MONOCONJUGATE ENZYME LINKED IMMUNOASSAY

Shiv PILLAI and B. K. BACHHAWAT
Indian Institute of Experimental Medicine, Calcutta-700 032, India

Received 3 April 1978

1. Introduction

Enzyme immunoassay is usually considered inferior to radioimmunoassay on grounds of poorer sensitivity and precision [1]. This failing stems from the relatively random techniques of protein–protein conjugation commonly employed in the labelling of antibodies and protein antigens. The majority of conjugation procedures employ bifunctional reagents and give rise to conjugates of varying stoichiometry. The presence of antibody–antibody conjugates or poorly labelled antibody may significantly interfere with the sensitivity of an enzyme immunoassay. If enzyme immunoassays of comparable sensitivity to radioimmunoassay are to be developed, a uniform, stoichiometric and reproducible labelling method is essential. Moreover, if such a method should prove practicable, it should be equally possible, in principle, to link a string of molecules of a given enzyme to a single molecule of antibody or antigen, giving rise to immunoassays of sensitivity well beyond that of conventional radioimmunoassay. No method of uniform enzyme antibody conjugation has hitherto been described. Of the methods that do not employ bifunctional reagents, two deserve mention. The periodate method [2] yields heterogeneous conjugates [3]. The dimaleimide method [4] produces conjugates of limited heterogeneity which are likely to be unstable [5]; these conjugates have however yielded the most sensitive enzyme immunoassays so far reported [6].

We have demonstrated that the 'spreading out' of an enzyme on a suitable dilute matrix could facilitate its conjugation in a uniform stoichiometric manner to another enzyme [7]. Using the same matrix principle we have conjugated an enzyme to an antibody and the resulting monoconjugate has been used in an immunoassay of high sensitivity.

2. Experimental

Protein was estimated as in [8] using bovine serum albumin (Sigma) as standard. Invertase was assayed by the one-step method in [9]. Antibodies to horse heart myoglobin (Sigma) were raised in rabbits (route: half intramuscular, half subcutaneous; Day 0, 1 mg in complete Freund's adjuvant. Boosters of 0.5 mg on day 30, 60, 90 and 120. Bled on day 70, 100 and 130). The pooled and decomplemented antiserum was purified on DEAE cellulose [10] and a myoglobin–Sepharose immunoadsorbent (1.8 mg/ml gel, divinylsulphone coupling, 0.5 mol/l NH_4OH as eluent [11]). The eluted protein was extensively dialysed against 0.05 mol/l sodium phosphate, pH 7.4, containing 0.15 mol/l NaCl (PBS). It was confirmed to be antimyoglobin IgG by immunodiffusion [12], immunoelectrophoresis against antirabbit serum (Wellcome) [13] and sodium dodecyl sulphate–polyacrylamide gel electrophoresis in a 5% gel [14]. Antimyoglobin (2.1 mg/ml) was coupled to squares of commercial nylon cloth of side 1 cm, as in [15]. The nylon pieces were washed extensively in 0.05% v/v Tween-20 in PBS. Pieces to which no protein was coupled and the antimyoglobin–nylon pieces were introduced into individual siliconised glass tubes each containing 0.4 ml of 2% w/v bovine serum albumin (BSA) in PBS.

Succinyl concanavalin A Sepharose 6B was prepared and equilibrated as in [7]. All the operations described below were performed at 4°C. The gel (4 mg protein ml), 10 ml, was suspended in 10 ml PBS. Invertase

(Type VI Sigma 0.9 mg/ml), 2 ml, was added dropwise and the mixture stirred overnight. The gel was packed into a column and the effluent was checked for the absence of enzyme activity. Glutaraldehyde (Sigma), 10 ml, 1% v/v, in PBS was passed into the column which was closed subsequently for 18 h. The column was washed with 7 bed vol. PBS and 10 ml antimyoglobin IgG (0.13 mg/ml) was introduced into the column which was then closed for 18 h. The column was washed with the above buffer untill the effluent was free of protein. Ethanolamine, 10 ml, 0.01% v/v, in PBS was introduced into the column which was closed for 4 h. The column was exposed to room temperature and eluted with 4% w/v α-methyl mannoside in PBS. Eight 2 ml fractions containing enzyme activity were pooled, concentrated using Aquacide III (Calbiochem) and dialysed against PBS. 4 ml of a 0.26 mg/ml protein solution was obtained. A 1.5 ml aliquot was diluted with 13.5 ml 2% w/v BSA in PBS. This was used in the immunoassay described below. Another 0.5 ml aliquot was subjected to gel filtration.

Succinyl concanavalin A–Sepharose 6B, 2 ml, was mixed with 3 ml antimyoglobin IgG (0.7 mg/ml), washed extensively with PBS and elution with α-methyl mannoside was attempted as above. The eluate was concentrated from 4–0.5 ml, dialysed, and tested for protein content.

Of each of the following solutions, 0.5 ml was successively chromatographed on Sepharose 6B:

(i) Blue dextran, 1%.
(ii) The succinyl concanavalin A–Sepharose column eluate.
(iii) Invertase, 0.7 mg/ml.
(iv) Urease (crystalline, Sigma) 1.9 mg/ml.

The fractions of the first invertase peak obtained on gel filtration of the succinyl concanavalin A–Sepharose eluate were pooled and the maximal velocities and Michaelis constants of this sample, as well as that of untreated invertase were determined.

The albumin solutions were aspirated from all the nylon containing tubes. Two untreated nylon and 2 antimyoglobin–nylon containing tubes served as controls; to each of these tubes 10 μl PBS diluted in 390 μl 2% BSA in PBS was added. A range of myoglobin solutions was prepared such that 10 μl aliquots contained from 20 pg to 200 ng of myoglobin. Each aliquot was diluted with 390 μl 2% w/v BSA in PBS, and added to separate antimyoglobin–nylon containing tubes set up in duplicate. After 20 h at 4°C, each tube was washed with 25 ml 0.05% v/v Tween-20 in PBS. Diluted conjugate, 0.4 ml, was added to each tube. After a further 20 h at 4°C the tubes were washed as before and assayed for invertase. All readings were against an assay blank (containing substrate, dye, glucose oxidase and peroxidase). The assay period was 60 min and the substrate solution contained 0.5% v/v Tween-20. The assay was repeated at myoglobin concentrations of 0 pg, 200 pg, 400 pg, 600 pg and 2000 pg in duplicate.

3. Results and discussion

No detectable binding of antimyoglobin IgG to the succinylated lectin matrix occurred under the above conditions.

On gel filtration the succinyl concanavalin A–Sepharose column elutate yielded 2 peaks, the second peak coinciding with that of free invertase and the first peak eluting close to a urease peak on the same column. The sum of the molecular weights of IgG and invertase approximates closely to the molecular weight of urease, which is 483 000 [16]. The purified conjugate and untreated enzyme were found to have identical maximal velocities and Michaelis constants.

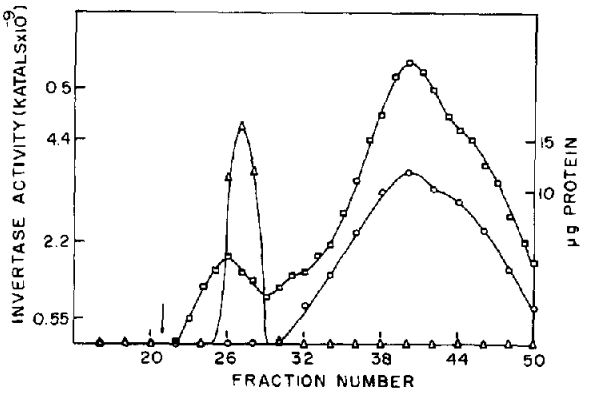

Fig. 1. Gel filtration on Sepharose 6B. Equilibration in PBS. Bed vol. 75 ml. Internal diameter 1.7 cm. Flow rate 28 ml/h. Fraction vol. 1.3 ml. (–○–) Enzyme activity of untreated invertase/0.1 ml. (–□–) Invertase activity in succinyl concanavalin A–Sepharose eluate/fraction. (–△–) Protein content of urease/fraction. Arrow indicates position of leading peak of blue dextran.

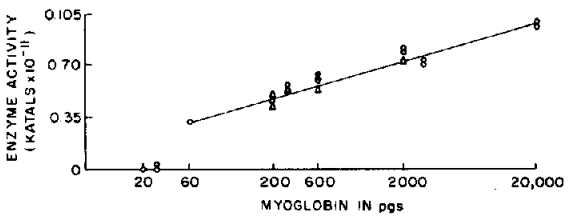

Fig. 2. Enzyme immunoassay of myoglobin. Myoglobin in pg is plotted on a log scale (○) and (△) represent enzyme activities in separate assays. No enzyme activity was detectable in the control antimyoglobin–nylon and untreated nylon tubes.

The commercial invertase selected showed negligible non-specific binding to nylon under the above conditions; the lectin eluate was used in the immunoassay without attempting separation of unconjugated enzyme on gel filtration. Although a major portion of the enzyme remained unconjugated under the above conditions, we have found that by using a suitably high concentration of the 'second' protein in the conjugation system, invertase can be almost quantitatively monoconjugated to another protein [7]. The principle of 'spreading out' one protein on a matrix is generally applicable to any protein–protein conjugation. A succinylated antibody matrix has also been found to facilitate enzyme–antigen monoconjugation (S.P. and B.K.B., unpublished).

The estimation of horse myoglobin was log linear over 4 log cycles and the minimum detectable amount was 60 pg (3.4 fmol). The immunochemistry of myoglobin from many species has been studied exhaustively and the number of its antigenic sites is known [17]. Human myoglobin has been estimated by radioimmunoassay [18,19] and the lowest detectable amount reported is 0.5 ng. Two site (sandwich) enzyme immunoassays of greater sensitivity than ours have been reported, but only for larger protein antigens which are likely to have many more antigenic sites than myoglobin. The use of more sensitive methods of enzyme assay and Fab fragments of antibodies are factors that could significantly enhance sensitivity.

Acknowledgements

The use of laboratory facilities of the Kothari Centre of Gastroenterology is gratefully acknowledged. S.P. is a Senior Research Fellow of the Indian Council of Medical Research. This work was supported by the Department of Science and Technology.

References

[1] Landon, J. (1977) Nature 268, 483–484.
[2] Nakane, P. K. and Kawaoi, A. (1974) J. Histochem. Cytochem. 22, 1084–1091.
[3] Hevey, R., Bonacker, L. H. and Sparacio, R. (1975) in: Immunoenzymatic Techniques (Feldmann, G. et al. eds) pp. 191–198, North-Holland, Amsterdam.
[4] Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. (1975) FEBS Lett. 56, 370–372.
[5] Kato, K., Fukui, H., Hamaguchi, Y. and Ishikawa, E. (1976) J. Immunol. 116, 1554–1560.
[6] Hamaguchi, Y., Kato, K., Ishikawa, E., Kobayashi, K. and Katunuma, N. (1976) FEBS Lett. 69, 11–14.
[7] Pillai, S. and Bachhawat, B. K. (1977) Biochem. Biophys. Res. Commun. 75, 240–245.
[8] Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 193, 265–275.
[9] Messer, M. and Dahlqvist, A. (1966) Anal. Biochem. 14, 376–392.
[10] Fahey, J. L. and Terry, E. W. (1973) in: Hand Book of Experimental Immunology (Weir, D. M. ed) 2nd edn, vol. 1, 7.1–7.16, Blackwell, Oxford.
[11] Sairam, M. R. and Porath, J. (1976) Biochem. Biophys. Res. Commun. 69, 190–196.
[12] Ouchterlony, O. (1948) Acta Pathol. Microbiol. Scand. 25, 186–191.
[13] Grabar, P. and Williams, C. A. (1953) Biochim. Biophys. Acta 10, 193–194.
[14] Laemmli, U. K. (1970) Nature 227, 680–685.
[15] Sundaram, P. V. and Hornby, W. E. (1970) FEBS Lett. 10, 325–327.
[16] Reithel, F. J., Robbins, J. E. and Gorin, G. (1964) Arch. Biochem. Biophys. 108, 409–413.
[17] Atassi, M. Z., Tarlowski, D. P. and Paull, J. H. (1970) Biochim. Biophys. Acta 221, 623–635.
[18] Stone, M. J., Willerson, J. T., Gomez-Sanchez, C. E. and Wateman, M. R. (1975) J. Clin. Invest. 56, 1334–1339.
[19] Rosano, T. G. and Kenny, M. A. (1977) Clin. Chem. 23, 69–75.

Enzyme-linked immunosorbent assay (ELISA). Uses enzyme-antibody conjugates to quantify target molecules. Direct ELISA: Uses a single antibody to detect the presence of an antigen.Â Enzyme immunoassays (EIA) are used to visualize and quantify antigens. They use an antibody conjugated to an enzyme to bind the antigen, and the enzyme converts a substrate into an observable end product. The substrate may be either a chromogen or a fluorogen.