PROTEIN A–ENZYME MONOCONJUGATE AS A VERSATILE TOOL FOR ENZYME IMMUNOASSAYS

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1. Introduction

Enzyme immunoassay techniques, by reasons of economy and simplicity, show considerable promise in clinical as well as in research applications [1]. Synthesis of enzyme–antibody conjugates is the principal requirement of enzyme immunoassay method. For the quantitation of antigens, conjugate of specific antibody with an enzyme is necessary [2]. However, a monoconjugate of an enzyme with a molecule which reacts specifically with the IgG will have widespread applications. Staphylococcal protein A binds specifically to F_c region of a wide variety of IgG molecules from several species [3]. The specific binding of protein A to F_c region of immunoglobulin is finding an expanding use in immunology for both preparative and analytical purposes. However, potentialities of protein A as a universal tool for the quantitation of antigens and antibodies have not been explored systematically. In this communication, we describe the preparation of protein A–enzyme monoconjugate and its use as a sensitive and universal tool for the quantitation of different antigens and antibodies. Protein A–enzyme monoconjugate was taken up by IgG molecules immobilised on an excess solid phase antigen. In presence of free antigen, uptake of limiting amounts of IgG by an excess of antigen immunoadsorbent was inhibited leading to decrease in protein A–enzyme uptake. Protein A–invertase monoconjugate has been used for the quantitation of human chorionic gonadotropin and human IgG and was found that as little as 3 ng/ml and 6 ng/ml, respectively, can be detected.

2. Materials and methods

Protein A was purchased from Pharmacia Fine Chemicals, Sweden and human chorionic gonadotropin (HCG) was a gift from Professor N. R. Moudgal. Invertase (EC 3.2.1.26) type VI, glucose oxidase (EC 1.1.3.4) type II and peroxidase (EC 1.11.1.7) type II were purchased from Sigma Chemical Co., MO. Nylon pieces were purchased from John Stanier and Co., England. Whole rabbit antisera to HCG was prepared as in [4]. Chromatographically pure IgG fractions of rabbit antisera to human IgG as well as to HCG were isolated as in [5].

Proteins were estimated as in [6] using crystalline bovine serum albumin (BSA) as standard. Concanavalin A, prepared as in [7], was coupled to Sepharose 4B by cyanogen bromide activation method [8]. Concanavalin A in the gel (4 mg concanavalin A/ml gel) was then succinylated and found to contain no reactive amino groups by trinitrobenzene sulphonic acid [9]. Invertase being a glycoprotein, invertase–protein A conjugate was prepared as in [10]. The complete experiment was carried out at 4°C. Invertase (15 ml of 100 μg/ml solution) was mixed with 6 ml succinylated concanavalin A–Sepharose in a column and washed extensively with 0.02 M Hepes buffer (pH 7.5) containing 0.15 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. When no enzyme activity was detected in the washing, 16 ml 1% glutaraldehyde in the above-mentioned buffer was passed and simultaneously washed thoroughly to remove excess of the crosslinking reagent. Protein A (90 μg/ml, 10 ml) was then introduced into the column and kept

closed for 2 h. Ethanolamine (20 ml 0.01%) was then introduced followed by washing with 6 bed vol. Hepes buffer. The conjugate was eluted with 10% α-methyl mannoside in the same buffer; eluate was concentrated using Aquacide II and dialysed against 0.1 M sodium acetate buffer (pH 5.0). Invertase activity in α-methyl mannoside eluate was determined using a coupled glucose oxidase–peroxidase system [11]. One unit of invertase was defined as the quantity of enzyme which hydrolyzes 1.0 μmol sucrose to invert sugar per minute at pH 5 at 37°C.

HCG, BSA and IgG were coupled to 1 cm^2 nylon pieces as in [12]. The nylon pieces were then kept in 0.05% (v/v) Tween-20 in 0.05 M phosphate buffer (pH 7.5) containing 0.1 M NaCl (PBS) for 20 h at 4°C. After proper washing with PBS, nylon pieces were stored in the same buffer at 4°C.

Molecular weight of the invertase–protein A conjugate was determined on a Sephadex G-200 column (1.5 × 70 cm) pre-equilibrated with 0.15 M NaCl as in [13]. Soybean trypsin inhibitor (21 500 daltons), ovalbumin (45 000 daltons), BSA (monomer, 67 000 daltons), BSA (dimer, 134 000 daltons), γ-globulin (200 000 daltons), catalase (240 000 daltons) and β-galactosidase (500 000 daltons) were used as molecular weight standards.

Protein A in the conjugate was estimated using radial immunodiffusion against human IgG by a modification of the method in [14].

All assays were carried out in duplicate. Isotonic phosphate-buffered saline (0.05 M, pH 7.5) was used for all dilutions. For immunoassay of HCG, 0.1 ml anti-HCG (1:100), differing amounts of HCG, 0.2 ml BSA (20 mg/ml) were incubated with a piece of HCG–nylon and made to 0.5 ml final vol. with PBS. After incubation for 12 h at 4°C, the nylon pieces were washed thoroughly with PBS containing 0.1% BSA. Each nylon piece was then incubated with 0.2 ml 20 mg/ml solution of BSA, 0.26 ml PBS and excess of protein A–invertase conjugate (0.1 ml) at 4°C for 3 h. Nylon pieces were then washed thoroughly with the same buffer and the enzyme activity was measured as above. Maximum binding of the conjugate at the concentration of IgG used for assays was determined by incubating antibody and nylon piece containing excess of solid phase antigen in the absence of free antigen. Antigen free nylon and BSA–nylon incubated with anti-HCG in the presence and absence of HCG served as controls.

For the immunoassay of human IgG, a limiting amount of conjugate was used since both free and solid phase IgG can bind to this conjugate. Nylon pieces containing IgG were incubated with 0.2 ml 20 mg/ml solution of BSA, increasing amount of free IgG and 0.04 ml protein A–invertase conjugate, made up to 0.5 ml final vol. with the same buffer. Same principle as discussed above was applied for maximum uptake of the conjugate as well as for the controls. After proper washing enzyme activity was measured in each of the nylon pieces.

3. Results

Protein A does not contain significant amounts of carbohydrate [15] and its inability to bind concanavalin A was confirmed by immunodiffusion technique in agarose.

Invertase is 270 000 daltons and protein A is 42 000 daltons. Invertase–protein A conjugate on gel filtration on Sephadex G-200 gave a peak corresponding to the conjugate in the molar ratio of 1:1. This peak was pooled separately from the free enzyme peak. Stoichiometry of 1:1 for the two proteins was confirmed by estimating the ratio of enzyme activity with the amount of protein A in the conjugate by radial immunodiffusion.

Preliminary studies showed that a 1:100 dilution of the HCG rabbit antisera and a 1:50 dilution of the conjugate were optimal. Figure 1 shows a typical calibration curve. Free HCG at ~60 ng gave 50% inhibition of the antibody binding as measured by protein A–invertase monoconjugate uptake. As little as 3 ng/ml final conc. antigen can be detected and the range is in 3 orders of magnitude. Controls in which either conjugate or solid phase antigen, or specific immunoglobulin was left out gave no measurable invertase activity. The assay was found to be reproducible. The effect of using a crude preparation of anti-HCG was examined and found to be identical with the purified anti-HCG. The amount of protein A–invertase monoconjugate required for the assay was found to be immaterial provided it saturates the antibody–antigen immunoadsorbent complex.

The ability of free IgG when preincubated with the solid phase IgG to prevent binding of limiting

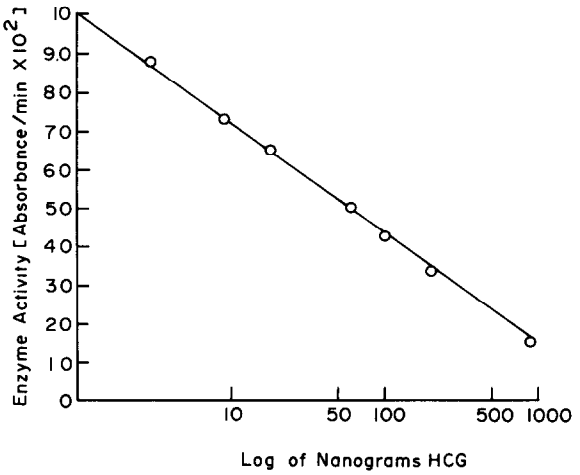

Fig. 1. Binding of protein A–invertase monoconjugate to anti-HCG bound to HCG–nylon in presence of free HCG: HCG–nylon was incubated with varying amounts of free HCG (12 600 IU/mg). The assay was performed as in the text with 0.1 ml anti-HCG (1:100) and 0.1 ml of protein A–invertase monoconjugate (1:50).

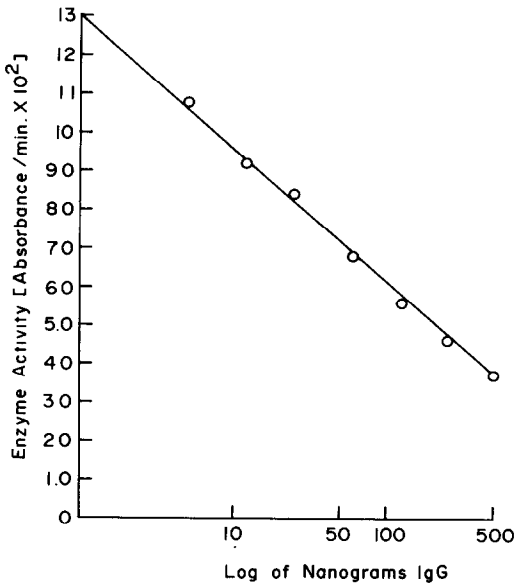

Fig. 2. Binding of protein A–invertase to IgG–nylon in presence of free human IgG: Nylon–IgG was preincubated with varying amounts of free human IgG; amount of protein A–invertase (0.04 ml, 1:50) was taken for the assay.

about of protein A–enzyme conjugate is shown in fig 2. IgG at ~80 ng gave 50% inhibition of the uptake of protein A–invertase monoconjugate and ~6 ng/ml IgG could be detected. BSA–nylon or nylon pieces as such which served as control did not show a significant enzyme activity.

4. Discussion

The unique property of protein A to bind specifically to the F_c region of a wide variety of IgG molecules without impairing the ability of antibody to bind antigen served as the basis for this general immunoassay system. This method has several advantages over conventional enzyme immunoassays such as:

(i) The assay is simple to perform and the results are reproducible due to the usage of a monoconjugate;
(ii) Purification of antibody is not essential because only the specific antibody will be taken up by solid phase antigen–immunoadsorbant;
(iii) Sensitivity is comparable to that of radioimmunoassay. Moreover sensitivity of the assay described here can be increased further by using a more active enzyme;
(iv) Same conjugate can be used as a universal tool for the quantitation of different antigens and antibodies.

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References

[1] Schuurs, A. H. W. M. and Van Weemen, B. K. (1977) Clin. Chim. Acta 81, 1–40.
[2] Wilson, G. B. (1976) Clin. Chem. 22, 1243–1255.
[3] Forsgren, A. and Sjöquist, J. (1966) J. Immunol. 97, 822–827.
[4] Geiger, W. (1971) in: Radioimmunoassay methods (Kirkham, K. E. and Hunter, W. M. eds) pp. 169–189, Livingstone, Edinburgh.

[5] Fahey, J. L. and Terry, E. W. (Weir, D. M. ed) Handbook of Experimental Immunology, 3rd edn, ch.8.
[6] Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 193, 265–275.
[7] Surolia, A., Prakash, N., Bishayee, S. and Bachhawat, B. K. (1973) Indian J. Biochem. Biophys. 10, 145–148.
[8] Porath, J., Axen, R. and Ernbalk, S. (1967) Nature 215, 1491–1492.
[9] Failla, D. and Santi, D. V. (1973) Analyt. Biochem. 52, 363–368.
[10] Pillai, S. and Bachhawat, B. K. (1977) Biochem. Biophys. Res. Commun. 75, 240–245.
[11] Surolia, A. and Bachhawat, B. K. (1977) Biochim. Biophys. Acta 497, 760–765.
[12] Sundaram, P. V. and Hornby, W. E. (1970) FEBS Lett. 10, 325–327.
[13] Andrews, P. Methods Biochem. Anal. 18, 1–54.
[14] Becker, W. (1969) Immunochemistry 6, 539–546.
[15] Sjöquist, J., Meloun, B. and Hjelm, H. (1972) Eur. J. Biochem. 29, 572–578.