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EFFICIENT METHOD FOR VISUALIZATION AND ISOLATION OF PROTEINS RESOLVED IN POLYACRYLAMIDE GELS

RALPH T. FRANCIS, Jr.*, JAMES R. DAVIE*, MIKE SAYRE**, ELISABET ROCHA***, FOREST ZIEMER and GEORGIA RIEDEL

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331 (U.S.A.)

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SUMMARY

Polyacrylamide gel electrophoresis is a popular method used to purify proteins for reconstitution experiments, amino acid composition and sequence determinations. In this report we describe methods that will be of general use in the isolation and characterization of proteins and the benefits of substituting boric acid for glycine in the electrophoresis tray buffers. We also describe how proteins resolved in a variety of gel systems (including those containing sodium dodecyl sulfate) may be rapidly visualized with 8-anilino-1-naphthalene sulfonic acid and efficiently transferred to a second gel for two-dimensional gel analysis, or isolated by electroelution for subsequent characterization.

INTRODUCTION

In the course of attempting protein separation and purification, many problems may arise regarding adequate resolution. The use of gel filtration and ion-exchange column chromatography often leaves much to be desired when applied to a system with proteins of similar charge-to-mass ratios and/or sizes. The use of polyacrylamide gel electrophoresis (PAGE) systems for separation of proteins via electrophoretic mobility in either a non-denatured or denatured state [*e.g.* sodium dodecyl sulfate (SDS) gels] has been very popular and widely used. In most cases, the acrylamide gels offer significant enhancement of protein separation over the above-mentioned chromatographic methods.

We have used various electrophoretic techniques to purify and analyze various protein species. However, during the course of this work we have found some methods to be unsuitable regarding the purity of an isolated protein. For example,

* Present address: Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada.

** Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093, U.S.A.

*** Present address: Departamento Quimica Marcromolecular (C.S.I.C.), Universidad Politecnica de Barcelona, Diagonal 647, Barcelona 28, Spain.

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we have found that proteins resolved and isolated from polyacrylamide SDS gels prepared as described by Laemmli[1] contained a large amount of glycine when their amino acid compositions were determined. This high background of glycine resulted from the tray buffer. In this report we demonstrate that a relatively low concentration of boric acid may be substituted for glycine as the stacking anion without any loss of resolution of the proteins.

Hartman and Udenfriend[2] and Nerenberg *et al.*[3] have reported that 8-anilino-1-naphthalene sulfonic acid (ANS) may be used to visualize rapidly proteins in non-denaturing polyacrylamide or agar gels. However, this staining technique has not been applied to gels containing SDS[4]. In this report, we demonstrate that ANS may be used for a variety of polyacrylamide gels including those that contain SDS. Furthermore, we demonstrate that ANS staining does not interfere with the recovery of the protein by electroelution, or its migration into a second dimension gel such as a polyacrylamide acid–urea gel.

EXPERIMENTAL

Preparation of human plasma

Human plasma was prepared as follows. Whole blood was taken up in 0.9% sodium chloride–heparin solution and centrifuged at 2000 *g* for 20 min at 4°C. The plasma supernatant (liquid) was removed and dialyzed against 0.10 *M* Tris–HCl, pH 7.4, for 24 h at 4°C.

Preparation of histones

Testes at intermediate stages of development were obtained from naturally maturing rainbow trout (*Salmo gairdnerii*) and stored at −80°C until use. The testes were homogenized in 10 volumes of Buffer A [1 *M* hexylene glycol, 10 m*M* piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPES), pH 7.0, 2 m*M* magnesium dichloride, 1% thiodiglycol, 30 m*M* sodium butyrate]. The scissor-minced tissue was homogenized in a Virtis homogenizer at the lowest speed for 1 min. The homogenate was filtered through four layers of cheesecloth and centrifuged at 750 *g* for 10 min (Sorval SS-34 rotor). The pellet was resuspended in Buffer A and again centrifuged. The resulting pellet was then resuspended in 3 volumes of Buffer B (10 m*M* Tris–HCl, pH 8.0, 0.6 *M* sodium chloride) and centrifuged at 12,000 *g* for 30 min. The pellet was resuspended in 3 volumes of Buffer B and centrifuged. These steps removed the majority of the non-histone chromosomal proteins and histone Hl. The pellet was extracted with 0.2 *M* sulphuric acid (30 min on ice) and insoluble material was removed by centrifugation (12,000 *g* for 30 min). The acid extract was dialyzed overnight at 4°C against 0.1 *M* acetic acid, lyophilized and redissolved in distilled water. The extracted proteins consisted of histones H2A, H2B, H3, H4, and some histone H1. Calf thymus and chicken erythrocyte histones were prepared as described by Davie *et al.*[5].

Polyacrylamide gel electrophoresis

Analytical gel electrophoresis. The minislab apparatus was purchased from Idea Scientific (Corvallis, OR, U.S.A.). The polyacrylamide SDS minislab gels were prepared as described by Davie[6], except that in some cases riboflavin (0.0004%) was

used as the catalyst. The "native" or non-denaturing gels were made up using the Davis method[7], except that riboflavin (0.0004%) was used as the catalyst. For the SDS gels, either Tris–glycine–SDS[6] or Tris–borate–SDS tray buffers were used. The borate tray buffer was made up as follows: 0.05 *M* Tris, 0.06 *M* boric acid, 10^{-4} *M* EDTA with 0.1% SDS. For the native gels, only the Tris–borate tray buffer was used (without the SDS). The acetic acid–urea (AU) minislab gels were prepared as described by Davie[6].

Staining. After electrophoresis the gel was stained with 0.25% Coomassie blue G-250 in 45% methanol and 9% acetic acid. The gel was destained briefly (30–60 min) by diffusion in methanol–acetic acid–water (2:1:5, v/v) and then destained further in 7.5% acetic acid and 5% methanol. Alternatively, the gel was stained with ANS (magnesium salt, Kodak). SDS gels were prewashed with 20 m*M* Tris–acetate, pH 8.8, for 30 min. This step removed the unbound SDS which would also stain with the ANS. This step was not required for the native or AU gels. The gel was then stained for 10–30 min with 0.003% (w/v) ANS[2,3] in a 0.1 *M* sodium phosphate buffer, pH 7.0. Destaining was not necessary. The band was visualized with ultraviolet light. A comparison of Coomassie blue-stained duplicate gels with or without previous ANS staining indicated that this procedure did not result in noticeable protein losses.

Electroelution. Following staining, the track of interest was cut out from the gel and equilibrated for 20–30 min in Buffer 0 of O'Farrell[8] (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 m*M* Tris–HCl, pH 6.8). The gel slice was placed into a dialysis bag containing either the Tris–glycine–SDS or the Tris–borate–SDS tray buffer. Electrophoresis was performed at 100 V for 24–48 h at 4°C.

Removal of dye and SDS. Following electroelution, the contents of the dialysis bag (except the gel slice) were dialyzed against distilled water, lyophilized and redissolved in 40 μl of distilled water. The sample was treated by ion-pair extraction using solvent system A as described by Henderson *et al.*[9].

RESULTS AND DISCUSSION

Boric acid may be substituted for glycine in tray buffers

As the presence of glycine interfered with the determination of a protein's amino acid composition (*i.e.* a protein isolated from a gel where glycine was present in the tray buffer), we wanted to know if a low concentration of boric acid could be effectively substituted for the glycine (borate buffers were first used in electrophoresis by Consden and Stanier[11] for carbohydrate separation). The results suggested that, indeed, boric acid could be used in both SDS and native gels to resolve the different proteins (Fig. 1) and exhibited the same stacking capabilities as glycine. In addition, the resolution of various proteins on the gels containing either boric acid or glycine in the tray buffer was similar (compare trout histone pattern (Fig. 1, lane c) to calf thymus histone pattern (Fig. 2, lane c)). However, borate complexes with and changes the charge on certain carbohydrates[11], so the migration of glycoproteins in native gels may be noticeably altered.

There are several benefits in using boric acid instead of glycine in PAGE. First, when keeping the Tris component (50 m*M*) of the tray buffer the same as with the conventional glycine buffer, far less boric acid was needed to obtain the same pH as with glycine (*e.g.* 0.38 *M* glycine *versus* 0.06 *M* borate). This is beneficial, as the gel

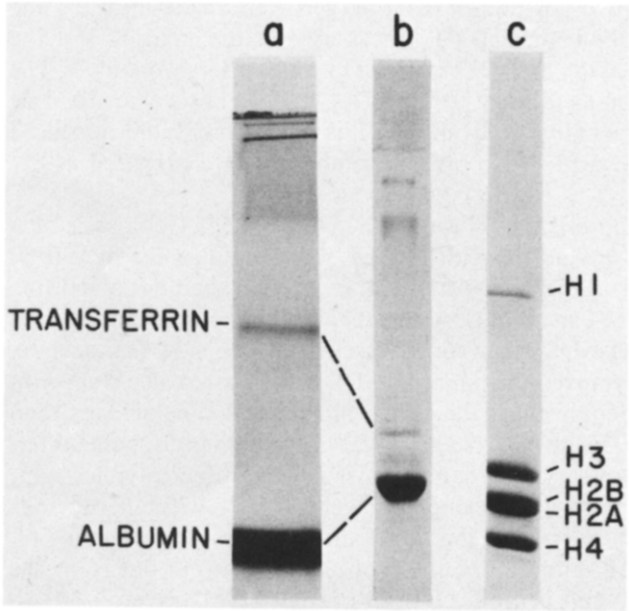

Fig. 1. (a) Human plasma proteins resolved on a 7% polyacrylamide native minislab gel with Tris–borate for the tray buffer; (b) human plasma proteins on a 8% polyacrylamide SDS minislab gel with Tris–borate–SDS for the tray buffer; (c) trout testis histones resolved on a 15% polyacrylamide SDS minislab gel with Tris–borate–SDS for the tray buffer. All gels were stained with Coomassie blue.

runs with less current at the same voltage and the gel temperature stays lower. This allows the same system to be run at a high voltage (*e.g.* 200 V), thus resulting in faster protein separation. Second, the most conspicuous advantage in using boric acid is the absence of the anomalous glycine background in the amino acid analysis data of an excised protein. This advantage is especially significant when attempting quantitation of very low levels of amino acids (*e.g.* 10^{-12} *M*).

ANS may be used to stain rapidly a variety of gels

ANS has been used for the visualization of proteins in non-denaturing polyacrylamide[2] or agar gels[3]. ANS will fluoresce when attached to proteins, presumably by the binding of the fluorophore to the hydrophobic portions of the molecule. However, it will not fluoresce alone in water[2,3]. We wanted to determine if ANS could be used for staining SDS or AU polyacrylamide gels. The results indicated that for both of these gel systems, ANS could be used to visualize the proteins (Fig. 2). In addition, ANS would stain proteins resolved on native or SDS gels that contained boric acid instead of glycine in the tray buffer (Fig. 2, lanes g and i). In the case of the SDS gels, the unbound SDS (*i.e.*, SDS not associated with the proteins) had to be removed from the gel as ANS will also fluoresce in the presence of SDS. This was done by soaking the gel in 20 m*M* Tris–acetate, pH 8.8, which allowed the unbound detergent to diffuse out of the gel. As was stated in the Experimental section, this prestain wash did not cause any detectable losses of any of the proteins included in this study. For

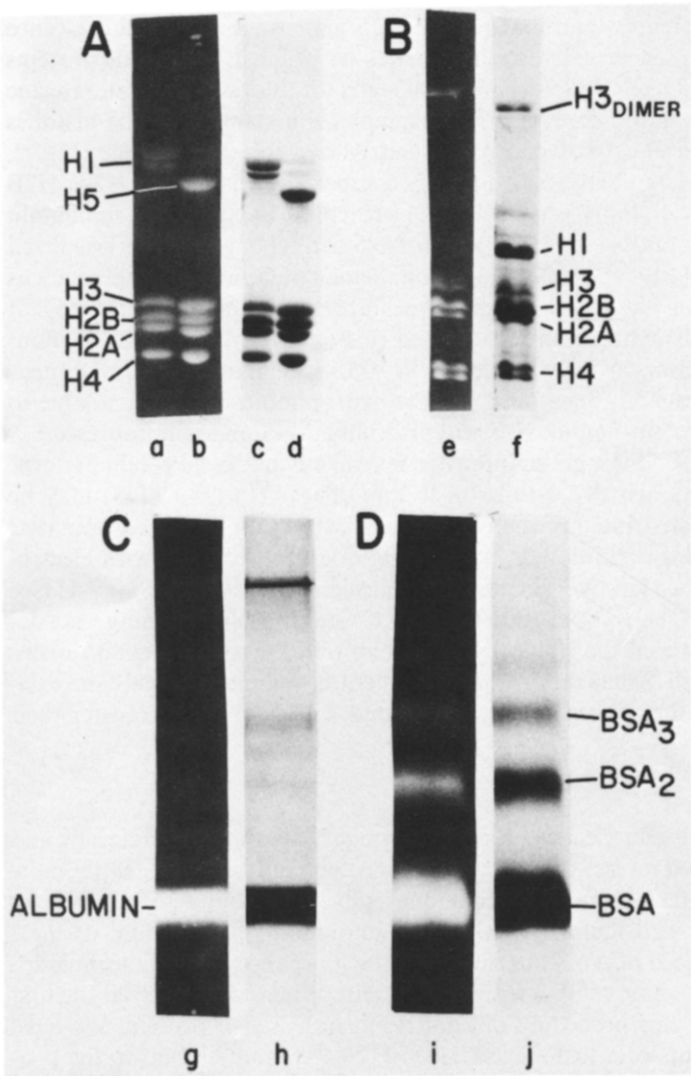

Fig. 2. (A) 15% polyacrylamide SDS minislab gel containing calf thymus histones (lanes a and c) and chicken erythrocyte histones (lanes b and d). The tray buffer was Tris–glycine–SDS; (B) 15% polyacrylamide AU minislab gel containing calf thymus histones (lanes e and f); (C) 7% polyacrylamide native minislab gel containing human plasma proteins (lanes g and h). The tray buffer was Tris–borate; (D) 7% polyacrylamide native minislab gel containing 12 μg of BSA (oxidized) (lanes i and j). Tray buffer again was Tris–borate. a, b, e, g and i were stained with ANS and c, d, f, h and j were stained with Coomassie blue.

native gels, the gel can be stained directly with ANS as has been previously described[2,3].

For polyacrylamide SDS gels, a band containing 0.5 μg of protein could be visualized. This sensitivity of staining with ANS was *ca.* 2–3 times less than that of Coomassie blue. For native or AU gels, the amount of protein required for visual-

ization with ANS was greater than that for the SDS gels. As the ANS fluorescence is dependent on the presence of hydrophobic sites to which it can bind, proteins resolved on native or AU gels will vary in their degree of fluorescence and, in some cases, proteins will not fluoresce at all. For example, the staining of the histones H2A, H2B, H3, H4 and H1, resolved on AU gels, varied for each histone (Fig. 2, lanes e and f). Histone H1 and H3 stained poorly compared with histones H2A, H2B and H4. However, high mobility group (HMG) proteins 14 and 17, which contain little in the way of hydrophobic sites[10] to which ANS can bind, cannot be visualized in ANS-stained AU gels (not shown). In addition, heme-containing proteins, such as hemoglobin, resolved on native gels quench the fluorescence of ANS and do not stain. However, most proteins we have examined (histones, HMG 14 and 17, albumins, hemoglobin and transferrin) will stain with ANS when resolved on SDS gels. This is due to (1) the protein is denatured and the hydrophobic sites are available to the ANS and (2) the protein-bound SDS will also bind ANS and will fluoresce.

The utilization of ANS for gel staining has several advantages. Protein patterns can be visualized rapidly, usually within 10–40 min. The ANS (and SDS) may be easily removed from electroeluted protein by ion-pair extraction whereas Coomassie blue cannot (see below). In addition, ANS staining does not interfere with electrophoretic transfer of proteins in two-dimensional gel electrophoresis (SDS → AU (see Davie[6]). In the SDS → AU system (not shown), Coomassie blue staining and destaining significantly lowered the efficiency of protein transfer to the second dimension[6]. Furthermore, ANS visualization for subsequent first dimension gel lane excision is preferred to blind excisions as we have found wider gel slices transfer their contents less efficiently.

Recovery of protein(s)

Following excision and electroelution of a stained band(s) (see Materials and methods) which recovered no less than 80% of the protein band, we wanted to know if, first, would the ion-pair extraction technique (solvent system A) described by Henderson *et al.*[9] work satisfactorily with small amounts of protein (*e.g.* 10 μg)? Second, would the presence of ANS interfere with the ion-pair extraction technique? This technique is used to remove SDS from the protein. When we addressed the first question, we found that this procedure did not result in losses of protein. We have subjected various amounts of a histone sample (10, 20, 100 and 200 μg) to the procedure and did not observe detectable losses of the histones or their associated modified forms when resolved on AU gels (not shown). When we addressed the second question, we found that ANS did not interfere with the recovery of the protein when using the ion-pair technique. Furthermore, we have found it to be advantageous to use ANS rather than Coomassie blue to detect the protein bands (see above) as ANS, but not all of the Coomassie blue was removed by ion-pair extraction. The presence of Coomassie blue in the sample led to the formation of a water-insoluble precipitate which contained both dye and protein.

Through the use of the above procedures (SDS or acid–urea polyacrylamide gel electrophoresis, ANS staining, electroelution, and ion-pair extraction), we have been able to purify a variety of proteins including histone H4 and the unacetylated and monoacetylated species of histone H4 (not shown).

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