Exercise No. 14

Preparation of Peroxidase-Labeled Antibody

The first procedure binding antibody to an enzyme was published by Nakane and Pierce (1966). These authors established covalent linkage between the two components by p,p'-difluoro-m,m'-dinitro-diphenyl sulfone. A peroxidase enzyme was used to detect the bound antibody both at light and electron microscopic levels. Nakane and Kawaoi later (1974) developed a new procedure to bind peroxidase to antibody by oxidizing the carbohydrate moiety in the enzyme with periodate, thus forming aldehyde groups which react readily with primary amino groups in the antibodies or Fab' fragments. The periodate treatment of the enzyme does not interfere with its enzymatic activity and a high percentage of the oxidized enzyme can be bound to antibodies without interfering with the reactivity of the antibody. This procedure is given here, based on the description of Nakane and Kawaoi (1974).

Materials and Equipment

Horseradish peroxidase (available from Sigma Chemical Co., St. Louis, MO 63178).

Anti-mouse IgG. (Commercially available from Cappel Labs. Inc., Downingtown PA 19335)

1% Dinitrofluorobenzene dissolved in ethanol

0.3 M NaHCO₃. Dissolve 2.52 g in 100 ml water

0.01 M Na₂CO₃/NaHCO₃ buffer, pH 9.6. Mix 320 ml 0.01 M Na₂CO₃ (1.06 g in 1000 ml) and 680 ml 0.01 M NaHCO₃ (0.84 g in 1000 ml)

0.08 M sodium metaperiodate (NaIO₄) in water. Dissolve 1.71 g NaIO₄ or 2.14 g NaIO₄ · 3H₂O in 100 ml water

0.16 M ethylene glycol. Dissolve 9.93 g CH₂OHCH₂OH in 100 ml water

Sodium borohydride (NaBH₄). Dissolve 50 mg in 10 ml distilled water

Phosphate-buffered saline (PBS). (See Exercise No. 1)

10 and 25 ml Erlenmeyer flasks

Tiny magnetic spin bar (8 mm)

Magnetic stirrer

Procedure

1. Dissolve 5 mg peroxidase in 1 ml 0.3 M NaHCO₃. Add 0.1 ml 1% dinitrofluorobenzene and stir with a magnetic stirrer at room temperature for 1 h.

2. Add to the mixture 1 ml 0.08 M NaIO₄ in distilled water. Continue mixing the solution for 30 min at room temperature.

3. To stop the oxidation, add 1 ml 0.16 M ethylene glycol. Continue mixing for 1 h at room temperature.

4. Dialyze the mixture against 0.01 M carbonate-bicarbonate buffer, pH 9.6, in the cold room for 48 h, changing the outer fluid twice daily.
5. Transfer the contents of the dialysis bag into a 25 ml Erlenmeyer flask. Add 5 mg IgG (anti-mouse IgG prepared in goat) dissolved in 2 ml carbonate-bicarbonate buffer. Mix the solutions with a magnetic stirrer for approximately 3 h at room temperature.

6. Add 2 ml NaBH₄ solution. Mix the solutions and transfer the flask to the cold room overnight.

7. Dialyze the mixture at 4°C against PBS. Dialysis should last for two days, changing the outer fluid twice daily. If a precipitate forms, remove it by centrifugation.

8. If further purification is required, the dialyzed sample should pass through a Sephadex G100 column (2 x 50 cm size) as described in Exercise No. 2. The first protein peak leaving the column after the void volume contains the enzyme-labeled IgG preparation. The chromatographic effluent should be monitored at 280 nm.

Evaluation

The preparation you have now is an anti-IgG immunoglobulin covalently linked to peroxidase. The dilution of this preparation may be adequate in this form for detecting antibodies on cell surfaces or in histological sections, but for some purposes it may be desirable either to dilute it or to concentrate it. In the latter case, the complex should be precipitated at cold room temperature by mixing it with equal volumes of saturated ammonium sulfate. Centrifuge the precipitate sharply at 5000 g for 30 min. Discard the supernatant and redissolve the sediment in PBS so that the desired protein concentration will be achieved. For the determination of the protein concentration, the procedure in Exercise No. 48 or No. 50 can be used.

The enzymatic activity of the preparation has to be determined next. If the immunoglobulin has an inert enzyme attached to it, it will react with the antigen but this reaction will not be detectable due to the lost activity of the enzyme. Moreover, if the preparation contains a mixture of active and inactive enzyme complexes, the inactive ones will compete for receptors and will considerably reduce the number of detectable sites. The enzyme activity must be determined and the procedure of Chance and Maehly (1955) can be used for this measurement.

The amount of enzyme used for the complexing being known (5 mg), the total activity of the starting material can be measured and calculated. After the activity of the immunoglobulin-enzyme complex has been determined, one must calculate the percent of enzyme activity present in the complex. This should not be less than 75% of the starting activity.

It is also necessary to measure the antibody reactivity in the complex. If the reactivity of the antibody is lost, no attachment will take place. We know the amount of antibody we complexed with the enzyme and now we must compare the reactivity of the complex with the reactivity of the same amount of free immunoglobulin.

For this purpose, probably the simplest method is the semiquantitative procedure described in Exercise No. 76. To carry this out, adjust the mg/ml concentration of a free immunoglobulin solution so that it will contain the same amount of immunoglobulin we added to the periodate-oxidized peroxidase. Make 12 double dilutions from both preparations, using PBS and the microtitrator kit, as