Producing and Applying Fluorescent Antibodies

With the fluorescent antibody (FA) technique, the antigen-antibody complex can be viewed directly under the microscope. This allows the researcher to detect and identify microorganisms simultaneously. FAs are useful for rhizobial strain identification in ecological research. In this chapter, antisera are purified by ammonium sulfate precipitations and dialysis. The protein content of the dialysate is determined. The immunoglobulin fraction is then conjugated with fluorescein isothiocyanate (FITC). The FITC-antibody conjugate is separated from the unreacted FITC by column chromatography (gel filtration). It is then used to identify rhizobia in nodules by the direct FA technique. A modification of this method, referred to as the indirect FA technique is described in Appendix 13.

KEY STEPS/OBJECTIVES

1. Precipitate the immunoglobulins.
2. Precipitate the immunoglobulins for a second and third time.
3. Dialyze the serum globulins.
4. Determine the protein content of the dialysate.
5. Conjugate the immunoglobulins with FITC.
6. Purify the FA by column chromatography.
7. Test the quality of the FA.
8. Type nodules with the FA technique.

a. Precipitating Serum Globulins (Key Steps 1 and 2)

Place a 250-ml beaker filled with crushed ice onto a magnetic stirring plate. Immerse a 50-ml centrifuge tube containing 15 ml of antiserum into the ice and clamp the tube to a ring stand. Drop a 12-mm (0.5 in) stirring bar into the tube. To the same ring stand,
attach a 30-ml burette filled with cold 3.9 M ammonium sulfate solution. The tip of the burette should be close to the surface of the antiserum. Add 15 ml of ammonium sulfate solution to the antiserum at the approximate rate of one drop per second while stirring continuously. Allow the resulting cloudy mixture to stand overnight (or for at least 2 h) at 4°C.

Separate the globulins by centrifugation in a refrigerated centrifuge at 10,000 \( \times \) g for 30 min. Discard the supernatant and dissolve the precipitated globulins in enough saline to bring the solution back to the original serum volume (15 ml). Repeat the precipitation and centrifugation steps twice as previously described, but without the intermediate step of overnight refrigeration. Instead, allow the precipitates to settle for 5 min at 4°C before centrifugation. Three precipitations are usually sufficient to render the globulins completely white and free of hemoglobin.

b. Purifying the Serum Globulins by Dialysis (Key Step 3)

The next step is to remove the excess ammonium sulfate from the immunoglobulin solution by dialysis. Use a dialysis membrane-filter tubing of approximately 2 cm in diameter. The molecular cut-off rating should be at 16,000. This will keep the large immunoglobulin molecules inside the tubing while the small ammonium sulfate molecules can pass through the pores freely. Cut off a length of approximately 20 cm and soak it in distilled water for 2 h or overnight. Just before using, make a tight knot at one end of the tubing. Wear surgical gloves. Do not touch the tubing with bare hands.

Dissolve the final precipitate in approximately 7.5 ml of saline (half of original volume). Using a 10-ml pipette, transfer the immunoglobulin solution to the dialyzing bag. This is best accomplished by holding the dialyzing tubing in a beaker of water with one hand while pipetting with the other. The pipette must be inserted into the tubing until it is almost to the closed end. Slowly pull out the pipette while the solution is discharged. For this operation, the walls of the tubing must remain wet so that the pipette can slide in and out freely. Close the dialysis tubing with a knot or with a tubing clip. Trap approximately 1 ml of air inside the tubing. This will cause the dialysis bag to spin upright near the surface during dialysis.

Dialyze against 2 liters of dialyzing fluid (saline adjusted to pH 8 with 0.1 N sodium hydroxide) in a cold room with frequent changes of fluid until the ammonium sulfate is no longer detectable in the saline. Three changes of saline at intervals of 4, 10 (overnight), and 4 h again, with continued dialysis for another 4 h, is usually sufficient. Merthiolate may be added to the saline as a preservative at a concentration of 0.01% (w/v).

To determine the presence of sulfate, mix a few drops of the dialyzing fluid with an equal volume of a saturated barium chloride solution. If the mixture does not become cloudy, the dialysis can be considered complete.

If phosphate has been used as buffer for the dialyzing fluid, use Nessler’s reagent to detect ammonium (Appendix 4) because phosphate will interfere with the sulfate precipitation. In a small test tube, mix a few drops of the dialyzing fluid with an equal