Original papers

David W. Sammons,
Lonnie D. Adams
and Edward E. Nishizawa

Diabetes and Atherosclerosis Research, The Upjohn Company, Kalamazoo

Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels

A color development system for staining polypeptides in one- and two-dimensional polyacrylamide gel electrophoresis is described. The basis of the process involves the complexing of silver with polypeptide reactive centers. The reaction is initiated by placing a polypeptide-containing gel, previously equilibrated with an appropriate concentration of silver nitrate, into a reducing solution that contains sodium hydroxide, sodium borohydride, and formaldehyde. After an appropriate time in the reducing solution, the gel is equilibrated through two changes of an enhancing solution that contains sodium carbonate. The sodium carbonate is necessary for optimal color development and prevention of excessive swelling of the gel. Optimal colors appear in the polypeptide-silver complexes after several hours in the enhancing solution and are best appreciated while viewing over a fluorescent light box that radiates light at 5000 °K. The color of each polypeptide-silver complex is clearly visible above the light background of the stained polyacrylamide gel. Colors of stained polypeptides are blue, green, yellow, and red. Subtle shades of colors also appear and thereby allow easy discrimination of overlapping spots of polypeptides in a two-dimensional gel. To illustrate the method's relative sensitivity, a two-dimensional pattern of human fibroblast polypeptides is compared with patterns of a duplicate gel that is stained with Coomassie Blue and developed by autoradiography. The sensitivity of the silver stain process is superior to Coomassie Blue and is comparable to autoradiography after incorporation of conventional levels of ^{35}S-methionine. The utility of the procedure for identifying and characterizing human proteins is illustrated by staining human plasma and platelet polypeptides after two-dimensional gel electrophoresis. The gel electrophoresis color development system consists of steps that are simple, reproducible, and sensitive, and most importantly, which yield colored polypeptide-silver complexes that are reproducible from gel to gel and tissue to tissue.

1 Introduction

Combining isoelectric focusing (IEF) in one direction and sodium dodecyl sulfate (SDS) electrophoresis in the other [1] has stimulated a flurry of recent developments that include the two-dimensional (2-D) gel ISO-DALT system [2–4]. Advances in one technology naturally lead to advances in another. Thus, as 2-D gel electrophoresis demonstrated high resolving power not previously realized by prior techniques, the deficiencies of staining methodologies became more apparent, thereby calling for new ultrasensitive methods for staining polypeptides. Monochromatic staining dyes such as Coomassie Blue are insensitive and stain only the most abundant polypeptides. Ultrasensitive polypeptide detection is possible with incorporation of radiolabeled amino acids or other radiolabeled precursors of proteins; however, the procedure requires active protein synthesis. Often the signal of the incorporated radiolabeled precursor is too weak, and many of the cells' protein gene products are not detected. In addition, proteins present in body fluids and in human biopsy samples cannot be practically radiolabeled. A series of procedures has evolved that utilizes the binding of silver to protein reactive centers [5–13]. The silver staining methods were borrowed from histology and photography, are monochromatic, and are ultra-sensitive if performed on gels less than 1 mm thick. Serious disadvantages peculiar to these methods include time-consuming multistep processes, cost, special lighting or temperature requirements. Goldman *et al.* [14] have described some shades of color after silver staining spinal fluid and plasma proteins; however, the method that was described suffers many of the same disadvantages of the other monochromatic silver stains.

This paper describes a new gel electrophoresis color development system* for silver staining of polypeptides in one- and two-dimensional polyacrylamide gels that is simple to use, efficient (batch-wise staining of gels), reproducible, ultrasensitive, inexpensive, and is optimized for gels 1.5 mm thick. Most importantly, however, the polypeptides complex with silver in a characteristic manner and the colored polypeptide-silver complex is readily visualized while backlighting with white light.

Correspondence: David W. Sammons, Diabetes and Atherosclerosis Research, The Upjohn Company, Kalamazoo, Michigan 49001, USA

Abbreviations: IEF: Isoelectric focusing; SDS: Sodium dodecyl sulfate; 2-D: Two-dimensional; ISO: The first-dimension separation; DALT: The second-dimension separation; TEMED: N,N,N',N'-Tetramethylenediamine

* The silver-based color development system was first described at the First Annual Meeting of the Electrophoresis Society, Charleston, South Carolina, April 7–10, 1981 [15].

0173-0835/81/0306-0135$2.50/0

2 Methods and materials

2.1 Chemicals

Acrylamide, N,N'-methylenebisacrylamide, and sodium dodecyl sulfate (SDS) were reagents of Serva Chemical Company. N,N,N',N'-Tetramethylenediamine (TEMED) and Standard Low-Mr Agarose were products of Bio-Rad. 2-Mercaptoethanol was purchased from Eastman Kodak. The Tris base and glycine were obtained from Sigma Chemical. Ammonium persulfate was obtained from J. T. Baker. Sodium hydroxide, phosphoric acid, acetic acid, and anhydrous sodium carbonate were obtained from Mallinkrodt. Silver nitrate and sodium borohydride were purchased from Alfa Products. Chemicals for photography, Kodak X-Omat R film, and Ektachrome E6 Duplicating film 6121 were purchased from Eastman Kodak. L- [^{35}S]-methionine (1260 Ci/mmol) from Amersham was used for radiolabeling of cultured fibroblasts. Ampholines (pH 3–10) were obtained from LKB.

2.2 2-D Electrophoresis

Two-dimensional gel electrophoresis was performed with the ISO-DALT system and solutions and procedures were prepared and used according to directions [16]. DALT tank buffers were changed immediately prior to electrophoresis and were not reused. All solutions except ammonium persulfate, TEMED, and DALT tank buffer were filtered through 0.22 micron filters prior to use.

DALT plates were washed in Micro detergent (International Products), rinsed with water, finally rinsed with 95 % ethanol, and air-dried. Cathode and anode electrode solutions in IEF were sodium hydroxide and phosphoric acid, respectively. Ethylenediamine is not recommended as a cathode solution since a dark greenish interfering background will result during staining with silver. Similarly, it was observed that the use of ethylene dichloride solvent in repair of the DALT tanks contaminated the buffer and subsequently the DALT gels, thereby resulting in an interfering background. For best results the IEF gels were equilibrated with fresh equilibration buffer which contained 2 % β-mercaptoethanol. Bromophenol Blue was deleted from the equilibration buffer. Equilibration was done for exactly 30 min, and the IEF gels were frozen at –70 °C until needed. A minimum of agarose was used to bond the IEF gels to the second dimension. The SDS second dimension consisted of a 10–20 % gradient of acrylamide. The polyacrylamide gel is 1.5 mm thick with a total gel volume of 54 ml.

2.3 Preparation of protein samples

Protein samples were derived from human fibroblasts, plasma, and platelets, rat heart, and Chinese hamster liver and kidney. Human fibroblasts, originally established from a skin biopsy, were cultured to confluency in F-12-DMEM with 10 % fetal calf serum. Immediately prior to L-[^{35}S]-methionine (1260 Ci/mmol) addition, the cells were rinsed several times with serum-free culture medium (MEM without methionine). Serum-free medium which containded 10 µCi/ml of ^{35}S-methionine was then added to the cells and culture was continued for 20 h. The labeled cells were scraped from the plastic culture flask (Falcon, T–25) with a rubber policeman and pelleted in Hank's buffer with a Beckman Microfuge. The Hank's medium was aspirated from the pellet and the cells were immediately vortexed in 100 µl ISO-UREA mix [16].

Human plasma from blood collected in sterile sodium citrate (9 parts blood to 1 part 3,8 % citrate) was centrifuged for 3 min in a Beckman Microfuge Model B. The plasma was stored frozen at –70 °C. The plasma was diluted 1:10 into ISO-UREA and 20 µl were loaded onto each ISO gel.

Human platelets were isolated according to [17] and were filtered on a Sepharose 2 B gel (Sigma) column prepared according to the method of Tangen *et al.* [18] using a plastic 50 ml syringe barrel and eluting with Lyndon's buffer [19]. The platelets (3 X 10^9) were then centrifuged and the pellet dissolved into 200 µl of ISO-UREA [16]. Twenty microliters were loaded onto the ISO gel.

Rat heart standards were prepared as previously described [20] and diluted 1:25 with hot agarose. The warm rat heart protein-agarose solution was solidified by cooling a filled tip of a Pasteur pipette in an ice bath. After extrusion of the agarose rod from the pipette tip, a 15 mm piece was placed on top of an SDS polyacrylamide (10–20 % gradient) and fixed in place with a warm agarose solution.

One hundred milligrams of wet weight liver and kidney from the same animal were minced with a razor and homogenized into 1 ml of ISO-UREA mixture [16]. After centrifugation at 223 000 x g for 2 h at 20 °C, 15 µl of sample were loaded onto each ISO gel.

3 Results and discussion

3.1 Silver-based color development system

Table 1 summarizes the steps required for staining polypeptides with the silver-based color development system. Groups of five gels are placed into the various solutions in a Pyrex tray (12" x 17" x 3") and are gently agitated on a platform shaker. The steps are performed at room temperature and lighting. During the procedure, surface evaporation is prevented by covering the trays with Saran Wrap. After elec-

Table 1. Staining procedure for the silver-based gel electrophoresis color development system

Steps	Solutions	Duration of agitation
Fix	50 % ETOH 10 % HAC	2 h or more
↓	↓	
Wash	50 % ETOH 10 % HAC	2 h
	↓	
	25 % ETOH 10 % HAC	1 h 2 x
↓	↓	
	10 % ETOH 0.5 % HAC	1 h 2 x
	↓	
Equilibrate gel	$AgNO_3$ (1.9 g/l)	2 h or more
↓	↓	
Rinse	H_2O	10–20 s
↓	↓	
Reduce silver	$NaBH_4$ (87.5 mg/l) HCHO (7.5 ml/l) in 0.75N NaOH	10 min
↓	↓	
Enhance color	Na_2CO_3 (7.5 g/l)	1 h
	↓	
	Na_2CO_3 (7.5 g/l)	1 h, store

trophoresis the polypeptides are fixed in the polyacrylamide gel with a mixture of 50 % ethanol and 10 % acetic acid (gel volume: solution volume = 1:5.5). Generally the gels are left overnight in the fixative whereupon the washing steps are completed the next day. Fairly extensive washing is recommended to completely remove SDS and carrier ampholytes. The gels may be stored in the last wash step of 10 % EtOH and 0.5 % acetic acid until silver staining can be initiated.

It is essential that solutions used in the staining procedure be degassed prior to use. During the equilibration of the gels with silver nitrate, a balance between concentration of silver, gel thickness, and protein concentration must be made for optimal color. An ideal concentration of $AgNO_3$ for 1.5 mm thick gels is 1.9 g/l and equilibration should be done for 2 h. The optimum ratio of gel volume to solution volume is 1:3; however, some laxity can be accepted in this ratio. The gels are briefly rinsed in degassed H_2O to remove excess surface silver and then submersed in a reducing environment. The reducing solution consists of 0.75 M NaOH, 7.5 ml/liter formaldehyde (USP 37 %), and 87.5 mg/liter $NaBH_4$ (gel volume: solution volume = 1:5.5). The formaldehyde should be added to the reducing solution immediately prior to submersion of the gels. Addition of $NaBH_4$ enhances the color slightly but is not absolutely necessary. Protein spots begin to appear within 5–6 min; however, the spots are only yellowish-brown on a yellowish-orange background. For best results the gels should be removed from the reducing solution within 10 min and placed into the color enhancing solution (gel volume: solution volume = 1:5.5).

The color develops as the sodium carbonate diffuses into the gel and continues for the next several hours. Two changes at 1 h intervals are necessary to remove the excessive NaOH. After one hour of agitation of the gel in the last Na_2CO_3 step, the gel may be set aside. Generally the colors are optimal within 6 h and are best viewed with backlighting on a standard fluorescent viewer, 5000 °K, daylight. The colors are stable in the Na_2CO_3 for several days; in fact, the background tends to lighten as time passes, without significant fading of colors. The silver stain procedure is designed to optimize several advantageous features such as ease of use, time efficiency, reproducibility, and inexpensiveness.

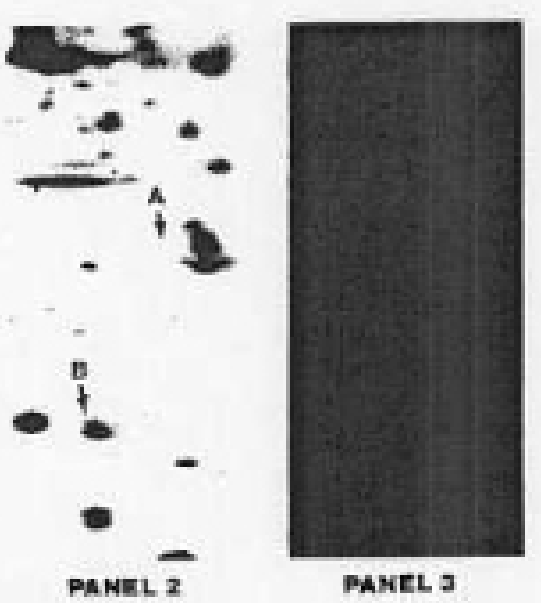

Figure 1. Comparison of Coomassie Blue, autoradiography, and silver-based color development system for sensitivity of protein staining. Equal amounts of fibroblast protein were separated on each gel. Panel 1, proteins stained with Coomassie Blue; Panel 2, autoradiograph of the ^{35}S-methionine labeled proteins; Panel 3, proteins stained with silver. (A) indicates a protein more readily seen after silver staining. (B) indicates another protein more readily visualized in the autoradiograph.

3.2 Relative sensitivity

Fig. 1 illustrates the sensitivity of the silver stain process relative to Coomassie Blue and ^{35}S-autoradiography. Equal amounts of fibroblast protein were loaded onto the silver-stained 2-D gel as the Coomassie Blue stained gel. It is obvious that the silver stain is far superior to Coomassie Blue staining. The autoradiograph was obtained by developing X-Omat film that was exposed to the Coomassie Blue 2-D gel for 9 days. Careful comparison of the silver-stained gel to the autoradiograph shows comparable levels of sensitivity. There are some spots visible on the silver-stained gel (designated A) that are not observed in the autoradiograph. These spot did not have enough ^{35}S-methionine incorporated into their primary sequence. In contrast, some spots clearly visible in the autoradiograph (designated B) do not appear or are barely visible in the silver-stained gel. Apparently these proteins incorporate a disproportionately larger amount of methionine than other proteins. These proteins are dim on the silver stain because they are less abundant or bind less silver than others. In order to more easily visualize the protein, a more concentrated protein sample should be added to the IEF gel.

3.3 Applicability toward one-dimensional gels

Fig. 2 shows the applicability of the silver stain to one-dimensional gels. The rat heart standard was prepared and run exactly as described by Giometti *et al.* [20] except fiftyfold less protein was applied to the SDS gel. The same protein bands were observed as previously reported with Coomassie Blue staining. The colors of each protein may serve as internal color standards as well as molecular weight markers. In this way corrections can be made for slight variations in color intensity that may be due to unavoidable variations in stain from day to day.

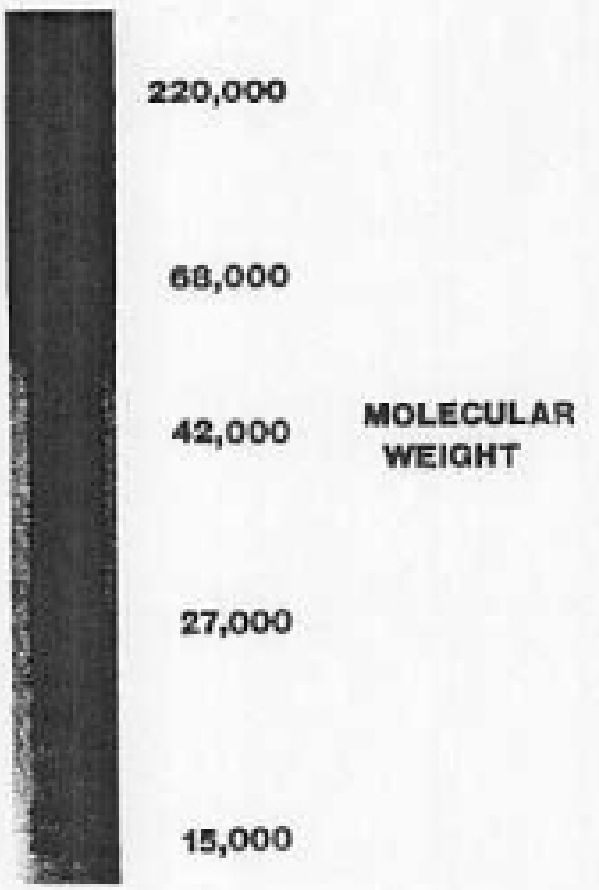

Figure 2. Stained one dimensional SDS gel of rat heart standards.