Ultrasensitive Silver-Stain Method for the Detection of Proteins in Polyacrylamide Gels and Immunoprecipitates on Agarose Gels

M. PORRO,[1] S. VITI, G. ANTONI, AND M. SALETTI

ISVT Sclavo, Research and Development, Biologicals, 53100 Siena, Italy

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The highly sensitive silver-stain procedure for the detection of proteins in polyacrylamide gels has been revised and simplified using a single-step silver ion reduction after suitable treatment of proteins with bifunctional aldehyde. Washing steps were eliminated and excellent reproducibility of results was achieved. Sensitivity obtained using this procedure was at least equal to that obtained with the original one. Use of the present silver-staining methods has been extended to the quantitative analysis of immunoprecipitates on agarose gels, with a good increase of sensitivity and excellent increase of resolution when compared to the Coomassie blue stain.

Several modifications have been introduced (1–3) to the highly sensitive silver-stain method since its first application in the detection fo very small amounts of proteins in polyacrylamide gels (4,5). Despite its sensitivity, this technique originally appeared complicated and an unsatisfactory reproducibility of results was obtained in our laboratory even when the subsequently published modifications were adopted. Similar findings were also reported by Morrissey (6) and Wray *et al.* (7) in their improvements in this staining. By comparison of these methods in our laboratory, we found that reproducibility could be obtained by further studying the reaction between silver ion and protein or by a better control of the amount of chemicals left in the gels after the washing steps. Because the first point is poorly understood, we undertook to carefully study the second point which appeared fundamental in order to obtain a more reproducible method. We achieved excellent reproducibility of results by using a single-step reaction after an appropriate treatment of the proteins with glutaraldehyde. The method also proved to be suitable for immunoprecipitates on agarose gels, where the other silver-stain methods (1,5,7) failed.

MATERIALS AND METHODS

Phosphorylase *b*, bovine serum albumin,[2] ovalbumin, soybean trypsin inhibitor, and α-lactalbumin were obtained from Pharmacia (Uppsala, Sweden).

Pneumococcal capsular polysaccharide type 6A was purified in our laboratory as a component of the polysaccharide polyvalent vaccine for human use (lot VPP/IS-ISVT Sclavo, Siena, Italy). Rabbit antisera to bovine serum albumin was obtained in our laboratory, and rabbit antisera to pneumococcal capsular polysaccharide type 6 were furnished by the Statens Seruminstitut (Copenhagen, Denmark).

Electrophoresis

SDS electrophoresis on polyacrylamide gels was performed according to the Läemmli

[1] To whom all correspondence should be addressed.

[2] Abbreviations used: BSA, bovine serum albumin; PP_s6A, pneumococcal capsular polysaccharide type 6A; SDS, sodium dodecyl sulfate.

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TABLE 1

GLUTARALDEHYDE TREATMENT FOR SILVER STAINING OF IMMUNOPRECIPITATES IN AGAROSE GEL

Antiserum in agarose (% v/v)	Required concentration of glutaraldehyde solution (% w/v)	Staining of "rockets" obtained after diammino silver treatment
0.05/0.5	1	Negative form
>0.5/1	1/3	
0.05/0.25	3	Positive form
>0.25/1	5	

procedure (8) using an acrylamide gradient (3–9% w/v) and Tris–HCl buffer at pH 8.3. Gels were 0.75 and 1.5 mm thick with 4-mm slots.

Immunoelectrophoresis

Rocket immunoelectrophoresis on agarose gels was performed according to the Weeke procedure (9), using agarose gel (1% w/v) and Tris–barbiturate buffer 0.02 M at pH 8.8.

When bovine serum albumin was used as antigen, antiserum employed in the agarose ranged between 0.05 and 1% (v/v) which corresponded to values between 0.075 and 1.5 $\mu l/cm^2$ gel area. The electrophoretic run was performed at 70 V/cm for 45 min. Similar conditions were used for pneumococcal rabbit antiserum when pneumococcal polysaccharide type 6A was used as antigen.

Silver-Stain Procedures

(a) Polyacrylamide gels. The procedures described by Oakley *et al.* (1), Merril *et al.* (5), and Wray *et al.* (7) were used to stain reference gels. The present silver-stain method was performed as follows.

After electrophoresis, proteins in the gel were fixed as usual in 45% methanol–10% acetic acid mixture for at least 30 min and then washed with distilled water for 30 min to remove excess organic solvents. The gel was then soaked in 200 ml of glutaraldehyde solution (Merck-Schuchardt, Germany) overnight (15 h) at room temperature with magnetic stirring. Optimal concentration of glutaraldehyde solution is a critical parameter, and control for absence of aspecific or occasional reducing chemicals present in water used for the solutions employed was performed by the modified method of Park–Johnson (10).

A glutaraldehyde solution of 1% w/v was necessary for a slab of polyacrylamide gel with dimensions of 12 × 14 cm and 0.5% w/v for a slabslice with dimensions of about 3 × 4.5 cm. The glutaraldehyde solution cannot be changed during the time period reported (15 h). These concentrations of glutaraldehyde were suitable for gels with a thickness of 0.75 to 1.5 mm. Magnetic stirring may be substituted with a shaker, but efficient stirring of the solution is required.

Gel was transferred into 200 ml of a solution of diammino silver (I) 0.2% w/v (21 ml sodium hydroxide 0.36 w/v + 1.4 ammonium hydroxide 28% + 1 ml silver nitrate 20% w/v and water to 100 ml; the reagent was stable at room temperature for several days). The diammino silver solution is not substituted in the time period indicated (about 30 min). Precipitation of reduced silver occurs but does not present a problem since efficient stirring prevents silver deposits on the slab gel. Occasionally, overstained backgrounds can be selectively destained with a solution of 10% v/v of acetic acid in a few minutes.

After extensive washings with distilled water or with buffered saline at pH 7.2, if acetic acid solution was used for destaining, gels were photographed and stored in plastic bags.

(b) Agarose gels. Following electrophoresis, agarose gel (on a plastic plate support with dimensions of 8.5 × 9.5 cm) was moistened with distilled water or saline, pressed with filter paper, and dried at 37°C for 30 min. Afterward, the gel was treated with 100 ml of glutaraldehyde solution at a suitable concentration (see Table 1) for 15 min depending on the amount of antiserum casted in the agarose gel and on the selected staining

of the "rockets" (positive or negative form in respect to the gel background, for both kinds of immunocomplex: proteic antigen antibodies and polysaccharide antigen–antibodies).

After glutaraldehyde treatment, the plastic plate was transferred to 100 ml of diammino silver (I) solution (prepared as described above) for 5 to 10 min. In all the steps, thorough stirring of the solutions must be performed, regardless of the type of stirring (magnetic or shaker). A change of the solution in each step cannot be performed. As for polyacrylamide gel, overstained background can be selectively reduced by 10% v/v acetic acid solution in 1 to 2 min or less.

After repeated washings with distilled water, the plates were dried at 37°C and then photographed or immediately stored.

Coomassie Blue Stain

Polyacrylamide gels were soaked overnight in 0.2% Coomassie blue R-250 in 45% methanol, 10% acetic acid, and then destained in 10% methanol and 7% acetic acid.

After electrophoresis, agarose gels were moistened with distilled water, pressed with filter paper, dried at 37°C for 30 min, and then stained for 15 min with 0.1% Coomassie blue R-250 in 45% ethanol, 10% acetic acid, and destained with 45% ethanol and 10% acetic acid solution.

RESULTS

As shown in Fig. 1 for polyacrylamide gels, proteins were detected using the present method, with a minimum concentration of about 0.025 ng/mm^2 for phosphorylase *b* (lane 5, Fig. 1C), 0.5 ng/mm^2 for ovalbumin (lane 4, Fig. 1C), and 0.03 ng/mm^2 for bovine serum albumin (lane 5, Fig. 1C). These values are comparable to those reported by Oakley *et al.* (1) and were obtained in our laboratory using their method. With the proteins employed, the sensitivity obtained with the present method was at least 100 times

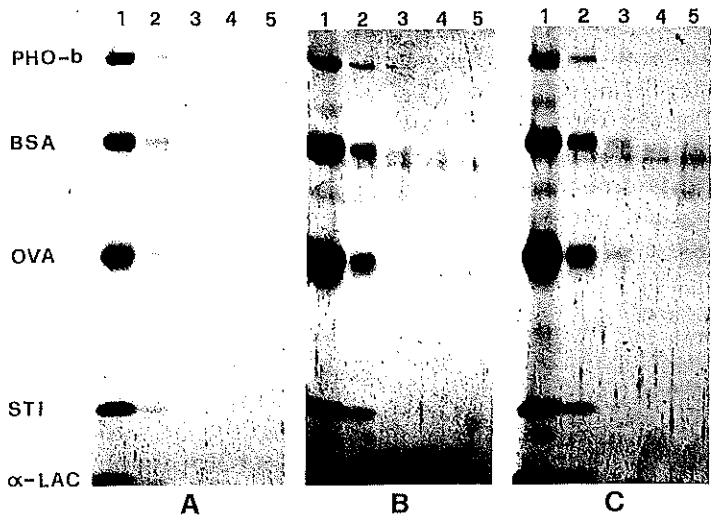

FIG. 1. Comparison of staining procedures on SDS–polyacrylamide gel. Gel A was stained by Coomassie blue R-250, gel B by the silver-stain method according to Oakley *et al.* (1), and gel C by the present silver-stain procedure. PHO-b, phosphorylase *b*: slot 1, 1600; slot 2, 160; slot 3, 16; slot 4, 1.6; and slot 5, 0.15 ng. BSA, bovin serum albumin: slot 1, 2000; slot 2, 200; slot 3, 20; slot 4, 2; and slot 5, 0.2 ng. OVA, ovalbumin: slot 1, 3600; slot 2, 360; slot 3, 36; slot 4, 3.6; and slot 5, 0.36 ng. STI, soybean trypsin inhibitor: slot 1, 2000; slot 2, 200; slot 3, 20; slot 4, 2; and slot 5, 0.2 ng. α-LAC, α-lactalbumin: slot 1, 3000; slot 2, 300; slot 3, 30; slot 4, 3; and slot 5, 0.3 ng.

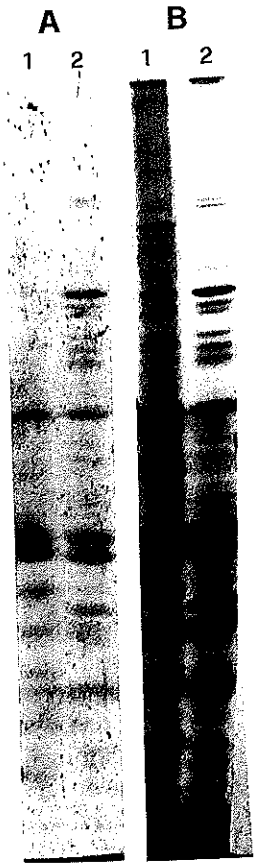

FIG. 2. Comparison between (A) Coomassie blue R-250 staining and (B) the present silver-staining method in detecting an electrophoretic behavior of proteins of the outer membrane of *N. meningitidis* group A (1) and group B (2). Proteins were released in aqueous solution after treatment at 50°C of the cultured microorganisms using a sodium acetate buffer, pH 5.8, containing 0.2 M lithium chloride. SDS–polyacrylamide electrophoresis was performed as described under Materials and Methods, loading 500 ng of proteins for all the samples.

higher than that found by staining with Coomassie blue. Compared to the previous methods, this simplified silver-stain method has the advantage of giving very reproducible results, and can be performed in about 30 min in a single step after the overnight reaction between glutaraldehyde and proteins in the gels. An application of the present silver-staining method to analyze the electrophoretic behavior on polyacrylamide gel of proteins of the outer membrane of *N. meningitidis* group A and B, in comparison with Coomassie blue staining, is shown in Fig. 2. The results obtained on agarose gel for proteic antigen–antibody complexes are shown in Fig. 3. The sensitivity attained in the first case gave a detectable rocket with 0.8 ng of antigen (BSA) (well 7, Fig. 1B), while the minimum detectable amount of BSA was 6.3 ng when using Coomassie blue, which corresponds to an increased sensitivity of about 10-fold, with the present silver-stain procedure. In the case of polysaccharide antigen–

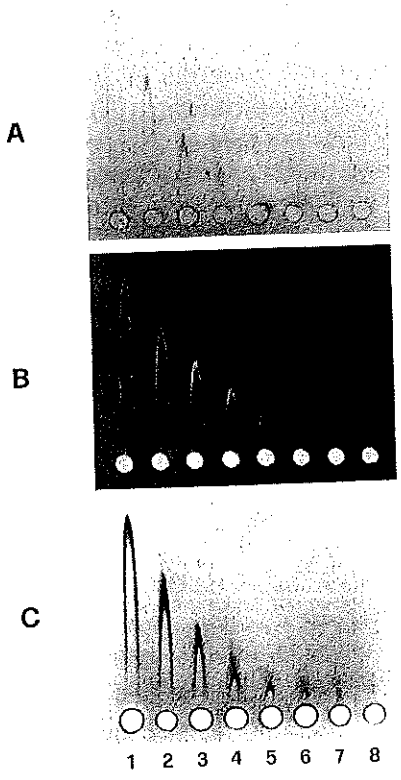

FIG. 3. Comparison of staining procedures on agarose gel for proteic antigen–antibody immunocomplexes. Gel A was stained by Coomassie blue R-250 and gels B and C by the present silver-stain procedure, showing the "rockets" in (B) negative and (C) positive form with respect to the gel background. The amount of BSA as antigen was line 1, 50; line 2, 25; line 3, 12.5; line 4, 6.3; line 5, 3.1; line 6, 1.6; line 7, 0.8; and line 8, 0.4 ng in 10 μl of loaded solution. Agarose gel contained rabbit antiserum to BSA in the quantity of 0.075 μl/cm^2 gel area.

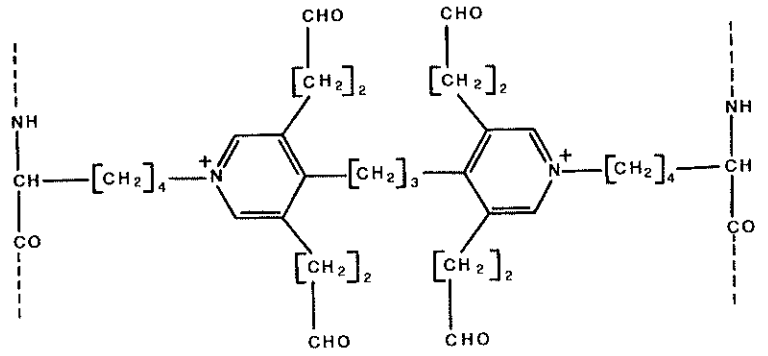

FIG. 4. Quaternary pyridinium compound in glutaraldehyde-treated ovalbumin (Hardy *et al.* (11)).

antibody complexes, a minimum detectable rocket corresponded to 0.1 ng of polysaccharide (PP_s6A), while the value of 0.4 ng of antigen was obtained using Coomassie blue staining (data not shown). However, it is important to note that these amounts of immunocomplexes stained with Coomassie blue showed a less marked definition of the rockets in respect to the silver-stain procedure, and this represents a serious limitation to the correct evaluation of form and height of the rockets because in this procedure, the measurement of the height of the rockets by quantitative analysis is an approximation of the more exact criterion of the comparison of the area enclosed by the precipitates (9). In other words, the difference of sensitivity between the two staining methods on agarose gels is much higher than apparently observed, because it is not correct to compare the area of the immunocomplexes obtained by Coomassie blue staining in similar amounts. Both negative and positive forms of silver-stained rockets were comparable in terms of sensitivity for both kinds of immunocomplexes, but negative forms showed a more favorable color contrast and were preferred for the accuracy of the measure.

DISCUSSION

The silver-stain method offers the possibility of detecting very small amounts of proteic material, comparable to those detected by labeling with radioactive isotopes. However, the early applications of this method for detecting peptides on polyacrylamide gels (4,5) were very laborious, and the procedure appeared insufficiently reproducible according to our experience. The subsequently published modification (1) simplified the procedure, making it less expensive but still with low reproducibility of results, as also reported in more reliable silver-staining methods later published (6,7). Because the basic reaction of this process is the reduction of silver ions to their own metallic form by an organic reducing agent, we studied the possibility of staining the proteins in a single step, considering the fact that glutaraldehyde may act as a reagent for proteins and as reducing agent for silver ions. The reaction of the bifunctional glutaraldehyde on proteins has been applied in various fields for a long time. Recently, an interesting contribution on the nature of protein crosslinking by glutaraldehyde with proteins has been made by Hardy *et al.* (11,12), wh. identified quaternary pyridinium compounds in glutaraldehyde-treated ovalbumin (Fig. 4).

Because free aldehyde groups exist in the protein after glutaraldehyde treatment, we considered the possibility that these reducing groups contribute to selectively reduce silver ions on the glutaraldehyde-treated proteins. The main problem appeared to be to reduce the gel background to an acceptable level, in respect to the staining of the protein bands.

due to the aspecific reducing effect of glutaraldehyde. This was resolved by using a low concentration of the glutaraldehyde solution and extending the reaction time (at room temperature) between the aldehyde and proteins trapped in the polyacrylamide gel. In fact, if shorter incubation time with glutaraldehyde solution was used (1–2 h), only a poor staining of the gel background occurred, whereas if higher concentrations of glutaraldehyde solution were employed, unacceptable background levels appeared simultaneously with the staining of the protein bands. These observations suggested that the action of bifunctional aldehyde on the proteins could be controlled using the reaction time as a variable to discriminate between background and the staining of protein bands. Thus, after the suitable time of reaction with the glutaraldehyde solution, the subsequent addition of silver ions in alkaline solution allowed for the selective staining of protein bands showing a more extensive silver ion reduction on the protein bands, rather than in the gels.

The substitution of glutaraldehyde for formaldehyde in similar conditions did not discriminate between staining of protein bands and gel background. This observation suggested that the role of glutaraldehyde is really important in selective staining of proteins by silver reduction.

Although glutaraldehyde is reported to react in a few minutes with proteins in solution (13), a significant difference in the reaction time was seen between proteins casted in polyacrylamide or agarose gels. In addition to the different concentrations of the glutaraldehyde solutions, an explanation may be found in the fact that the two gel media have completely different chemical and physical properties and these factors could be important in the action of the glutaraldehyde with the proteins.

Of the several proteins tested, different sensitivity levels have been found and the maximum sensitivity for a typical protein would be investigated every time that it is required. In conclusion, the present silver-stain method shows that the correct amount of bifunctional glutaraldehyde and an accurate reaction time permitted the fast, simple, highly sensitive, and inexpensive procedure of protein staining, which gave reproducible results on both polyacrylamide and agarose gels.

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