the routine procedures used for the particular gel type.

Results

Representative immunofixation patterns of agarose gel electrophoretic and agarose-acrylamide gel isoelectric focusing separations are shown in figures 1 and 2 and figure 3, respectively. It can be seen that direct side-by-side comparison of mobilities of genetic variants of $\alpha_1$-antitrypsin and group-specific component (Gc globulin or Gc) is possible with relatively high resolution following agarose gel electrophoresis, and even higher resolution following isoelectric focusing.

In the case of relatively low concentration proteins such as Gc, the identification of variants is also aided by the fact that all other proteins of similar mobility are washed out of the gel. In addition, the enhanced visualization permits phenotyping body fluids such as amniotic fluid with even lower concentrations than serum, as shown in figure 1.

Discussion

Electrophoresis and isoelectric focusing have been used extensively for the study of protein polymorphisms and conversions, such as activation and complexing. These separatory procedures alone do not permit the unequivocal identification of more than a few bands in serum or other mixed protein solutions and are relatively insensitive for the localization of many low-concentration proteins or fragments thereof.

Previous immunochemical methods used to enhance the phenotyping of proteins and to study conversion include, primarily, immunoelectrophoresis and crossed immunoelectrophoresis. The former has been used extensively for the study of Gc-globulin, among other proteins. However, even with prolonged electrophoretic separations, the long period of diffusion results in such long precipitin arcs that minor differences in mobility are difficult to ascertain.

![Figure 2. Patterns of serum $\alpha_1$-antitrypsin variants as shown by immunofixation following prolonged agarose gel electrophoresis at pH 8.6. (From Johnson, A. Myron: Genetic typing of $\alpha_1$-antitrypsin by immunofixation electrophoresis. Identification of subtypes of Pi M. J. Lab. Clin. Med. 87:152-163, 1976.)](image-url)
In crossed immunoelectrophoresis, a strip is cut from an electrophoretic or isoelectric focusing gel. The separated proteins in this strip are driven by a second electrophoresis, with the axis perpendicular to the first, into agarose gels containing antibody. This is a much more sensitive procedure. However, it does not permit accurate comparison of mobilities because of the nature of the second electrophoresis. A known marker protein may be added to each sample to improve comparisons. If minor differences in mobility are not important, crossed immunoelectrophoresis remains one of the best methods for detecting low concentration conversion products, such as C3c.

Immunofixation is of major usefulness in the genetic phenotyping of proteins, particularly low-concentration ones which are not readily detectable by specific characteristics such as enzyme activity. Its application to very high resolution techniques such as isoelectric focusing has just begun and will undoubtedly increase with time.

Most immunofixation procedures require 20 to 200 μl of high-titer, monoclonal specific antiserum per sample. The cost is still competitive with that of other procedures with less resolution, sensitivity, or both.

**Sources of Error**

Unacceptably wide bands may result from excessive diffusion of antigen. It is essential that the separatory step be set up and run as quickly as possible. Likewise, the antiserum must be spread evenly over the area of interest.

Antigen excess, which may result from inadequate sample dilution or too low titer antiserum, also results in diffusion and wider bands. In greater extreme, anti-
gen excess will result in central clear spots or “holes.” This is a particular problem with isoelectric focusing, in which antigen is highly concentrated into a narrow band. Samples must be diluted more than for agarose gels, therefore. In addition, for the patterns in the acrylamide gels themselves, direct application of antiserum is less likely to result in antigen excess than is application with a cellulose acetate strip. The latter is recommended only if an immunoprint is desired or if several different antisera are to be applied to samples in the same gel.

Antiserum raised in horses may be unsatisfactory because of the narrow range of antigen-antibody ratios giving insoluble precipitates. If only such antiserum is available, a wide range of antigen and antibody dilutions should be tried.

Unwanted bands will confuse interpretation. For this reason, monospecific antiserum is generally preferred. In addition, labile proteins or protein mixtures should be stored with a preservative, frozen below −65°, or both.

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References