Rapid Micromethod for Preparation of Enzyme-antibody Conjugates

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A rapid method is described for labeling antibody with alkaline phosphatase by a one-step glutaraldehyde linkage. The method involves the centrifugation of a small volume of an enzyme and antibody mixture through a minicolumn packed with hydrated Sephadex. This procedure rapidly removes ammonium sulfate and glutaraldehyde from the enzyme-antibody mixture and results in the efficient recovery of conjugated antibody without significant dilution.

Enzyme immunoassay procedures are increasing in popularity for the detection of many antigens and antibodies. Although many of these assay systems are quite similar to radioimmunoassay procedures, their increasing usage can be attributed to the fact that the detector antibody is labeled with an enzyme rather than a radioisotope. This eliminates the hazards and problems associated with the preparation, measurement, and disposal of radioactive material.

Many methods for coupling enzymes to antibodies have been described (4). One of the most widely used is the one-step glutaraldehyde linkage described for the conjugation of alkaline phosphatase to antibody by Avrameas (1). The method involves mixing the enzyme preparation with the antibody to be labeled in the presence of glutaraldehyde. Although it is effective, this method requires two extensive, time-consuming dialysis steps, one before conjugation to remove the ammonium sulfate used to stabilize the alkaline phosphatase and a second to remove the glutaraldehyde after the conjugation reaction. Gel filtration with Sephadex G-50 has been used for the removal of excess glutaraldehyde from the enzyme-antibody conjugate. Although it is less time-consuming than dialysis, this procedure invariably results in the dilution of the final preparation (7).

We have devised a rapid, easy procedure for the complete removal of both ammonium sulfate and glutaraldehyde from enzyme-antibody conjugates based on a method for desalting protein solutions initially described by Neal and Florini (6) and recently modified by Christopherson et al. (3). Our procedure involves centrifugation of a small volume of protein mixture through a minicolumn (3 by 1.0 cm) packed with hydrated coarse-grade Sephadex G-50. Low-molecular-mass molecules (less than 500 daltons) rapidly enter the pores of the hydrated gel and are retained, whereas globular proteins with molecular masses greater than 30,000 daltons are excluded and pass through the column upon centrifugation without dilution. The minicolumn consisted of a 3.0-ml plastic disposable syringe barrel (5 by 1 cm; Monoject, Sherwood Medical Industries Inc.; Fig. 1). Glass-fiber disks (6-mm diameter) were cut from mini-MASH filter mats (M. A. Bioproducts) with a paper hole punch. A glass-fiber disk presoaked in 10% fetal calf serum in phosphate-buffered saline, pH 7.2, was placed in the bottom of the syringe barrel and centered over the needle attachment aperture. The assembly was filled with 3.0 ml of a thick slurry of Sephadex G-50 in 10% fetal calf serum in phosphate-buffered saline. A 1.5-ml conical polypropylene microcentrifuge tube (Bel-Art Products) was dropped into a glass centrifuge tube (10 by 1.3 cm), and the minicolumn assembly was positioned with the adapter tip in the microcentrifuge tube. The combination was centrifuged at 100 x g for 1 min in a centrifuge equipped with a horizontal rotor (radius, 15.24 cm). After centrifugation, additional Sephadex was added to maintain a bed height of 3.0 cm. The column was then washed three times with 2.0 ml of phosphate-buffered saline to remove excess fetal calf serum. Before each sample was loaded onto the column, the assembly was centrifuged again for 2 min at 100 x g to ensure the removal of all buffer.

Optimum centrifugation speeds and times were determined by centrifugation of a 0.2-ml sample of a 0.2% phenol red and blue dextran solution. These optimum conditions resulted in the recovery of 0.2 ml of a solution containing only blue dextran and the retention of the phenol red in the upper portion of the Sephadex bed.

The 3.0-cm bed height was determined by an experiment. A 200-μl portion of 3 M ammonium sulfate was layered onto minicolumns of varying...
After glutaraldehyde fixation in the prepared column of 1 M BaCl₂, containing 200 μl of 0.2 ml of phosphate-buffered saline, was added to the enzyme precipitate. After resuspension of the enzyme, the remaining ammonium sulfate was removed by layering 0.2 ml of the antibody-enzyme mixture onto the 3.0-cm minicolumn prepared as described above. The column was centrifuged for 2 min at 100 × g, and 16 μl of glutaraldehyde was added to the eluted fraction containing the desalted antibody-enzyme mixture to give a final concentration of 0.2%. After incubation at 25°C for 2 h, the glutaraldehyde was removed from the conjugate by again applying the mixture to a 3.0-cm minicolumn and centrifuging as described above.

Total volume, protein, and enzyme activities of the initial enzyme-antibody mixture were measured at each centrifugation step throughout the minicolumn centrifugation procedure. Total protein was measured as described by Lowry et al. (5). A 100% recovery of total protein was attained (Table 1). The volume of the sample recovered was consistently within ±5% of the initial volume added to the minicolumn. No ammonium sulfate could be detected after the first centrifugation step. The activity of the alkaline phosphatase was measured using a modification of the method of Bowers and McComb (2). A total of 70.3% of the alkaline phosphatase activity was recovered after the complete removal of ammonium sulfate. An additional 22.6% of the enzyme activity was lost after the conjugation step and removal of the glutaraldehyde, resulting in a final specific activity of 109.4 U/mg of total protein. In our laboratory, the labeling of antibody with alkaline phosphatase by following the original method of Avrameas (1) has routinely resulted in specific enzyme activities of between 106.0 and 156.5 U/mg.

The major advantages of this system for labeling antibody with alkaline phosphatase are as follows. (i) The conjugates can be prepared rapidly. This method requires only 3 to 4 h of preparation time compared with 72 to 96 h with labeling methods requiring dialysis, gel filtration, or both to remove reactants. (ii) The minicolumns are easily constructed from inexpensive components readily available in most clinical and research laboratories. (iii) Small volumes and microgram amounts of antibody can be labeled efficiently. Preadsorbing the column components with fetal calf serum enhances the high recovery of total protein by reducing the nonspecific adsorption of sample protein to column components. This high efficiency of recovery in the absence of sample dilution makes this method suitable for the labeling of small amounts of antibody without the addition of a contaminating carrier protein.

Glutaraldehyde can be used in the one-step procedure for coupling a variety of enzyme-protein systems and for a two-step procedure for the preparation of peroxidase-protein conjugates (4). The use of minicolumn gel centrifugation for

**TABLE 1. Preparation of alkaline phosphatase-labeled anti-horse immunoglobulin G by Sephadex G-50 minicolumn centrifugation**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (U)</th>
<th>Enzyme sp act (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>2.48</td>
<td>550.4</td>
<td>229.3</td>
</tr>
<tr>
<td>1st centrifugation</td>
<td>2.48</td>
<td>387.2</td>
<td>161.3</td>
</tr>
<tr>
<td>2nd centrifugation</td>
<td>2.50</td>
<td>275.8</td>
<td>109.4</td>
</tr>
</tbody>
</table>

*One international phosphatase unit (U) will yield 1 μmol of p-nitrophenol per min from p-nitrophenylphosphate at 37°C.*
the removal of unwanted reactants from these reactions should have wide application for the rapid preparation of enzyme-labeled antigens and antibodies commonly used in immunohistochemical techniques and enzyme immunoassays.

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LITERATURE CITED