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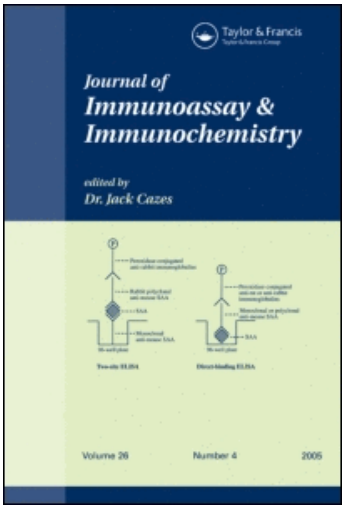

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COMPARISON OF GLUCOSE OXIDASE AND PEROXIDASE AS LABELS FOR ANTIBODY IN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

Horse-radish peroxidase and glucose oxidase were each separately conjugated to identical aliquots of goat IgG containing anti-human IgG antibodies. We used a minor modification of the periodate method of Nakane and Kawaoi to covalently bind each enzyme to IgG. Glucose oxidase conjugates proved superior to peroxidase conjugates based on the following qualities. The glucose oxidase conjugates had 1) usable dilutions 2 to 10 times greater than peroxidase conjugates, 2) much lower background or control non-specific activities, and 3) nearly twice the sensitivity as expressed by absorbance change vs. change in antigen. Comparison of the antibody titers showed the glucose oxidase conjugate with 80% and the peroxidase conjugate with 20% of the original goat antibody.

INTRODUCTION

The usefulness of an enzyme-antibody conjugate in detecting antigen depends upon the degree to which that conjugate can be diluted and bind quantitatively to the antigen in question or can exhibit proportional loss of binding to fixed antigen by known amounts of free antigen. The response must also be significantly higher than background activity shown by controls. The ultimate success of assays that involve enzyme labeled antibody adsorption depends on the quality of the conjugate which, in turn, is a

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function of: 1) the coupling or conjugative process; 2) the particular enzyme used; 3) the purity and immunologic properties of the antibody; and 4) the stability of the linkage. Given that the quality and quantity of the antibody to be used are identical, one can compare the usefulness of different enzymes as labels.

Several enzymes have been proposed (1) as labels in immunoassays. Here we compare two of these, peroxidase and glucose oxidase. Both are glycoproteins which can be obtained in high purity, are remarkably stable in solution, can be conjugated to other proteins by similar means, and are assayed using comparable techniques. This report is a summation of experiences with several conjugate preparations.

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MATERIALS AND METHODS

Antiserum, Enzymes and Chemicals

Antiserum to human IgG was raised in a goat from the colony of Scripps Clinic and Research Foundation by weekly injections for four weeks. The IgG was isolated according to the octanoic acid method of Steinbuch and Audran (2), concentrated to a protein content of 53.5 mg/ml (3) and showed a precipitating antibody titer of 1:16. Donkey anti-goat IgG was purchased from Miles Laboratories. Antibodies cross reacting with human IgG were removed by adsorption with solid phase human IgG (4).

Human IgG was isolated from pooled serum (2) and further purified by QAE chromatography (5). A 100 µg aliquot was labeled with ^{125}I using chloramine T (6).

All enzymes came from Sigma Chemical Co., St. Louis, MO; ACA-34 came from LKB Instruments, Inc., Rockville, MD, and ABTS

(2.2^1-Azino-di [3-ethyl-benzthiazolin sulfonate (6)] diammonium salt) came from Boehringer-Mannheim Biochemicals, Indianapolis, IN. All other chemicals were reagent grade.

Peroxidase was assayed by observing the change in absorbance at 642 nm with a buffer substrate consisting of 2 mg ABTS in 20 ml 0.015% H_2O_2 (v/v) in 0.1M phosphate buffer, pH 5. Glucose oxidase was assayed by observing the change in absorbance at 642 nm with a solution prepared by combining 3 ml of 18% aqueous glucose (well equilibrated) with 2 mg ABTS, 1 ml of 20 mg% peroxidase and 25 ml 0.1M phosphate buffer, pH 6. Some enzyme assays were performed using phenol-amino antipyrene as described for peroxidase in the Worthington Enzyme Manual (7). This latter gave only 20% as much absorbance change at 500 nm as the ABTS system gave at 642 nm.

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Preparation of Conjugates

We prepared conjugates of goat IgG with peroxidase and with glucose oxidase by the method of Nakane and Kawaoi (8) with a slight modification (9). Briefly, 12 mg of glucose oxidase or 5 mg of peroxidase dissolved in 1 ml 0.3M $NaHCO_3$ was reacted (22^oC, 1 h) with 100 µl fluorodinitrobenzene (10 g/L absolute ethanol) then oxidized with an equal volume 0.08 molar sodium periodate (20 min., 22^oC). The reaction was stopped with 1 ml 0.16 molar ethylene glycol and after 1 hour the mixture was dialyzed vs. 0.01 M Carbonate buffer, pH 9.5, 3 times, 1 hour each. 10 mg goat IgG was added, mixed and after any required adjustment of pH to 9.5 this mixture was left at 22^oC in dark, 18

hours. Sodium borohydride, 5 mg, was added, mixed and allowed to react 7 hours, 5°C. After extensive dialysis vs. saline, the material was concentrated in a colloidin tube to 1 ml by negative pressure. Certain peroxidase conjugates were purified by chromatography (AcA-34, 2.5 X 90 cm column, 13 ml/h). The 1 ml of concentrated final material, both peroxidase and glucose oxidase derived, was preserved with 1 ml glycerol and kept at -12°C.

Evaluation of Conjugates

Glucose oxidase and peroxidase were conjugated to identical amounts (10 mg from the same pool of goat IgG) so that only the type of enzyme differed in the resulting conjugates. The qualities used to characterize these conjugates included 1) the percent of enzyme covalently bound; 2) the enzymatic activity retained after conjugation; 3) the loss of antibody activity due to conjugation; 4) the optimal dilution required for use in immunoassay; 5) the non-specific enzyme product formation as a percent of the absorbance at a stated assay level; and 6) the maximum change in absorbance with change in antigen at optimal dilution.

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RESULTS AND DISCUSSION

Percent of Enzyme Covalently Bound

Physical evidence of the binding of peroxidase to IgG was obtained by AcA-34 chromatography (Fig. 1). Acrylamide gel electrophoresis (10) provided similar but more subjective evidence about the glucose oxidase/IgG conjugation product. Rabbit anti-goat IgG was used to separate free and bound enzyme from one glucose oxidase and one peroxidase conjugate, each chosen because

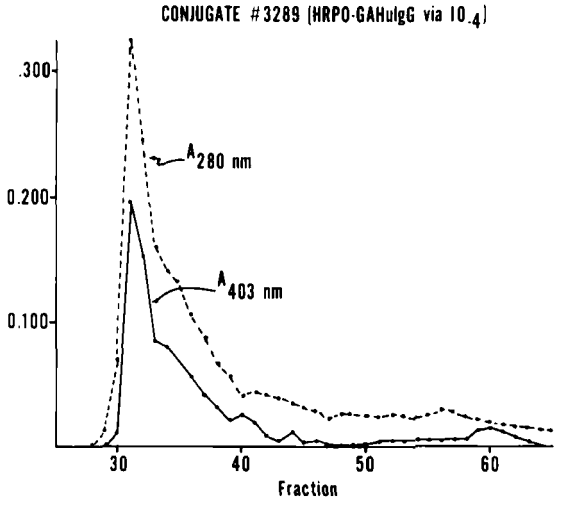

FIGURE 1 Exclusion chromatography of a peroxidase conjugate using a 90 x 2.5 cm. AcA-34 column shows high molecular weight material appearing soon after the void volume (fraction 29). Free human IgG would appear at fractions 45-55 and free peroxidase near fraction 60. This chromatographic purification step was abandoned after several trials showed routinely successful conjugations by the periodate procedure.

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of superior evaluating titers. Here, rabbit antibody co-precipitated carrier IgG from normal goat serum and the free and conjugated goat anti-human IgG leaving free enzyme in solution. Determination of the free and total enzymatic activities showed 21.9% of the peroxidase and 4% of the glucose oxidase to remain unconjugated. All peroxidase conjugates gave RZ values of 0.47-0.61 which indicate about 1 mole enzyme per mole IgG.

The Enzymatic Activity Retained after Conjugation

Equal aliquots of the same peroxidase and glucose oxidase conjugates examined above were analyzed for total enzymatic ac-

tivity. The peroxidase activity showed a change in absorbance at 642 nm of 1.145/3 min vs. 0.191/3 min for glucose oxidase. If 78% of the peroxidase were conjugated (see above) the bound enzyme activity was 0.890/3 min vs. 0.180/3 min for the bound glucose oxidase.

The Loss of Antibody Activity due to Conjugation

The relative titers of goat antibody (comparing equal amounts of goat IgG) before and after conjugation to peroxidase and to glucose oxidase were obtained using the enzyme-linked immunoassay by determining that dilution of unconjugated goat antibody which on binding to solid phase human IgG prevented the binding of a fixed dilution of enzyme-conjugated goat antibody. Briefly, we used dilutions of the goat antibody (originally 5 mg IgG per ml in conjugates) to incubate with 200 ng human IgG fixed in wells. After 3 washes with saline/Tween/BSA, a fixed dilution of conjugate (1-200 for glucose oxidase; 1-50 for peroxidase) was added to permit exposure of available antigen. Both enzyme conjugates showed a distinct increase in bound enzyme activity at a 1-250 dilution of the unconjugated goat IgG. That change was more dramatic with the glucose oxidase conjugate at a 1-200 dilution than with the peroxidase conjugate at a 1-50 dilution. From these results we estimate that the glucose oxidase conjugate is 1-200 or 80 percent of original; the usable titer of the peroxidase conjugate is 1-50 or 20 percent of original. This somewhat crude antibody titration avoided the severe and irremedial cross reactions of a second antibody in

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attempts to titrate via the binding of ^{125}I labeled human IgG.

The Optimal Dilution for use in Immunoassay

Graded amounts of human IgG (3,2,1,0.5,0.25,0.125, and 0.062 µg) were bound to a bovine serum albumin base by glutaraldehyde in flat-bottomed wells of a micro elisa plate (Dynatech Labs). After thorough washing with saline containing 0.5% Tween-20 and 0.5% Bovine serum albumin, duplicate dilutions of conjugate in the same wash fluid were allowed to react with gentle agitation at 22°C for 1 hour. After 3x washes with Tween-20/saline, 200 µl of buffer substrate appropriate for the enzyme was added in duplicate wells at 15 sec intervals. After a 30 min incubation at 22°C and at 15 sec intervals the fluids from duplicate wells were pooled into numbered test tubes for final absorbance measurement at 642 nm using microcuvettes. Further conversion of substrate to product stopped on transfer. That conjugate dilution showing both a continuously smaller absorbance with decrease in human IgG and near maximum response at high antigen levels is considered optimal for the conjugate. The dilution of 1 to 300 meets these criteria in the titration shown in Table 1. Best usable dilution for one peroxidase conjugate was 1-100 but for others 1-50. In comparison most glucose oxidase conjugates were useful at a 1-200 or greater dilution. Figure 2 graphically expresses optimum responses of conjugates to changes in antigen.

Non-Specific Enzyme Product Formation

Peroxidase conjugates showed enzyme reactions in blank wells

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TABLE 1

Titration of Conjugate #8229, GO_x-GA Hu IgM

Conj. Dilution	Mass of Human IgM							
	Blank	3 µg	2 µg	1 µg	500 ng	250 ng	125 ng	62 ng
1-50	0.045	0.416	0.392	0.423	0.417	0.326	0.219	0.131
1-100	0.014	0.398	0.412	0.426	0.362	0.313	0.194	0.102
1-150	0.015	0.395	0.385	0.413	0.373	0.275	0.164	0.089
1-200	0.000	0.411	0.407	0.428	0.365	0.270	0.126	0.064
1-250	0.000	0.411	0.422	0.428	0.388	0.229	0.138	0.061
1-300	0.000	0.412	0.420	0.403	0.318	0.230	0.127	0.072
1-500	0.009	0.391	0.432	0.347	0.307	0.218	0.133	0.051
1-600	0.010	0.406	0.404	0.289	0.295	0.188	0.130	0.065

which ranged from 22% to nearly 90% of the maximum gross absorbance obtained with 3 µg human IgG fixed. Glucose oxidase conjugates show blank reactions from 1 to 30 percent of maximum (3 µg fixed human IgG). Some non-specifically produced color is due to non-enzymatic reactions. Thus, if the H_2O_2 used in the peroxidase substrate was not freshly diluted from 30 percent stock, the chromagen in the substrate colors rapidly without enzyme. Under the best conditions, peroxidase substrate deteriorates faster than the glucose oxidase substrate. In addition, the peroxidase conjugate seems to adsorb non-specifically and in spite of numerous precautionary measures and vigorous washing.

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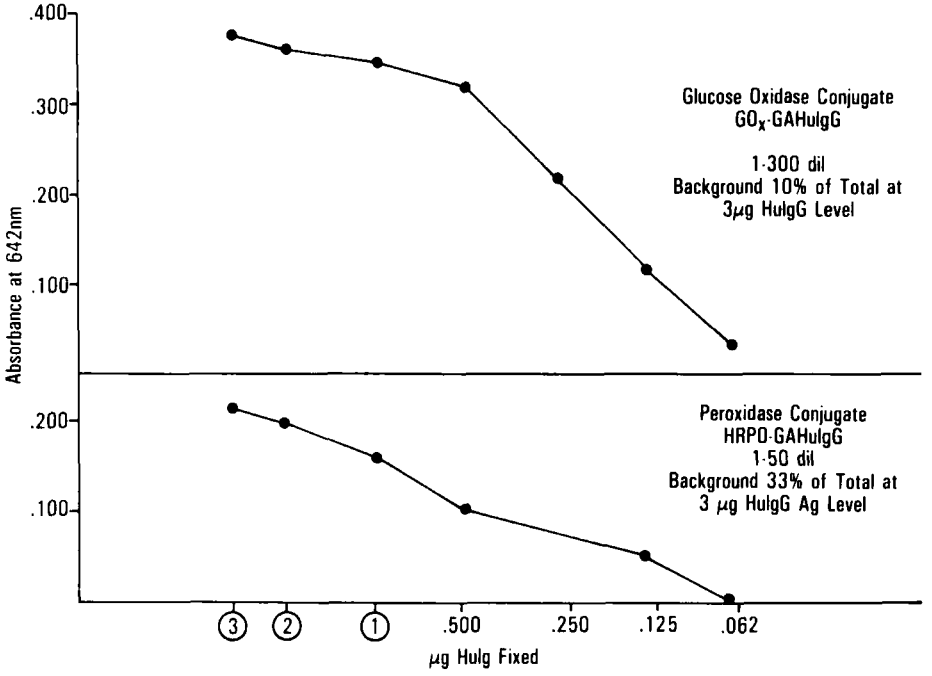

FIGURE 2 Responses to decreasing amounts of human IgG fixed in wells of a micro-titer plate by a glucose oxidase conjugate and a peroxidase conjugate to the same amount of goat IgG containing anti human IgG. Glucose oxidase shows a hint of pro-zone phenomenon above 500 ng human IgG. The horizontal scale is $\log_{10}$.

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Sensitivity as Given by ΔA vs. ΔAg

Out best peroxidase conjugate showed 0.058 absorbance change (1 μg to 500 ng) while glucose oxidase changed 0.100 in that region of antigen concentration.

This study reports specific measurements on the best of several conjugates prepared. The summary of these comparisons on peroxidase and glucose oxidase as shown in Table 2 indicates

TABLE 2

Quality Measured	Peroxidase	Glucose Oxidase
Percent Enzyme Bound	70-80%	90%
Enzyme Activity/3 min	1.145	0.191
Conjugate Antibody Titer	25% Original	80% Original
Optimal Dilution (ave)	40-100 (50)	100-500 (200)
Non-specific Activity (% of Max. gross)	40-90%	1-30%
Max. ΔA vs. Δ [Ag]	0.058 (1 μg-500 ng)	0.100 (1 μg-500 ng)

glucose oxidase to be the superior enzyme label. We are unable to explain the reason for the loss in usable peroxidase conjugate despite all indications that covalent bonding occurred between enzyme and IgG and with only small losses in enzyme activity.

Use of glycerol has permitted storage of conjugates at -12°C without freezing and, therefore, eliminated the destructive effects of freeze-thaw. Both peroxidase and glucose oxidase conjugates have remained stable over 6 mo with this treatment.

We found conjugates of glucose oxidase with specific goat anti-human immunoglobulins to be reliable in the detection and assay of Rubella antibodies. This study will be reported later.

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