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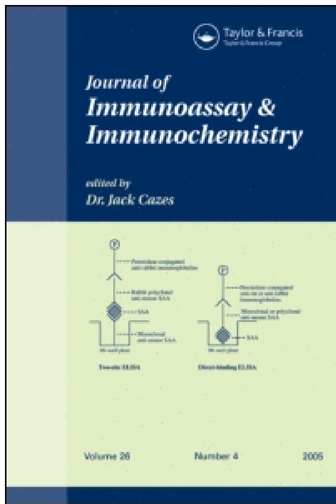

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Enzyme Linked Immunosorbent Assay Using Alkaline Phosphatase Conjugated with Streptococcal Protein G

Bo Nilson[a]; Lars Björck[ab]; Bo Åkerström[a]
[a] Department of Physiological Chemistry, University of Lund, Sweden [b] Department of Medical Microbiology, University of Lund, Sweden

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ENZYME LINKED IMMUNOSORBENT ASSAY USING ALKALINE PHOSPHATASE CONJUGATED WITH STREPTOCOCCAL PROTEIN G

Bo Nilson, Lars Björck[+] and Bo Åkerström

Department of Physiological Chemistry and Department of Medical Microbiology[+], University of Lund, Sweden

("Key words": Protein G; alkaline phosphatase; conjugate; ELISA; goat antibodies; mouse monoclonal antibodies.)

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ABSTRACT

Protein G, an IgG-binding protein, purified from the surface of group G streptococci, was coupled to alkaline phosphatase. The conjugate was used for detection of polyclonal goat and rabbit antibodies and monoclonal mouse IgG1, IgG2a and IgG2b in an enzyme-linked immunosorbent assay. A two-step coupling procedure was used, in which glutaraldehyde was allowed to react with the enzyme, excess glutaraldehyde was then removed by dialysis, and finally protein G added to the glutaraldehyde-activated and polymerized alkaline phosphatase. The activity and yield of the conjugates were then tested in an enzyme-linked immunosorbent assay. Coupling of 25 ug protein G to 5 mg alkaline phosphatase gave a conjugate which could be used for more than 10,000 determinations with maximal antibody binding giving an absorbance of 2.0. Under these conditions, there was no need for separation of the reactants before using the protein G-alkaline phosphatase complex.

INTRODUCTION

Protein G is an immunoglobulin G (IgG)-binding streptococcal surface protein. It has been purified by Björck and Kronvall (1)

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from human group G streptococci, and by Reis et al. (2) from group C streptococci. It was shown (3,4) that its IgG-binding was of a wider spectrum than protein A, the corresponding staphylococcal protein (5). Radiolabelled protein G has been applied to solid phase radioimmunoassay (6) and immunoblotting (3,7). Here, we have used protein G for measurement of IgG and antigens in an enzyme-linked immunosorbent assay (ELISA), which is, in many cases, more rapidly and conveniently evaluated than a solid phase radio-immunoassay. For this purpose we have produced a protein G-alkaline phosphatase conjugate.

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MATERIALS AND METHODS

Proteins and Antiserum

Protein G was prepared by papain-digestion of group G streptococci, strain G-148, as reported (8). Human alpha-1-microglobulin (alpha-1-m) and rabbit and goat anti-human alpha-1-m serum were produced as reported (9). Mouse monoclonal anti-alpha-1-m antibodies BN11.1 (IgG1), BN11.2 (IgG2a), BN11.3 (IgG2a) and BN8.1 (IgG2b) have been described (10). Protein G was radio-labelled with ^{125}I using the lactoperoxidase-glucose oxidase Enzymobead kit (BioRad Lab.) as instructed by the supplier. Protein G was thus labelled with radioiodine to 10^6 cpm/10-30 ng.

Coupling of Protein G to Alkaline Phosphatase

The two-step glutaraldehyde method was used as described by Engvall (11). Briefly, the alkaline phosphatase suspension (from

bovine intestinal mucosa, Sigma Chemical Co.) was centrifuged, dialysed against phosphate buffer, 10 mmol/L, + NaCl, 0.12 mol/L, + KCl, 3 mmol/L, pH 7.4 (PBS), then "activated" by dialysis for 16 hours at 4°C against glutaraldehyde, 2 g/L (Merck) in PBS, and finally washed by dialysis against PBS. Protein G (both as the ^{125}I-labelled protein, 0.5 x 10^{6} cpm, and as non-labelled protein) was added to the enzyme and incubated over night, and the volume was adjusted to 1.0 ml. Two-thirds of this reaction mixture was separated by gel-chromatography on Sepharose 6B (Pharmacia Fine Chemicals). The column (0.8 x 90 cm) was equilibrated and eluted with Tris-HCl, 20 mmol/L, pH 8, + NaCl, 0.15 mol/L, + Na-azide, 0.2 g/L, at a flow-rate of 1.5 ml/hour, and 1.5 ml-fractions were collected. These were analysed for protein G by analysing the ^{125}I activity, for alkaline phosphatase by incubation of 1 ul of the eluted fraction with 100 ul alkaline phosphate-substrate buffer (see below), and for protein G-alkaline phosphatase conjugates by ELISA. The one-step method (11) of coupling was done by mixing the centrifuged, dialysed alkaline phosphatase with protein G and glutaraldehyde, 2 g/L, in PBS, over night.

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Enzyme-Linked Immunosorbent Assay

The ELISA was performed as described for solid phase radio-immunoassay (6). Antigen, diluted in PBS, was coated to microtiter plates by adding 50 ul to 96-well microtiter plates (Falcon 3912, Becton Dickinson) and incubating for one hour. The wells were rinsed with Tween-20, 0.5 g/L in NaCl, 0.15 mol/L. 50 ul antibody, diluted in PBS + Tween-20, 0.5 g/L, was added and incubated for

one or two hours. After washing and incubating usually for two hours with the protein G-alkaline phosphatase conjugate diluted in PBS + Tween-20, 0.5 g/L, and again washing, 100 ul p-nitrophenyl-phosphate, 1 g/L in diethanolamine-HCl, 1 mol/L, pH 9.8, containing $MgCl_2$, 0.5 mmol/L, was added to the wells, and the absorbance at 405 nm was measured after 90 or 120 minutes in a Titertek Multiscan.

RESULTS

Coupling of Alkaline Phosphatase and Protein G

We tried first to couple 1 mg alkaline phosphatase with 0.2 mg protein G by the one-step method. The mixture was used in an ELISA to detect coated human polyclonal IgG. No activity was seen. The two-step method of coupling protein G and alkaline phosphatase was performed with three different ratios of the proteins: 1:5, 1:40, and 1:200. For the first coupling we used 0.2 mg protein G and 1 mg alkaline phosphatase, for the second 0.1 mg protein G and 4 mg enzyme, and for the third 0.025 mg protein G and 5 mg enzyme (see Table 1). The coupled products were analysed by gel-chromatography on Sepharose 6B (Figure 1). The activities of alkaline phosphatase and the protein G-enzyme complex in the ELISA were analysed, and the presence of protein G was monitored by ^{125}I-labelled protein G. As is evident from Figure 1, some of the radiolabelled protein G appeared in a high-molecular weight position, coinciding with the alkaline phosphatase- and ELISA-activity, most likely

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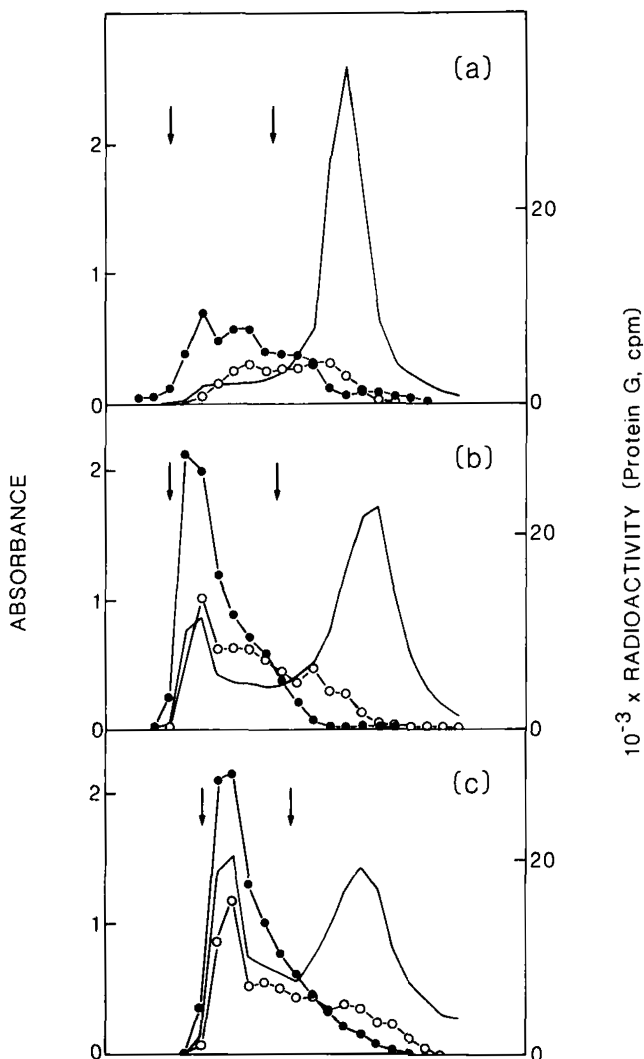

FIGURE 1. Gel-chromatography on Sepharose 6B of protein G-alkaline phosphatase conjugates, directly after performing the coupling. Protein G and the enzyme were mixed in the ratio 1:5 using 200 ug and 1 mg, respectively (Figure 1A), 1:40, using 100 ug and 4 mg (Figure 1B), and 1:200, using 25 ug and 5 mg (Figure 1C). The column (0.8 x 90 cm) was equilibrated and eluted with 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02 % Na-azide, at 1.5 ml/hour. The eluted fractions were analysed for alkaline phosphatase activity (open circles) enzyme-linked antibody-binding (filled circles) and radiolabelled protein G (lines) as described in the Materials and Methods section. The conjugates were pooled as shown in the figures.

TABLE 1

Incorporation of Protein G to Alkaline Phosphatase after Coupling at Different Ratios[a]

Amount Protein G (ug)	Amount Alkaline Phosphatase (mg)	Ratio Protein G: Alkaline Phosphatase	Incorporated Protein G[b] (%)
200	1	1:5	5
100	4	1:40	20
25	5	1:200	40

[a]Alkaline phosphatase was incubated with glutaraldehyde, excess glutaraldehyde removed by dialysis, and protein G was then added.

[b]Estimated from the eluted peaks of radiolabelled protein G from gel-chromatography of the coupled alkaline phosphatase and protein G.

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representing protein G conjugated to the enzyme. The degree of incorporation of radiolabelled protein G to the enzyme was estimated from each of the gel-chromatographies (Table 1). The ratio of protein G:alkaline phosphatase 1:5 gave approximately 5 % incorporation of protein G, the ratio 1:40 gave 20 % incorporation, and the ratio 1:200 gave 40 % incorporation.

Optimization of Protein G-Alkaline Phosphatase Incubations in ELISA

For optimal usage of the protein G-alkaline phosphatase conjugates in ELISA, different incubation times and temperatures were tested. Human alpha-1-m and several anti-human alpha-1-m antibodies were used. Human alpha-1-m was coated for one hour, and

rabbit or goat polyclonal antibody, or mouse monoclonal anti-alpha-1-m was then incubated for one hour. Figure 2 shows the result of different incubation times with a protein G-enzyme conjugate (1:200, not purified by gel-chromatography). It was concluded that two hours incubation was optimal, and this was used for the rest of the experiments. Figure 3 illustrates the result of incubations at different temperatures. High background occurs at $4^{o}C$, and there was lower specific binding at $37^{o}C$. Room-temperature appears optimal for the ELISA.

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Activity in ELISA of Different Protein G-Alkaline Phosphatase Conjugates

The products of the three couplings were tested by determining the binding to antibody-coated alpha-1-m of different dilutions of the three conjugates. The free, uncoupled protein G in the conjugation mixture could act as an inhibitor of the ELISA-activity. Purified protein G-enzyme complexes were therefore also tested after concentration down to the original volume before gel-chromatography. The result is shown in Figure 4. The six dilution curves suggest different amounts of similar conjugates, all useful for the detection of antigen-antibody complexes.

The samples tested in Figure 4 correspond to an original volume of the conjugation mixture of 1.0 ml, giving different concentrations of protein G. Quantitative evaluation of ELISA-activity of the conjugates requires correction for this. Thus, the conjugates, before and after separation by gel-chromatography, were adjusted to volumes giving a concentration of 0.1 mg/ml of

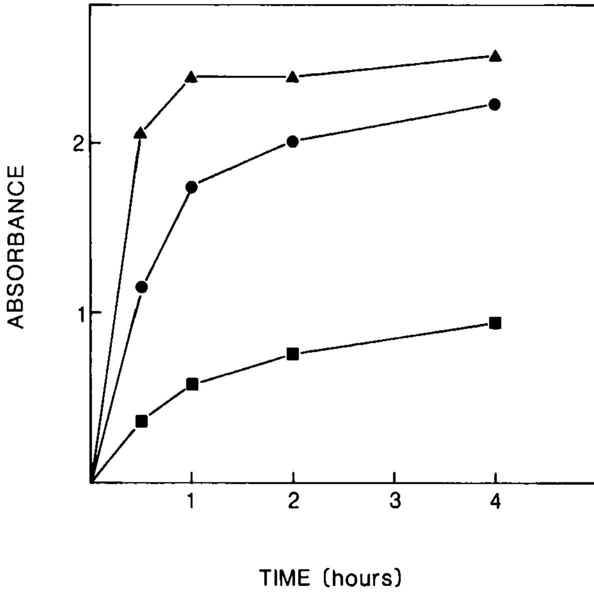

FIGURE 2. Time-dependence of incubation with protein G-alkaline phosphatase complex. Microtiter plates were coated with human alpha-l-m (4 ug/ml), incubated for two hours with rabbit anti-alpha-l-m serum (circles), goat anti-alpha-l-m serum (squares), or for one hour with mouse monoclonal anti-alpha-l-m BN11.3 (triangles), the last antibody followed by one hour incubation with rabbit anti-mouse Ig, all antibodies diluted 500 times. The following incubation with protein G-alkaline phosphatase complex, 1:200, non-separated by gel-chromatography, proceeded for different lengths of time. All incubations were done at room-temperature.

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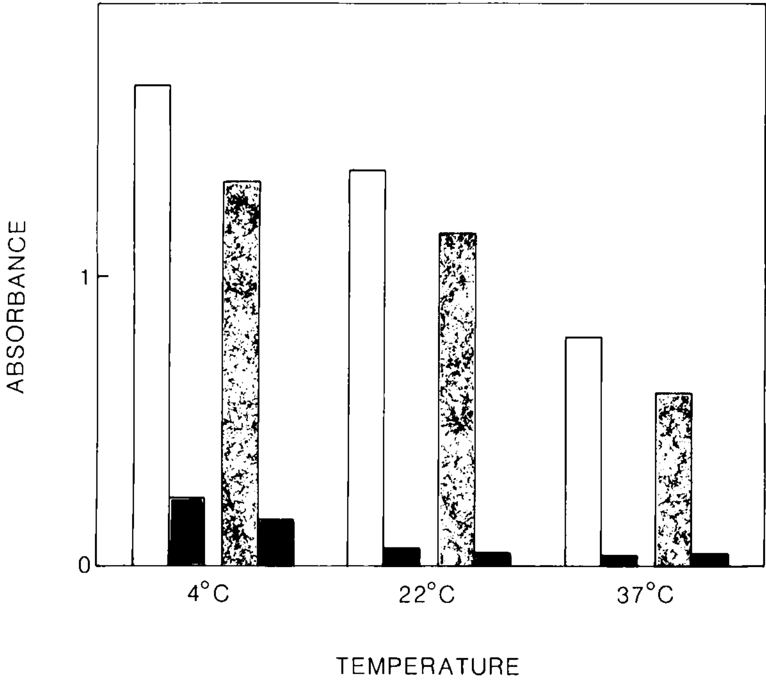

FIGURE 3. Influence of temperature on the ELISA. Microtiter plates were coated with 4 ug/ml human alpha-1-m, incubated with rabbit anti-alpha-1-m serum diluted 1000 times, and with protein G-enzyme conjugate, 1:40, non-separated (grey bars) or separated by gel-chromatography (empty bars) for two hours. Background binding was achieved by substituting antibodies with buffer (filled bars).

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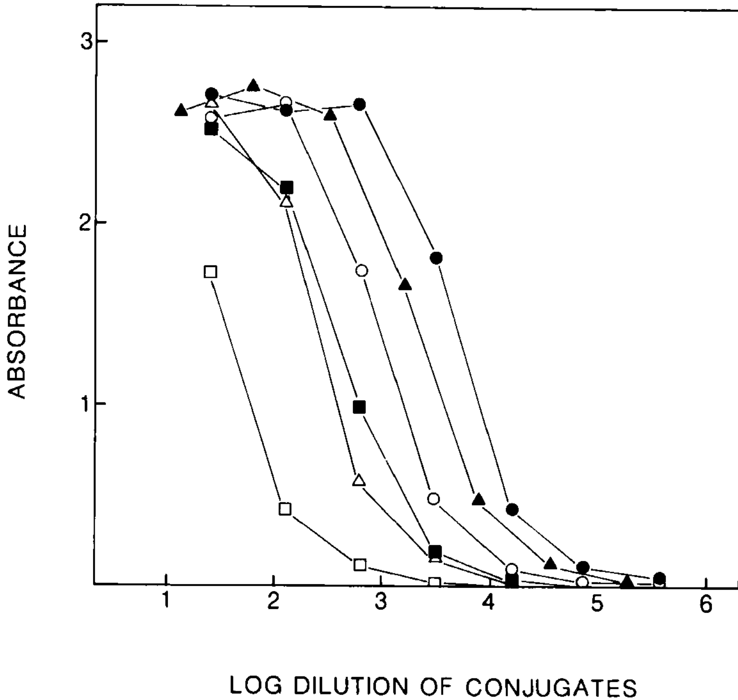

FIGURE 4. Activity in ELISA of protein G-alkaline phosphatase conjugates in different dilutions. Coating with 4 ug/ml human alpha-1-m, and incubation with rabbit anti-alpha-1-m serum diluted 500 times, was followed by incubation for two hours with the protein G-enzyme conjugates: 1:5, separated (■) or non-separated (□); 1:40, separated (●) or non-separated (○); 1:200 separated (△) or non-separated (▲). The conjugates were analysed directly or after separation by gel-chromatography of free and conjugated protein G as described in the Materials and Methods section. Samples of the protein G-alkaline phosphatase complexes were first adjusted to a volume corresponding to 1.0 ml of the original conjugation mixture, and then diluted as shown on the x-axis.

protein G originally added to the alkaline phosphatase. They were then tested in different dilutions as detectors of antibody-coated alpha-1-m in ELISA. Table 2 lists the absorbance values achieved after dilution 1000 times of all six conjugates. These figures thus represent the ELISA-activity per 5 ng protein G used for coupling to the enzyme. The 1:200 coupling gave the highest activity (absorbance 2.63) and the ratio 1:5 the lowest (absorbance 0.042). Comparing the values for the non-separated and the separated conjugates, it seems likely that the 1:5 complex was blocked by free protein G since a ten-fold increase in antibody-binding activity was achieved with the separation of free and complex-bound protein G by gel-chromatography. Also the 1:40 coupling seemed to gain by gel-chromatography. The last coupling, in ratio 1:200, however, did not increase its ELISA-activity by molecular sieving. These results could be expected from the low degree of incorporation in the 1:5 coupling and higher degree of incorporation in the 1:40 and 1:200 couplings (Table 1). The potential of the conjugates in the assay was also estimated by calculating the degree of dilution which gave the absorbance 1.0 under the experimental conditions mentioned. The highest value was reached by coupling in the ratio 1:200 without separation by gel-chromatography. It thus seems that 40 % incorporation of protein G makes it unnecessary to add an extra separation with its unavoidable losses of material. Hence, the coupling ratio 1:200 is optimal: high yield of protein G in the enzyme-conjugate, high ELISA-activity, and no need for separation of reactants before use.

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TABLE 2

Activity of Conjugates after Coupling of Protein G to Alkaline Phosphatase at Different Ratios

Ratio Protein G: Alkaline Phosphatase	Absorbance in ELISA[a] of Protein G-Conjugate, diluted 1000x[b]		Dilution of Protein G-Conjugate giving ELISA - absorbance 1.0	
	Non-separated	Separated[c]	Non-separated	Separated
1:5	0.042	0.41	32	315
1:40	1.39	2.41	1,580	7,900
1:200	2.63	1.85	15,850	3,630

[a]Incubation of microtiter plates first with human alpha-1-m (4 ug/ml) and then rabbit anti-alpha-1-m, diluted 500 times, was followed by incubation with protein G-alkaline phosphatase conjugates for two hours.

[b]The conjugation mixture, before or after separation by gel-chromatography, was adjusted to 0.1 mg/ml with respect to the amount of protein G originally mixed with alkaline phosphatase, and then diluted.

[c]Protein G-alkaline phosphatase conjugates were separated from free protein G by gel-chromatography, and then reconstituted to the same volume as before the separation.

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Evaluation of the Conjugates as Detectors of IgG

The usefulness of the protein G-alkaline phosphatase conjugate for detection of IgG on microtiter plates was determined by coating the plastic surface either directly with various IgG´s or with an antigen and its specific antibody. Figure 5 shows the result when the conjugate (1:200, non-separated) is incubated with coated rabbit or goat polyclonal IgG, or mouse monoclonal IgG1, IgG2a, or IgG2b, in serial dilutions. Comparable binding occured

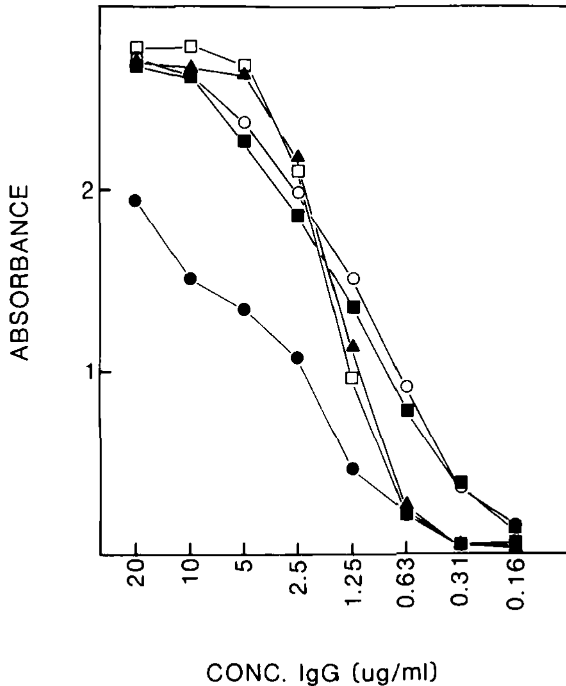

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FIGURE 5. Binding of protein G-alkaline phosphatase conjugates to IgG from various sources. Microtiter plates were coated over night with a dilution series of rabbit (open circles) or goat (filled squares) polyclonal IgG, or mouse monoclonal IgG1 (BN11.1, filled circles), IgG2a (BN11.2, open squares) or IgG2b (BN8.1, filled triangles). The protein G-alkaline phosphatase conjugate, 1:200, non-separated, was then incubated for two hours at a total protein G concentration of about 100 ng/ml.

to rabbit and goat IgG, and mouse IgG2a and IgG2b, with a weaker binding to mouse IgG1. Figure 6 illustrates the result of coating with alpha-1-m, followed by incubation with several anti-alpha-1-m antibodies and finally with protein G-alkaline phosphatase conjugates (1:40 or 1:200, non-separated). Very low binding was achieved with mouse monoclonal anti-alpha-1-m antibody (BN11.1 - IgG1, or BN11.3 - IgG2a, not shown), while goat and rabbit

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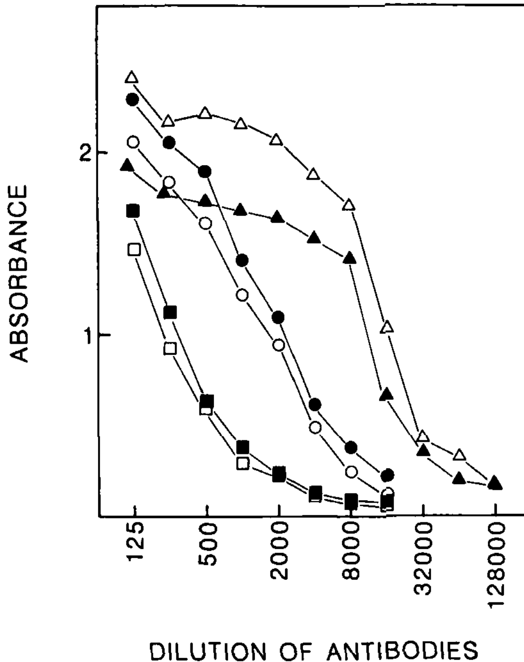

FIGURE 6. Detection of antigen with the use of specific antibodies and protein G-alkaline phosphatase conjugates. Human alpha-1-m (4 ug/ml) was coated on microtiter plates and incubated for two hours with different dilutions of anti-alpha-1-m serum from rabbit (circles) or goat (squares), or one hour each with different dilutions of mouse monoclonal anti-alpha-1-m (BN11.3, 11 mg/ml) and rabbit anti-mouse Ig (diluted 500 times). Protein G-enzyme conjugates, 1:40, non-separated (open symbols) or 1:200, non-separated (filled symbols) were then incubated for two hours at a total protein G concentration of approximately 100 ng/ml.

polyclonal anti-alpha-1-m antibody and monoclonal antibody BN11.3 amplified with rabbit anti-mouse Ig yielded an increasing binding of the protein G conjugates. Background binding, i.e. coating with buffer instead of IgG (Figure 5) or incubation with immune sera or monoclonal antibodies with other specificities (Figure 6), did not give detectable absorbance at any concentration (not shown).

DISCUSSION

Protein G binds to the Fc-part of many mammalian IgG's (4). The binding is more widely distributed among species and IgG-subgroups than protein A, its staphylococcal counterpart (3,8,12). Protein G thus complements protein A as a detector of IgG-antibodies in methodological applications. For example, group G streptococci were used for quantitative precipitation of antibodies from solutions (7) and purified protein G was succesfully applied for detection of goat and rabbit polyclonal and rat monoclonal antibodies in Western blotting (3,7), for preparation and characterization of mouse monoclonal antibodies (10) and in solid phase radioimmunoassay (6). Finally, protein G has also been used for detection of antibodies against HIV-proteins in human serum (13). In all these reports, the protein had been labelled with ^{125}I. We have expanded its usefulness by coupling it to an enzyme and developing an ELISA (14).

Mixing alkaline phosphatase and protein G with glutaraldehyde gave a protein G-enzyme complex with no activity in ELISA, and was thus not useful for our purpose. We tried instead the two-step method suggested by Avrameas and Ternynck (15), in which the enzyme first was "activated" by glutaraldehyde, and protein G then added after removal of free glutaraldehyde by dialysis. This yielded conjugates with higher ELISA-activity perhaps, as suspected by Engvall (11), because protein G does not become buried in the large enzyme complexes that result from the two-step method. Dialysis of free glutaraldehyde before the addition of

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protein G protects this molecule from high concentrations of the cross-linking agent, which may impair its IgG-binding properties.

After conjugation, free protein G was removed by gel-filtration because protein G not carrying alkaline phosphatase could also bind to the IgG, competing with the conjugated protein G. As expected, the activity in ELISA of the remaining protein G-bound enzymes was increased after the gel-filtration. However, when using the lowest ratio protein G:alkaline phospatase, 1:200, the incorporation of protein G into the enzyme complexes was high enough (approximately 40%) to make the influence of free protein G neglible, and gel-filtration was not necessary.

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The conjugate bound strongly to goat and rabbit polyclonal IgG, and to mouse monoclonal IgG1, IgG2a and IgG2b, although with a weaker binding to IgG1 than to the other IgG's. This agrees with earlier results concerning goat IgG and mouse IgG-subclass 1 (3), which are not bound by protein A (16,17). As antibodies, goat and rabbit polyclonal IgG's were readily detected by the protein G-alkaline phosphatase complex, and mouse monoclonal anti-alpha-1-m (IgG1), amplified with rabbit anti-mouse IgG, displayed a high titer. Coating with alpha-1-m and incubation with monoclonal anti-alpha-1-m alone did not, however, give any useful binding of protein G-enzyme. This surprising difference in binding between the polyclonal and monoclonal antibodies could be the result of enhancement: binding of one antibody to a single binding site increases the binding of other antibodies (18,19). Another possible explanation is that when the antigens are covered with several polyclonal antibodies, the protein G-enzymes are able to

bind at multiple points, leading to an amplification of the avidity of the protein G-conjugate.

To conclude, it is possible to produce a protein G-alkaline phosphatase conjugate, which is active in an ELISA as a detector of antibodies. Coupling of 25 ug protein G with 5 mg alkaline phosphatase gives a conjugate which can be used for more than 10,000 determinations of matrix-bound antibody, with maximal antibody binding giving an absorbance of 2.0.

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