

## Silver Stain for Proteins in Polyacrylamide Gels: A Modified Procedure with Enhanced Uniform Sensitivity

JAMES H. MORRISSEY\*

Department of Biology, B-022, University of California, San Diego, La Jolla, California 92093

Received May 4, 1981

The rapid, ultrasensitive silver stains that have been developed recently for detecting proteins in polyacrylamide gels show variation in staining from gel to gel and do not stain certain proteins at all. It was found that treatment of gels with dithiothreitol prior to impregnation with silver nitrate results in more reproducible staining patterns that are also qualitatively similar to those obtained with Coomassie blue. In addition, it obviates the need for treatment with intense light, and results in sensitivities at least as high as those obtained with previously published methods.

The original silver stain for detecting polypeptides in polyacrylamide gels (1) has been simplified and reduced in cost by several groups (2-4). These staining procedures are some 100-fold more sensitive than Coomassie blue, making them extremely useful for detecting proteins in trace quantities and for staining two-dimensional gels (3,4). I have found that there is considerable variation from gel to gel in sensitivity and that some proteins which stain well with Coomassie do not stain at all with silver stains. Other drawbacks to these procedures are that some require special high-intensity light sources (3,4), employ unstable, potentially explosive solutions (1,2), or result in surface staining of the gels, which causes gels to stick to both the container and to each other (1,2). This latter point means that gels must be stained in separate containers. Since development of these stains is stopped at an arbitrary point, different gels will be stained to different extents, making direct comparisons between gels difficult.

This report describes a silver-staining procedure that is more constant from gel to gel, uses stable solutions, is independent of light-

ing conditions, stains certain proteins not stained by other silver stains, and can be used to stain a number of gels in one container.

### MATERIALS AND METHODS

**Electrophoresis.** Sample preparations and electrophoresis were performed according to Laemmli (5) in slab gels containing 10% acrylamide. Bromphenol blue was the tracking dye.

**Proteins.** *Dictyostelium discoideum* (strain NC4) spores were lysed in a French pressure cell and the lysate was then boiled for 10 min in sample buffer. *D. discoideum* spore coat proteins were prepared according to Orłowski and Loomis (6). Molecular-weight standards were obtained from the following sources: rabbit muscle myosin,  $\beta$ -galactosidase, conalbumin, cytochrome *c*, and insulin from Sigma; ovalbumin from Miles. Discoidin was a gift from Dr. B. Murray.

**Coomassie blue stain.** Gels were soaked for several hours in 0.2% Coomassie blue R-250, 45% methanol, 10% acetic acid and then destained in 10% methanol, 7% acetic acid.

**Silver stains.** Gels were stained by the method of Oakley *et al.* (2) as published or

\* Address after Jan. 1: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, England.

by the method of Merrill *et al.* (4) using a No. 1 photoflood lamp equipped with an aluminum reflector illuminating the gel at a distance of 10 cm.

The new silver stain is performed as follows (gentle but thorough agitation is important throughout the procedure):

*Step 1:* Prefix the gel in 50% methanol, 10% acetic acid for 30 min, followed by 5% methanol, 7% acetic acid for 30 min.

*Step 2:* Fix the gel for 30 min in 10% glutaraldehyde (E. M. Sciences, biological grade).

*Step 3:* Rinse the gel in distilled water. It is most convenient to soak the gel in a large volume of water overnight, followed by a fresh water rinse the next day for 30 min. Alternatively, wash in running deionized water, or several changes of water, for 2 h.

*Step 4:* Soak in 5  $\mu\text{g}/\text{ml}$  dithiothreitol for 30 min.

*Step 5:* Pour off solution and without rinsing, add 0.1% silver nitrate. Treat for 30 min.

*Step 6:* Rinse once rapidly with a small amount of distilled water and then twice rapidly with a small amount of developer. Soak in developer (50  $\mu\text{l}$  of 37% formaldehyde in 100 ml 3% sodium carbonate) until the desired level of staining is attained. Staining is stopped by adding 5 ml of 2.3 M citric acid directly to the developer and agitating for 10 min. This solution is then discarded and the gel is washed several times in distilled water over a 30-min period. For storage, it is best to soak the gel for 10 min in 0.03% sodium carbonate (to prevent bleaching), and then either to seal in heat-sealable food storage bags or wrap in cellophane.

Gels are fixed and stained in glass or polyethylene containers; since silver is not deposited on surfaces, no special cleaning is required. The same container may be used throughout the procedure. For gels of 1-mm  $\times$  9-cm  $\times$  13-cm dimensions, all volumes are 100 ml per gel, except for the 10% glutaraldehyde, which is 50 ml. These volumes should be adjusted accordingly if different

size gels are used. Particular attention should be paid to the volumes of the carbonate and citric acid solutions, which must be balanced to bring the pH to neutrality. If the pH remains too high, the reaction will not stop, and if the pH falls too low, the gel will bleach.

Gels should be handled only with rinsed plastic gloves to avoid stained fingerprints. Another source of contamination that can cause spurious staining is dust in the gel solutions (Fig. 2). All solutions must be filtered through Millipore filters.

Gels were photographed on Polaroid type 55 film using a Wratten No. 45 filter for silver-stained gels or a No. 15 filter for Coomassie-stained gels.

## RESULTS AND DISCUSSION

In the course of using published silver stains for gels, I have found that certain proteins repeatedly fail to show any staining, while the staining of other proteins varies considerably from gel to gel. As may be seen in Fig. 1, the *D. discoideum* spore coat protein sp96 (6), identified in lanes 2 at the position marked with triangles, stains with Coomassie blue (panel A), but not with the silver-staining methods of Merrill *et al.* (4) (panel B) or Oakley *et al.* (2) (panel C). This protein has failed to stain with either silver stain in at least 20 attempts. When molecular-weight marker proteins are stained, both silver stains show reduced sensitivity to insulin B chain, and in this gel the method of Merrill *et al.* (4) (panel B) shows little sensitivity to cytochrome *c*. Occasionally, the method of Oakley *et al.* (2) has been found to give a staining pattern with insulin B chain and bovine serum albumin which consists of a ring of staining surrounding an unstained band. In some cases with this procedure conalbumin exhibits negative staining, in that an unstained area considerably lighter than the background is found in place of a stained band (not shown).

Variable and unreliable staining reactions

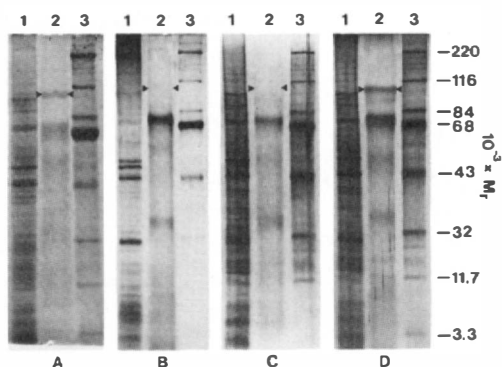


FIG. 1. Comparison of staining techniques. In A, lane 1 contains 50  $\mu\text{g}$  total *D. discoideum* spore proteins, lane 2 contains *D. discoideum* spore coat proteins, (sp96 is identified by triangles), and lane 3 contains 2  $\mu\text{g}$  each of the following proteins (molecular weight given in parentheses): myosin (220,000),  $\beta$ -galactosidase (116,000), conalbumin (84,000), bovine serum albumin (68,000), ovalbumin (43,000), discoidin I (32,000), cytochrome *c* (11,700), and insulin B chain (3300). This gel was stained with Coomassie blue. B-D contain the same samples diluted 1:33. Staining procedures are: B, Ref. (4); C, Ref. (2); D, this report. Proteins were loaded into 6-mm-wide slots.

have been observed with histological silver stains; however, Sun and Green (7) have found that treating tissues with 2-mercaptoethanol immediately prior to silver impregnation gives much more reproducible results. Their reasoning is that since silver staining likely involves reduction of silver ions to silver metal by proteins, full sensitivity will be obtained when the proteins are fully reduced. Variability of staining can thus be explained by postulating varying degrees of oxidation experienced by the tissues during handling. Accordingly, it was found that treating gels with dithiothreitol before silver nitrate impregnation results in staining patterns that are more reproducible (for the procedure of Merrill *et al.* (4), dithiothreitol treatment replaces the dichromate step). A surprising bonus is that such treatment makes the photochemical method of Merrill *et al.* (4) independent of lighting conditions: full sensitivity is obtained in complete darkness. It was also found that other reducing agents (2-mercaptoethanol and

chloral hydrate) had similar effects. However, only dithiothreitol was effective at sufficiently low concentrations as to keep background staining to a minimum.

These findings were used to develop a new procedure based in part on previously published methods (2,4) and employing dithiothreitol reduction. As may be seen in Fig. 1D, sp96 stains well with the new silver stain, as do all of the molecular-weight marker proteins. It may also be seen that the total cellular protein pattern qualitatively resembles that of the Coomassie blue-stained lane. This procedure has been found to be substantially more reproducible from gel to gel than the published procedures quoted above.

Among the advantages of this procedure are: (i) As with other rapid silver stains (3,4), it uses only small amounts of silver (as of April 1981 it cost 3.8 cents in silver nitrate to stain one gel). (ii) It does not require special high-intensity light sources. (iii) Unlike ammoniacal silver stains, it does not employ unstable or potentially explosive solutions. (iv) There is virtually no surface

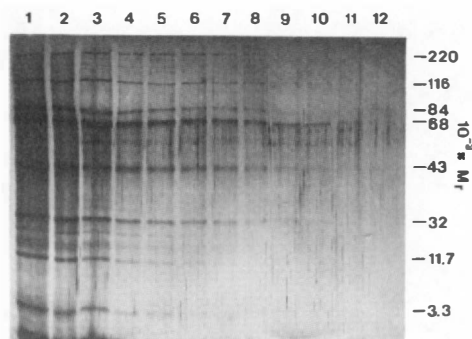


FIG. 2. Sensitivity of the new silver stain. Dilutions of the sample mixture used in lanes 3 in Fig. 1 were applied to this gel. The amount of each protein (in ng) loaded per lane is: lane 1 = 100, 2 = 50, 3 = 25, 4 = 10, 5 = 7.5, 6 = 5, 7 = 3.5, 8 = 2, 9 = 1.5, 10 = 1, 11 = 0.5, 12 = 0.25. The staining seen across the gel just below bovine serum albumin is due to a contaminant in the sample buffer which stains when gels are developed to maximum sensitivity. The thin vertical streaks visible in this gel and in Fig. 1D are from dust particles and can be avoided by filtering all of the gel solutions before use.

deposition of silver, substantially reducing background staining. Since surface staining causes gels to stick together, this improvement means that several gels may be stained together in one container. All of the gels are stained to the same extent, so that quantitative comparisons may be made between gels run in parallel.

As may be seen in Fig. 2, this method is at least as sensitive as the previously published methods. Sensitivities of selected proteins are: 0.042 ng/mm<sup>2</sup> for bovine serum albumin, 0.083 ng/mm<sup>2</sup> for ovalbumin, and 0.17 ng/mm<sup>2</sup> for cytochrome *c*. These values compare favorably to those of Oakley *et al.* (2) and Switzer *et al.* (1). I have found that silver-stained gels may be fluorographed successfully, using the commercially available preparation Enhance (New England Nuclear): even heavily overstained gels of [<sup>35</sup>S]methionine-labeled bacterial proteins gave fluorographic patterns only slightly reduced in intensity compared to unstained gels (data not shown). However, silver-staining severely quenches tritium fluorography.

#### ACKNOWLEDGMENTS

I would like to acknowledge the help and advice of Drs. J. Schmidt, K. Devine, and B. Murray and Dr. W. F. Loomis, in whose laboratory these experiments were conducted. This work was supported by a grant to W.F.L. from the National Science Foundation (PCM 79-02698). J.H.M. was supported by an institutional postdoctoral training grant from the National Institutes of Health (GM 07199-06).

#### REFERENCES

1. Switzer, R. C., III, Merrill, C. R., and Shifrin, S. (1979) *Anal. Biochem.* **98**, 238-241.
2. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Anal. Biochem.* **105**, 361-363.
3. Merrill, C. R., Dunau, M. L., and Goldman, D. (1981) *Anal. Biochem.* **110**, 201-207.
4. Merrill, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1981). *Science* **211**, 1437-1438.
5. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
6. Orłowski, M., and Loomis, W. F. (1979). *Develop. Biol.* **71**, 297-307.
7. Sun, T-T., and Green, H. (1976). *Cell* **9**, 511-521.