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VISUALIZATION OF ANTIGENIC PROTEINS BLOTTED ONTO NITROCELLULOSE USING THE IMMUNO - GOLD - STAINING (IGS) - METHOD

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SUMMARY. A new and simple method for the detection of antigenic proteins blotted onto nitrocellulose was developed. After transfer of spinach stromal proteins and purified phosphoribulokinase immunolabeling was performed with phosphoribulokinase antiserum, followed by a) Protein A-labeled colloidal gold particles, and b) by horseradish peroxidase conjugated Protein A and substrate mixture. The Protein A-Gold method is at least twofold more sensitive than the Protein A-peroxidase procedure. Incubation of immunolabeled nitrocellulose replicas with 0.1 M glycine, pH 2.2, removes the antibody-Protein A-Gold complexes quantitatively without influencing the antigenicity of the immobilized proteins. The replicas can be re-used for immunostaining with other antisera. The versatile applicability of the immuno-gold-staining method suggests that it is a true alternative to the peroxidase assay.

INTRODUCTION. During the past few years the technique of SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose or diazotized paper has been frequently used to characterize specific polypeptide bands with regard to their reactivity with e.g. antibodies, DNA, RNA or lectins (for review see e.g. 1). Especially the immunological probing of nitrocellulose membrane filters after electrophoretic protein transfer or after "native blot" (2) has proven a powerful tool for antigenic analysis. For detection of antigenic sites in transfered polypeptides usually the horseradish peroxidase assay is used or nitrocellulose membranes are incubated with iodinated

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Abbreviations used: PBS phosphate buffered saline; BSA bovine serum albumin; PA-Gold Protein A-Gold; PA-HRP horseradish peroxidase conjugated Protein A.

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probes. In this report we describe a new, very sensitive method to visualize antigenic sites in proteins immobilized on nitrocellulose using colloidal gold particles, coated with Protein A.

MATERIALS AND METHODS.

ANTISERUM. An antiserum against spinach phosphoribulokinase was raised in rabbits (Specific activity of the enzyme was 40 U/mg protein.).

GEL ELECTROPHORESIS AND BLOTTING. SDS-polyacrylamide gel electrophoresis (12,5 % running gel) was performed according to Laemmli (3). The gels were blotted onto nitrocellulose sheets (Schleicher & Schüll BA 85) essentially according to the method of Towbin et al. (4). Transfer was carried out for 5 h at 55 V using the BioRad Trans-Blot-System.

PREPARATION OF COLLOIDAL GOLD. Colloidal gold with an average particle size of 15 nm was prepared using tri-sodium citrate as reducing agent (5), 5 nm gold particles were prepared with tannic acid/citrate (6).

COUPLING OF PROTEIN A TO COLLOIDAL GOLD. Protein A (Sigma) was linked to colloidal gold according to the procedure of De Mey et al. (7) using BSA as stabilizing agent. The gold sols were stored at 4° C in PBS/1% BSA/0.02 % azide and showed no loss of immunoreactivity for at least four weeks. Before use the gold suspensions were briefly centrifuged to remove unstabilized, aggregated gold particles (250 x g for 15 nm particles, 4000 x g for 5 nm particles).

IMMUNOLOGICAL DETECTION OF PROTEINS ON NITROCELLULOSE. To prevent nonspecific background binding the nitrocellulose filters were quenched by incubation with 2% BSA (Sigma, RIA grade) in PBS, pH 7.4 (4 h, 30° C). The quenched filters were reacted with diluted antiserum or preimmune serum (1:100 in PBS containing BSA, 2 h, 30° C), and extensively washed with PBS/BSA. Incubation with Protein A-Gold suspensions (OD_{520}: 0.2-0.25, diluted with PBS/BSA) was done for 30-60 minutes, excess unbound gold particles were removed by several short buffer washes. Horseradish peroxidase-conjugated Protein A (Miles) was used at 1.2 µg/ml PBS containing BSA. The blots were incubated for 30-60 minutes at 30° C and washed as above. For the colour reaction the blots were soaked in a solution of 25 µg of o-dianisidine (Fluka) per ml, 0.01% H_2O_2, 10 mM Tris-HCl pH 7.4. The reaction was terminated after 30-60 minutes by rinsing the nitrocellulose strips with buffer or water.

ERASING OF IMMUNOSTAINED BANDS. Nitrocellulose strips were incubated in the following buffer for 30 minutes:
0.1 M glycine-HCl pH 2.2, 20 mM sodiumacetate, 50 mM potassiumchloride. The regenerated nitrocellulose was neutralized by a saline wash. (8)

PREPARATION OF A STROMA FRACTION FROM SPINACH CHLOROPLASTS. Intact spinach chloroplasts were isolated from hydroponically grown plants according to the method of Nakatani & Barber (9). To obtain the stroma, pelleted chloroplasts were lysed by resuspension in 10 mM Tris-HCl pH 7.75 or in bidistilled water. Thylakoid membranes were separated from the stroma fraction by centrifugation.

RESULTS.

When colloidal gold particles coated with Protein A are used to detect specific antigens immobilized on nitrocellu-

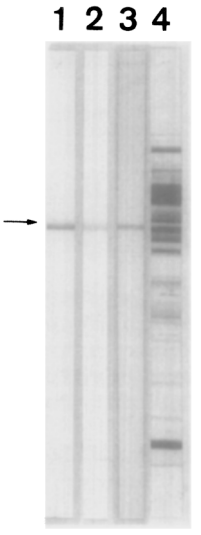

Fig. 1: Comparison of IGS-method and HRP-assay after transfer of spinach stromal proteins to nitrocellulose. Incubations with reagents as indicated in Material and Methods. Lane 1: Immunostaining with Protein A-labeled 5 nm gold particles. The arrow indicates a minor polypeptide band with antigenic properties. Lane 2: Immunostaining with Protein A-labeled 15 nm gold particles. Lane 3: Immunostaining with Protein A-conjugated horseradish peroxidase. Note the darker background. Lane 4: Amido black stained stromal proteins.

lose membrane filters, bands appear in bright red on a virtually unstained background. In contrast, PA-HRP treated bands are brown on a yellowish background. Fig. 1 shows nitrocellulose strips after electrophoretic transfer of stromal proteins, which were incubated with anti-phosphoribulokinase serum and subsequently reacted with PA-Gold or PA-HRP. When BSA has been used as blocking agent, one main polypeptide is stained with either reagent. With PA-Gold, however, one minor band appears in addition. 5 nm gold particles lead to more sharply focussed bands when compared to 15 nm particles, which yield slightly diffuse but broader bands. No bands are visible after treatment with preimmune serum. To determine the sensitivity of PA-Gold versus PA-HRP decreasing amounts of purified phosphoribulokinase (10 μg - 5 ng) were blotted and immunostained (Fig. 2). Under identical incubation conditions PA-Gold was at least twofold more sensitive

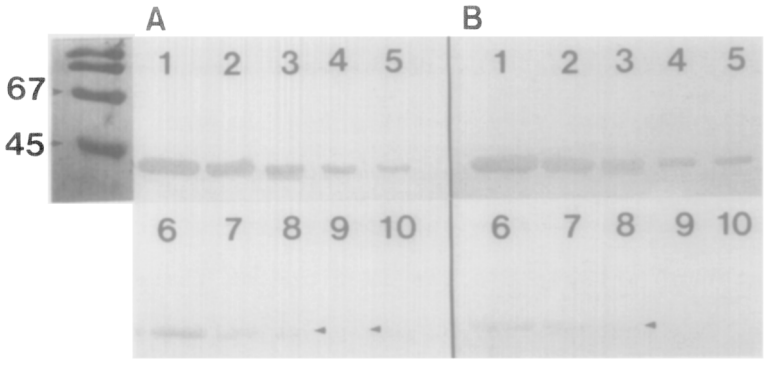

Fig. 2: Decreasing amounts of purified wheat PRK, which strongly crossreacts with the spinach PRK antiserum, were applied to a 12,5% SDS polyacrylamide gel and blotted onto nitrocellulose.
1= 10 µg, 2= 5 µg, 3= 2.5 µg, 4= 1 µg, 5= 0.5 µg
6= 100 ng, 7= 50 ng, 8= 25 ng, 9= 10 ng, 10= 5 ng.
A: nitrocellulose, stained with PA-Gold.
B: immunolabeling was performed with PA-HRP. When only small amounts of antigenic protein are present, 10 ng can still be detected with PA-Gold, while for PA-HRP the detection limit is 25 ng. On the left: amido black stained molecular weight markers (KD).

than PA-HRP. The light sensitive peroxidase stained preparations became pale after a few days of storage, while PA-Gold incubated preparations remained unchanged, thereby increasing the difference in sensitivity even further.

After application of PA-Gold for the detection of antigenic proteins, nitrocellulose replicas can be re-used for immunostaining with either the same or a different antiserum: Incubation of strips with low pH glycine buffer removes the antibody/PA-Gold complexes quantitatively without affecting the immobilized proteins, which fully retain their antigenicity. When those nitrocellulose strips were incubated with phosphoribulokinase antiserum and PA-Gold for a second time, bands reappeared in the same intensity as before. No bands were to be seen when erased nitrocellulose strips were incubated with PA-Gold alone, proving the absence of antibody-antigen bonds. The attempt to remove the immunostained bands from PA-HRP treated nitrocellulose failed .

DISCUSSION. A new application of colloidal gold coupled to Protein A is introduced. The gap between immunocytochemistry and biochemistry has only very recently been bridged by the use of gold-protein complexes as immunoprecipitation reagents (10). Here we describe another biochemical application of Protein A-Gold as indicator of antigenic sites in proteins transfered to nitrocellulose. The preparation and use of colloidal gold labeled Protein A is a simple and rapid procedure when compared to the preparation and application of horseradish peroxidase conjugates (11) or radioiodinated Protein A (12). One major advantage is that no chemical changes occur in the ligand during the labeling procedure due to the absence of the formation of covalent bonds between the gold and the protein (13), while e.g. radioiodination procedures may influence the biological activity of Protein A (12). Binding of PA-Gold to antigen-antibody-complexes gives rise to distinct red bands on an unstained background, when unoccupied binding sites on the filter are effectively blocked. Pronounced background staining after peroxidase treatment can be a problem if only small amounts of protein are to be visualized. In addition most of the chromogenes are suspected carcinogens, and the colour reaction products are light sensitive. PA-Gold labeled nitrocellulose is not influenced even by strong light and can be stored indefinitely. The immuno-gold-staining procedure is a simple and reproducible method for detecting nanogram quantities of antigenic proteins immobilized on nitrocellulose. It is considerably less expensive to use than iodinated probes and does not have any hazards or disposal difficulties. It is at least twofold more sensitive than PA-HRP.

We have shown that a nitrocellulose replica of stromal polypeptides separated on an SDS polyacrylamide gel can be re-used for immunostaining with PA-Gold. This has previously been

demonstrated for nitrocellulose after ^{125}I-Protein A labeling (8,14). It is not possible for HRP-treated nitrocellulose, providing additional evidence about the more versatile applicability of PA-Gold versus PA-HRP. The use of PA-Gold bears one more important potential: after blotting of more complex protein mixtures under non-denaturing conditions, endogenous peroxidases will not interfere with the staining reaction.Thus it is suggested that the use of PA-Gold for immunostaining of blotted proteins is an attractive alternative to peroxidase conjugated probes.

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REFERENCES

1. Gershoni, J.M., and Palade, G.E. (1983) Anal. Biochem. 131, 1-15.
2. Reinhart, M.P., and Malamud, D. (1982) Anal. Biochem. 123, 229-235.
3. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
4. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
5. Frens, G. (1973) Nature Phys. Sci. 241, 20-22.
6. Mühlpfordt, H. (1982) Experientia 38, 1127-1128.
7. De Mey, J., Moeremans, M., Geuens, G., Nuydens, R.,and De Brabander, M. (1981) Cell Biol. Intern. Rep. 5, 889-899.
8. Legocki, R.P., and Verma, D.P.S. (1981) Anal. Biochem. 111, 385-392.
9. Nakatani, H.N., and Barber, J. (1977) Biochem. Biophys. Acta 461, 510-512.
10. Goodman, S.L., and Newman, R.A. (1983) Exp. Cell Res. 144, 209-214.
11. Nygren, H., and Hansson, H.A. (1981) J. Histochem. Cytochem. 29, 266-270.
12. Mayers, G.L., and Klostergaard, J. (1983) J. Immunol. Methods 57, 235-246.
13. Warchol, J.B., Brelinska, R., and Herbert, D.C. (1982) Histochemistry 76, 567-575.
14. Erickson, P.F., Minier, L.N., and Lasher, R.S. (1982) J. Immunol. Methods 51, 241-249.