A COMPETITIVE ENZYME-IMMUNOASSAY USING LABELLED ANTIBODY

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1. Introduction

Enzyme immunoassay (EIA) is a useful method of measuring antigens; it is specific, sensitive and reproducible. Several different types of EIA have been described [1,2]. Those which use labelled antibody are attractive because:

(a) A standard method of labelling antibody with enzyme can be used for all assays;
(b) In some cases it is easier to prepare pure antibody than pure antigen.

However, the labelled antibody–EIAs which have been described all require at least two incubation steps (excluding the incubation used to determine the enzyme label's concentration) and the aim of the work described here was to develop a one-step EIA which used labelled antibody and which was both rapid and sensitive. In this EIA the antigen, human immunoglobulin G (IgG), was quantified by measuring its ability to inhibit the binding of labelled antibody to excess solid phase antigen.

2. Materials and methods

2.1. *Anti-IgG antibodies*

Human IgG was purified from normal serum by ammonium sulphate precipitation and DEAE-cellulose chromatography [3]. The IgG, which gave a single arc with anti-human serum protein and anti-IgG antisera in immunoelectrophoresis, was injected into rabbits by the intramuscular route. The antiserum produced also gave a single arc against purified IgG in immunoelectrophoresis. The anti-IgG antibodies were obtained by immunoaffinity chromatography using IgG immobilized on Ultrogel AcA 34 (LKB Instruments Ltd., South Croydon, Surrey) with glutaraldehyde [4].

2.2. *Alkaline phosphatase/anti-IgG conjugate*

Anti-IgG antibodies (0.5 mg) were linked to alkaline phosphatase (Sigma Chemical Co., St Louis, MO; type VII) (1.5 mg) with glutaraldehyde [5].

2.3. *Competitive EIA*

All assays were carried out in duplicate. Controls in which free antigen, solid phase antigen or conjugate was omitted were set up with each batch of assays.

1. The wells of M129A Microtiter plates (Dynatech Labs Ltd, Billingshurst, Sussex) were coated with 0.3 ml 0.05 M sodium carbonate buffer (pH 9.6) containing 0.02% NaN_3 and 5 μg purified IgG/ml (or in some experiments, the same amount of crude IgG). The plates were incubated at 35°C for 3 h.
2. The plates were washed 3 times by holding them under a distilled water tap flowing at ~500 ml/min. The contents of the wells were decanted by inversion and finally the inverted plate was tapped firmly on paper towelling.
3. Standard or unknown IgG solution (0.1 ml) in 0.02 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl, 0.02% NaN_3 and 0.05% Tween 20 was added to the wells. This was immediately followed by a 1:50 dilution of conjugate (0.1 ml) in the same buffer. The plates were incubated at 35°C for 1 h.
4. The plates were washed as in step 2.
5. Substrate solution (0.2 ml), *p*-nitrophenyl phosphate (2 mg/ml) in 0.05 M sodium carbonate buffer

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(pH 9.8) containing 1 mM $MgCl_2$, was added to each well. After 8–15 min incubation at room temperature the enzymic reaction was stopped by the addition of 0.1 ml 1 M NaOH and the solution A_{405} nm was determined in a flow-through microcuvette of 10 mm path length (MSE Scientific Instruments, Crawley, Sussex).

2.4. *Radial immunodiffusion*

Single radial immunodiffusion was performed in 1% agarose gel containing anti-IgG antiserum [6].

2.5. *Protein determination*

Protein was measured by the Lowry method [7].

3. Results

Preliminary studies showed that a conjugate dilution of 1:50 and a coating solution containing 5 μg antigen/ml were optimal. The incubation time for the antigen–antibody reaction was restricted to 1 h although, after this period, only 80% of maximum conjugate binding had occurred. The adsorbative capacities of the Microtiter plates were found to vary between and within batches and the plates were classified before use; those that showed low adsorption in sample wells were discarded.

A typical calibration curve is shown in fig.1. The sensitivity of the assay is ~50 ng/ml and the range is ~3 orders of magnitude. Controls in which either conjugate or solid phase antigen were left out (blanks) gave ~0.2 A_{405} readings. The reproducibility of the assay was determined at high, intermediate and low antigen concentrations; the results are presented in table 1. Accuracy was measured by comparing the EIA results for diluted IgG solutions with the results obtained by single radial immunodiffusion. The regression line gave a correlation coefficient of 0.99 and a slope of 1.1.

The effect of using a crude preparation of antigen for immobilization on the solid phase was examined. Human serum or the serum fraction precipitated by ammonium sulphate at 33% saturation diluted to contain 5 μg IgG/ml were used. The results are shown in fig.1.

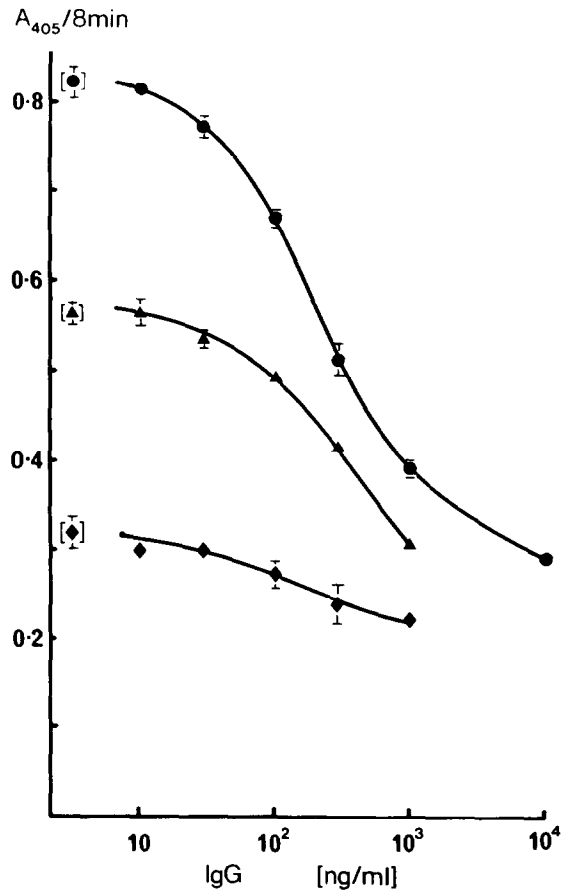

Fig.1. Calibration curves relating the amount of enzyme activity on the solid phase to IgG concentration. The mean and range of duplicate determinations are shown. Different preparations of IgG (5 μg/ml) were immobilized on the solid phase: (●) purified IgG; (▲) ammonium sulphate fraction of serum, 0.7 mg IgG/mg protein; and (◆) serum, 0.2 mg IgG/mg protein. The blank value was 0.17. Zero dose response is given within brackets.

Table 1
Reproducibility of EIA

IgG (ng/ml)	Within-day variation	Between-day variation
10	4.4	4.6
100	6.0	5.8
1000	4.0	5.0

Coefficients of variation are given for within-day experiments (9 replicates) and between-day experiments (3 replicates on each of 3 days)

4. Discussion

In the assay described, antigen competes with solid phase antigen for a limited amount of antibody. The assay uses the same reagents as the 'immunoenzymometric assay' [2], however, the latter is a non-competitive system and requires two incubation steps; in the first, antigen reacts with excess labelled antibody and, in the second step, the remaining labelled antibody reacts with excess solid phase antigen (e.g. [8]).

The advantages of the competitive EIA using labelled antibody are as follows.

(1) The single antigen–antibody reaction and the simple washing procedure allows a rapid assay.
(2) The assay is carried out in a single well or tube which can act as a solid phase for separation and as a cuvette for spectrophotometric measurement of the enzyme's product [9].
(3) Conjugates for the assay of any antigen can all be prepared by the same procedure.
(4) The conjugates are stable. (The conjugates employed in this work were used at a final dilution of 1:100 after storage at room temperature and 4°C for between 1 and 2 years.)
(5) Protein-coated Microtiter plates are relatively stable; they may be stored at −18°C for several weeks prior to use [10].
(6) Pure antigen is not essential for immobilization on the solid phase but when impure antigen is used the sensitivity is decreased. (Pure antibody may not be required for labelling if the antiserum's IgG fraction has sufficient avidity.)
(7) The sensitivity of the EIA may be easily increased by prolonging the antigen–antibody and enzyme incubations.
(8) The assay is sensitive, reproducible and accurate; it is also convenient and of wide applicability.

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