

Purification of Aster Yellows Agent from Diseased Lettuce Using Affinity Chromatography

Y. P. Jiang, J. D. Lei, and T. A. Chen

Graduate assistant, former postdoctoral fellow, and professor, respectively, Department of Plant Pathology, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ 08903.

We thank X. F. Jiang for her technical assistance and R. J. McLaughlin for a critical reading of the manuscript. This research was supported in part by the New Jersey Agricultural Experiment Station (publication D-11260-1-87) and BARD Grant US-1030-85.

Accepted for publication 7 January 1988 (submitted for electronic processing).

ABSTRACT

Jiang, Y. P., Lei, J. D., and Chen, T. A. 1988. Purification of aster yellows agent from diseased lettuce using affinity chromatography. *Phytopathology* 78:828-831.

An affinity chromatographic procedure was developed for purification of the mycoplasma-like organism (MLO) from lettuce with aster yellows (AY) disease. The affinity column consisted of Staphylococcus protein A covalently linked to a 6MB Sepharose matrix and coupled with monoclonal antibody specific against AY-MLO. AY-MLO was concentrated from clarified crude sap of diseased lettuce by differential centrifugation. After a low-speed centrifugation, supernatants containing AY-MLO were loaded on the affinity column. Extraneous unbound plant

materials from the crude sap were washed from the column after an incubation period. The aster yellows agent retained in the column was released by mechanical shaking and then eluted. The entire procedure took 3 hr to complete. Intact and undamaged cells were observed by electron microscopy, and the high purity of this preparation was revealed by electrophoretic protein profiles and the production of polyclonal AY-antiserum. In addition, the specificity of the monoclonal antibodies was confirmed in Western blots.

Aster yellows (AY), associated with mycoplasma-like organisms (MLOs), is one of the most intensively studied yellows diseases (13). To date, these organisms have not been cultivated in vitro. Previous attempts at isolation and purification of MLOs have also met with limited success (2). Recently, Jiang and Chen (8) partially purified MLOs from lettuce with AY disease by centrifugation in a discontinuous Percoll density gradient. Electron microscopy of thin sections of these preparations showed a high concentration of AY-MLOs. Specific monoclonal antibodies were then produced against AY-MLOs by using these partially purified preparations (11). However, polyclonal antibodies obtained from mice injected with similar preparations were not AY-specific because of plant materials in the antigen preparation. An AY-MLO antigen preparation of much higher purity is probably needed to produce specific polyclonal AY-antisera (12).

Protein A is a major cell wall component found in most strains of *Staphylococcus aureus* (5), where it is covalently associated with the cell wall as a component of the membrane (3). Protein A reacts with the Fc region of IgG. Affinity chromatography on a protein A-Sepharose matrix has been shown to be particularly useful for separation of Ig-bearing cells or cells for which specific antibodies to surface antigens are available (4,14). We report here on the use of monoclonal antibodies in an affinity chromatographic procedure to isolate highly purified AY-MLO from infected plants.

MATERIALS AND METHODS

Plant extracts. Methods for obtaining diseased lettuce plants (*Lactuca sativa* L. 'Mesa') and preparing crude extracts have been described previously (8). Briefly, the plant tissue was cut into pieces, comminuted with a Sorvall Omni-Mixer (Newtown, CT) in isolation medium, and centrifuged at low speed. The pellet was resuspended in suspending medium and centrifuged at high speed, and the supernatant was clarified by another low-speed centrifugation.

Preparation of the immunomatrix. The methods for preparation and characterization of monoclonal antibody against AY-MLOs have been previously reported (11). Purified monoclonal antibody (IgG2b) was coupled to protein A Sepharose 6MB (SpA) (Pharmacia, Piscataway, NJ) in the following manner: 5 ml of

SpA-Sepharose matrix was poured into a 0.9- \times 15-cm acrylic plastic column (Pharmacia). Forty milliliters of phosphate-buffered saline (PBS), pH 7.6, was passed through the SpA-Sepharose column, followed by 20 ml of suspending medium (pH 7.6). Four milliliters of monoclonal antibody IgG2b (8 mg/ml of protein) in PBS was then added to the SpA-Sepharose matrix and incubated for 20 min at room temperature. The column was washed with 50 ml of suspending medium and soaked in one bed volume of that medium. The column matrix contained approximately 25 mg of bound IgG2b.

Immunoabsorption. Crude extract from lettuce plants (5 ml per column) was loaded onto the affinity column and incubated for 20 min at room temperature. Unbound materials were washed off the column with 100 ml of suspending medium. The flow rate was approximately 250 ml/hr. Bound AY-MLOs were eluted by a method similar to that of Ghetie et al (4). After washing, the column matrix was agitated for 30 sec with a Vortex-Genie mixer (Scientific Industries Inc., Springfield, IL). Immediately after shaking, 5 ml of suspending medium was added, and the gel was gently resuspended by agitation with a coiled stainless steel wire. During resuspension, the column was eluted with an additional 5 ml of suspending medium. The eluate was immediately diluted with suspending medium (1:20) and centrifuged for 40 min at 35,000 g. Pellets were saved for electron microscopy and electrophoresis. As a control, another column was treated in the same way, except that it was loaded with an extract from a healthy plant.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A discontinuous gradient SDS gel was used, having 4.0% acrylamide in the stacking gel and between 5-20% in the linear gradient of the resolving gel (6). The SDS-PAGE was performed in a Bio-Rad Protean dual slab cell (Richmond, CA) by the method of Laemmli (9). The concentration of protein extracts from purified AY-MLO was adjusted to 0.4 mg of protein per milliliter in PBS. Before electrophoresis, each sample was mixed with sample buffer (1:3, v/v) and heated at 100 C for 3 min (6). Two other samples, prepared from crude healthy and diseased lettuce extracts, were treated in the same way. Samples were run at 40 mA (stacking gel) and 60 mA (resolving gel) for a total of 4 hr at 10 C. Gels were stained in 0.05% Coomassie Brilliant Blue R 250 (Sigma Chemical Co., St. Louis, MO) for 1 hr (7).

Electrophoretic blotting. A transfer procedure similar to that of Towbin et al was used (15). After SDS-PAGE, the slab gel was laid

on one layer of thick filter paper (0.1 cm; Bio-Rad), which was supported by two layers of sponge pads and a porous polyethylene grid (Bio-Rad). A sheet of nitrocellulose (0.3 μm pore size; Bio-Rad) was put on the gel. Care was taken to remove all air bubbles. Two more layers of sponge pads and a polyethylene grid were placed on top of the sheet. All components that were placed in contact with the slab gel were prewetted in the electrophoresis buffer (1). The assembly was put in an electrophoretic destaining chamber with the nitrocellulose toward the anode. The chamber was filled with electrophoresis buffer, and 6 V/cm was applied for 8–10 hr using a Bio-Rad 250/2.5 power supply.

Binding of antibody to nitrocellulose-immobilized protein.

After protein transfer to the nitrocellulose sheet, a modification of the method reported by Towbin et al (1) and Burnette (15) was used. The sheet was immersed in carbonate-bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) containing 3% (w/v) crystalline grade bovine serum albumin (BSA) and incubated for 1 hr at 37 C. The sheet was washed in PBS with 1% BSA for 15 min on a reciprocating shaker and was then transferred to fresh PBS containing 0.7 mg of monoclonal antibody protein and 1% BSA. After incubation for 3 hr at 37 C and another wash in PBS, biotinylated anti-mouse IgG (Sigma) was added and incubated for 40 min. After another wash, a dilute avidin-biotin peroxidase complex solution (Vector Laboratories, Inc., Burlingame, CA) was added, followed by incubation for 30 min at 37 C and another wash. For the color reaction, the sheet was soaked in a solution containing 25 $\mu\text{g}/\text{ml}$ of o-dianisidine, 0.01% H_2O_2 , and 10 mM Tris-Cl (pH 7.4). The nitrocellulose paper was rinsed with water to stop the reaction when the color band appeared.

Electron microscopy. The procedure for the preparation of AY-MLO for electron microscopy has been previously described (8). Briefly, the eluate from an affinity column was pelleted by centrifugation and then prepared for embedding. Stained, ultrathin sections were examined on a Philips 301 transmission electron microscope (Philips Electronic Products, Mahwah, NJ).

Preparation of polyclonal antiserum to a purified AY-MLO. AY-MLO preparation used for immunization of BALB/c mice was subjected to two cycles of affinity chromatography before concentrated by centrifugation. The pellets were diluted with phosphate-buffered saline (pH 7.0) and homogenized by ultrasonication. Each mouse was administered three intraperitoneal injections, 10 days apart, containing 0.1 mg each of AY-MLO protein with an equal volume of Freund's complete adjuvant. Mice were bled 5 days after a final intravenous injection containing 0.01 mg of total AY-MLO protein in saline. Pools of sera were collected and diluted with saline (1:10, v/v). The supernatant was saved as polyclonal AY-antiserum after centrifugation at 1,000 g for 10 min.

Enzyme-linked immunosorbent assay (ELISA). Both diseased and healthy lettuce leaf midribs were ground and diluted 10-fold (w/v) in 0.1 M carbonate buffer (pH 9.6). The saps were squeezed through three layers of cheesecloth and were used to coat a polystyrene 96-well microtiter plate (50 μl per well). The plate was incubated at 37 C for 3 hr or overnight at 4 C. After washing, the plate was blocked with 0.5% BSA. Serially diluted antisera and monoclonal antibodies were added to the wells and incubated at 37 C for 1 hr. Biotinylated goat anti-mouse IgG and IgM were used as a second antibody, followed by streptavidin conjugated with peroxidase. The color reaction developed with substrate was then read with an ELISA spectrophotometer at 490 nm (11).

RESULTS

Immunoabsorption and electron microscopy. Electron micrographs of thin sections from pellets obtained after affinity chromatography from a diseased plant showed a very high concentration of AY-MLOs (Fig. 1A), indicating that the monoclonal antibody-coupled affinity column was quite efficient for trapping intact cells of the organism. As shown in Fig. 1A, MLOs of various sizes and shapes were recovered. Most of the recovered cells maintained their cellular integrity, showing little or

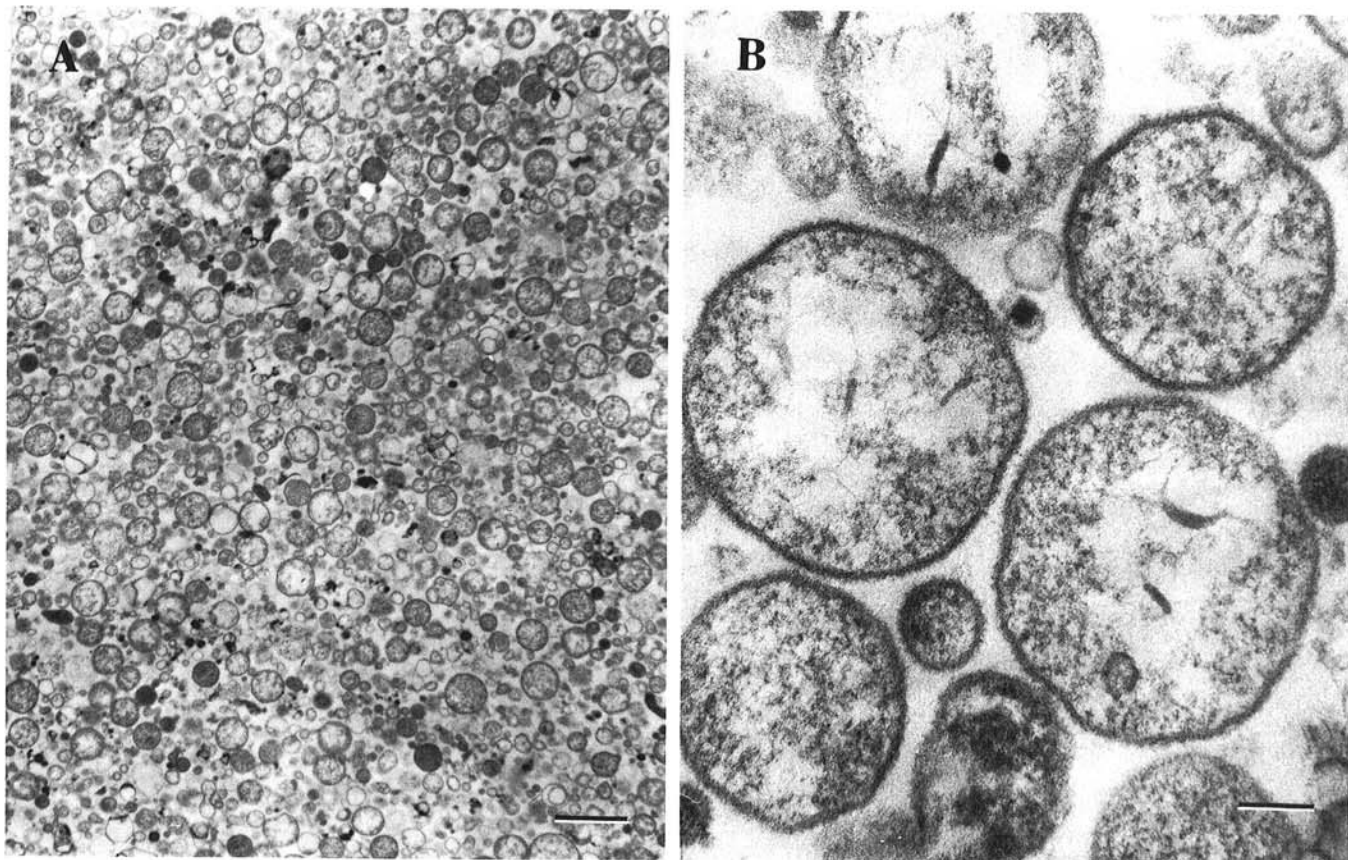


Fig. 1. A, Electron micrograph of mycoplasma-like organisms purified from aster yellows-diseased lettuce (AY-MLO) showing high concentration of cells. Bar = 1.30 μm . B, High resolution of AY-MLO showing ribosomal granules, DNA fibrils, and trilaminar unit membrane. Bar = 0.12 μm .

no damage. These cells exhibited a typical unit plasma membrane, ribosomal granules, and a fibrous network of DNA (Fig. 1B). No MLOs were found in eluates prepared from healthy control plants.

SDS-PAGE. An SDS-PAGE profile of proteins from AY-diseased and healthy lettuce is shown with purified AY-MLOs obtained by immunoaffinity chromatography in Figure 2. Peptide bands in AY-diseased lettuce (Fig. 2A, lane 3) were similar to that in healthy lettuce plants (Fig. 2A, lane 2). However, a major peptide band (18.5 kDa) was noted in the diseased lettuce that was not as prominent and wide in extracts from healthy plants (Fig. 2A, lane 2). This band was also seen in the purified AY-MLO preparation (Fig. 2A, lane 1), indicating that this protein was of MLO origin. Moreover, the band in the purified AY-MLO preparation was more prominent than in diseased plants, indicating a higher AY-MLO concentration. In addition to this major band, two minor protein bands were observed in both AY-MLO preparations, but not in the healthy plant preparation. These proteins may also be proteins of AY-MLO origin.

Western blotting and immunoprobings. When protein profiles from SDS-PAGE were transferred to nitrocellulose and probed with monoclonal antibody, the same major band was seen in gel profiles from the diseased plant and purified AY-MLO preparations (18.5 kDa, Fig. 2B). No other peptide bands were detected from either healthy or diseased plants, or from purified MLOs. Apparently, this band contains the epitope that our specific monoclonal antibody recognizes.

ELISA with polyclonal AY-antiserum. Lin and Chen (12) reported that polyclonal antisera from mice immunized with partially purified AY-MLO failed to distinguish diseased and healthy lettuce plants because they cross-reacted with healthy plant proteins. In this study, the specificity of polyclonal AY-antiserum was much increased with the use of highly purified AY-MLO as antigen. In ELISA, antiserum discriminated between diseased and healthy lettuce plants. As shown in Table 1, A_{490nm} values of diseased plants were about 7 times that of healthy plants. Similar results, but with much higher A_{490nm} values, were obtained with

monoclonal antibody at comparable dilutions. The titer of polyclonal antiserum was decreased with increasing dilutions.

DISCUSSION

SpA-Sepharose 6MB has been used previously to separate mouse B lymphocytes pretreated with rabbit anti-mouse Ig (4). Based on the same principle, monoclonal antibody against AY-MLO that was immobilized on SpA-Sepharose 6MB provided an effective matrix for isolation of AY-MLO from a crude plant preparation. The effectiveness of affinity chromatography was verified by the presence of high concentrations of AY-MLO in electron micrographs and the AY-MLO protein profile on the polyacrylamide gel.

The use of Western blots has provided additional proof that the isolated cells were indeed AY-MLOs. Western blots of protein extracts from diseased plants also showed a particular protein band that specifically reacted with the monoclonal antibody, which was also the predominant protein from purified AY-MLO extracts. The SDS-PAGE profile of AY-MLO proteins shows several faint protein bands that are located below the 18.5-kDa major protein band in the purified AY-MLO preparation. These bands are not found in the healthy lettuce preparations and are hardly found in the diseased lettuce preparations. Perhaps this is because of a much lower concentration of AY-MLO in the diseased lettuce than in the purified AY-MLO preparation.

Preparation of an affinity column with monoclonal antibodies may be more advantageous than with polyclonal antibodies because of potential cross reactions between proteins that share common antigenic determinants. The use of monoclonal antibodies may therefore eliminate such problems, particularly when the isolated sample is a complex crude mixture.

In preparing monoclonal antibody-protein A Sepharose, we found that it is important to keep the PBS and suspending medium slightly alkaline (pH 7.6). At this pH, binding of monoclonal antibody (IgG2b) to protein A was sufficient to prevent elution of antigen despite extensive washing (10). Mechanical treatments (mild resuspension and vortexing) of the column matrix before elution apparently disrupt the binding of monoclonal antibody to the surface antigen of AY-MLO without affecting the reaction between the Fc region of the monoclonal antibody and protein A. Thus, very little of the bound monoclonal antibody was lost from the column matrix, allowing the column to be used repeatedly. Mechanical treatment may also be less harsh for cell release than if a drastic pH change is employed for elution of the cells.

Biochemical and serological studies of the aster yellows agent, a noncultivated MLO, have been limited for lack of a technique for purification from infected plant tissue. Although a procedure for isolation of AY-MLOs from diseased lettuce plant by density gradient centrifugation was recently developed (8), contamination with plant material was unavoidable, causing antibodies (polyclonal antibodies) produced against this antigen to lack specificity toward the AY-MLO. Nevertheless, the concentration of AY-MLO in this preparation was adequate for use as an immunogen for production of monoclonal antibodies (11). Therefore, this improvement in purification of AY-MLO, i.e., affinity chromatography, has facilitated production of a polyclonal AY-antiserum that discriminated AY-infected from noninfected plants. The titer of polyclonal AY-antiserum was

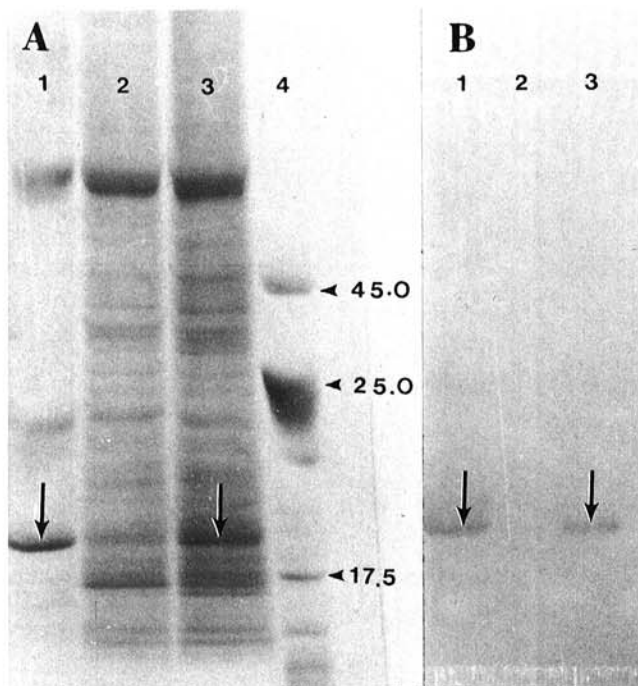


Fig. 2. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of proteins (samples containing 0.4 μ g of protein per milliliter); lane 1, purified AY-MLO; lane 2, crude extract from healthy lettuce; lane 3, crude extract from diseased lettuce; lane 4, relative molecular masses of standard proteins (45, 25, and 17.5 kDa). The arrow points to a major protein band (18.5 kDa) of AY-MLO origin. B, Monoclonal antibody probing (Western blot) of the SDS-PAGE protein profiles of A. Arrows indicate that AY-MA specifically recognizes the same protein bands from diseased plant and purified AY-MLO.

TABLE 1. Enzyme-linked immunosorbent assay reactions (at 490 nm) of serially diluted polyclonal aster yellows (AY) antiserum (PA) and monoclonal AY antibody (MA) with AY-diseased and healthy lettuce tissues

	Antibodies					
	100 μ g/ml protein		50 μ g/ml protein		25 μ g/ml protein	
	PA	MA	PA	MA	PA	MA
AY-infected lettuce	0.456	0.786	0.287	0.800	0.148	0.724
Healthy lettuce	0.054	0.023	0.021	0.004	0.012	0.002

lower than that of monoclonal antibody at the same protein content probably because the protein content in the polyclonal antiserum was heterogeneous.

The drawback of monoclonal antibody is its monospecific recognition of a single epitope. Thus, certain monoclonal antibodies may sometimes fail to diagnose plants showing aster yellows symptoms (12). Whether this is due to narrow specificity of the monoclonal antibody used for diagnosis or to the possibility that different mollicutes are infecting these plants still needs to be determined. Further studies of reactions of various yellows agents with polyclonal AY-antiserum and monoclonal antibodies should answer this question. In addition, study of the biological properties of AY-MLO will be facilitated now that purified preparations of the organisms are available.

LITERATURE CITED

1. Burnette, W. N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
2. Davis, R. E., and Whitcomb, R. F. 1970. Evidence on possible mycoplasma etiology of aster-yellows disease. *Infect. Immun.* 2:201-208.
3. Ghetie, V., Fabricius, H. A., Nilsson, K., and Sjoquist, J. 1974. Movement of IgG receptors on the lymphocyte surface induced by protein A of *Staphylococcus aureus*. *Immunology* 26:1081-1091.
4. Ghetie, V., Mota, G., and Sjoquist, J. 1978. Separation of cells by affinity chromatography on SpA-Sepharose 6MB. *J. Immunol. Methods* 21:133-141.
5. Goding, J. W. 1978. Use of Staphylococcal protein A as an immunological reagent. *J. Immunol. Methods* 20:241-253.
6. Hamer, B. D. 1981. Introduction of polyacrylamide gel electrophoresis. Pages 23-42 in: *Gel Electrophoresis of Proteins: A Practical Approach*. B. D. Hamer and D. Rickwood, eds. IRL Press, Oxford, England.
7. Jiang, Y. P. 1986. Purification of mycoplasma-like organisms from lettuce affected with aster yellows agent. M.S. thesis, Rutgers Univ., NJ.
8. Jiang, Y. P., and Chen, T. A. 1987. Purification of mycoplasma-like organisms from lettuce with aster yellows disease. *Phytopathology* 77:949-953.
9. Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
10. Langone, J. J. 1982. Application of immobilized protein A in immunochemical techniques. *J. Immunol. Methods* 55:277-296.
11. Lin, C. P., and Chen, T. A. 1985. Monoclonal antibodies against the aster yellows agent. *Science* 227:1233-1235.
12. Lin, C. P., and Chen, T. A. 1986. Comparison of monoclonal antibodies and polyclonal antibodies in detection of aster yellows mycoplasma-like organism. *Phytopathology* 76:45-50.
13. McCoy, R. E. 1979. Mycoplasmas and yellows diseases. Pages 229-259 in: *The Mycoplasmas*, Vol. III. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
14. Nash, A. A. 1976. Separation of lymphocyte sub-populations using antibodies attached to Staphylococcal protein A-coated surfaces. *J. Immunol. Methods* 12:149-161.
15. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.