Protein A-Bearing *Staphylococcus aureus* as the Solid Phase in an Enzyme Immunoassay and Its Application to Determination of Urinary Albumin

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Protein A-bearing *Staphylococcus aureus* was used as a solid-phase matrix in a sandwich-type enzyme immunoassay for urinary albumin. Heat-inactivated, formalin-fixed bacteria were coated with affinity-purified goat anti-human albumin, exposed to solutions containing standard or unknown concentrations of albumin, then challenged with an alkaline phosphatase/anti-human albumin conjugate obtained by periodate oxidation. Alkaline phosphatase activity bound to the bacteria was a function of albumin concentration from 25 to 1000 µg/L. This assay was applied to determinations of urinary albumin concentrations between 1.25 and 1000 mg/L. Between-run CV was 2.5% (63.9 mg/L concentration). Within-run CVs for albumin concentrations of 1.9, 38.1, and 638.0 mg/L were 3.7, 3.7, and 2.4%, respectively. Analytical recovery was 95 to 107% across the full working range of the assay. Bence Jones proteins and hemoglobin had no significant effect on the assay. Nonspecific binding of the enzyme–antibody conjugate was 1.3% (SD = 0.7%). Values agreed well with those by radial immunodiffusion.

**Additional Keyphrase:** alkaline phosphatase

"Protein A," a cell-surface component of *Staphylococcus aureus*, has a high affinity for immunoglobulins from many mammalian species (1, 2). It has been isolated previously from the bacterial membrane, labeled with reagents such as fluorescein or ¹²⁵I, and used in its soluble form as a specific probe for IgG (3–5). Sheep erythrocytes have also been coated with protein A and used in rosetting assays for quantifying and localizing various cellular antigens (4, 6). More recently, protein A-bearing *S. aureus* has proved useful in both preparative immunoprecipitation of antigens (7, 8) and precipitation of immune complexes in radioimmunoassays (9–13).

*S. aureus* that has first been fixed with formalin and heat-inactivated has several advantages over more commonly used solid-phase matrices in radioimmunoassays. The Cowan I strain of *S. aureus*, which is rich in protein A, can be easily grown and prepared for use in the laboratory, or purchased from commercial sources. In addition, IgG from certain mammalian species can be immobilized on *S. aureus*, which has a capacity of 100 µg of IgG per 10¹⁰ organisms, without need for chemical coupling procedures (7).

In view of recent efforts to develop sensitive, nonisotopic immunoassay techniques, we used protein A-bearing *S. aureus* as a solid phase in a sandwich-type enzyme immunoassay. Human serum albumin was used as the antigen, its assay in urine having been shown to aid in diagnosis and monitoring of glomerular membrane disorders after renal transplants.

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**Materials and Methods**

**Preparation of Materials**

*Treatment of S. aureus Cowan I.* The method of growth and preparation of *S. aureus* was adapted from Kessler (7). After centrifugation of the *S. aureus* suspension for 5 min at 3000 rpm, the supernate was removed and the bacteria were resuspended in a solution of anti-HSA IgG (4.5 g/L in PBS) to a final bacterial concentration of 50 g/L.² The bacteria were stored at 4°C in the IgG solution until use in the enzyme immunoassay. The bacteria were used after a minimum coating time of 1 h and could be stored in the IgG solution as long as six months at 4°C.

*Preparation of antiserum.* Antiserum to HSA was obtained from goats that had been previously immunized with a subcutaneous injection of pure HSA (1 mg) in incomplete Freund’s adjuvant and boosted three times with 1 mg each at 10-day intervals without adjuvant. After 40 days, plasma was removed from the animals and coagulated by addition of thrombin.

*Affinity purification of goat anti-HSA antibodies.* Pure HSA (Pentex, Kankakee, IL 60901) was covalently coupled to CM-Biogel A (Bio-Rad Labs., Richmond, CA 94804) for use as an immunosorbent in antigen purification. We dissolved 600 mg of HSA in 20 mL of 10 mmol/L pyridine HCl buffer, pH 4.8, and dialyzed this solution against the same buffer overnight at room temperature. The protein solution was then used to resuspend 30 mL of packed CM-Biogel A that had been previously washed on a Böchner funnel with 100 mL of 10 mmol/L phosphate-buffered saline (PBS), pH 7.6, containing an additional 1.4 mol of NaCl per liter. After 1 h of gentle stirring at room temperature, 200 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Travenol Lab., Deerfield, IL 60015) was added with continuous stirring, and the mixture was incubated overnight at room temperature. The resin was then used to pack a 1 × 22 cm chromatographic column, which was then successively washed with 100 mL of the 10 mmol/L PBS containing 1.4 mol/L NaCl; 100 mL of 5 mol/L guanidine HCl in PBS; and 150 mL of PBS. We then applied 100 mL of goat anti-HSA serum to the column and eluted the unbound proteins with PBS at a rate of 20 mL/h. When the eluate showed no absorbance at 280 nm, the antibodies were eluted with 3.5 mol/L NaSCN in PBS, at a rate of 20 mL/h. Fractions that contained protein, as detected by monitoring the absorbance at 280 nm, were pooled and immediately dialyzed against five changes of PBS (1 L each, at 2-h intervals). The procedure yielded 472 mg of specific anti-HSA IgG.

*Conjugation of alkaline phosphatase to specific goat anti-HSA IgG.* The enzyme–antibody conjugate was prepared by a variation of a procedure described previously (14). One milligram of alkaline phosphatase from calf intestine (Sigma

² Nonstandard abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; and IgG, immunoglobulin G. "Tween 20" is polyoxyethylene sorbitan monolaurate.
Chemical Co., St. Louis, MO 63178) was dissolved in 0.5 mL of carbonate buffer (10 mmol/L, pH 9.5) and dialyzed against the same buffer overnight at 4°C. The carbohydrate moieties of the enzyme were oxidized by adding 50 μL of 100 mmol/L NaIO4 (Matheson, Coleman and Bell, Cincinnati, OH 45212) to give a final periodate concentration of 10 mmol/L. After incubating the mixture at room temperature for 8 h, we added 4 mg of IgG (in 250 μL of 100 mmol/L carbonate buffer, pH 9.5) and 250 μL of polyethylene glycol 6000 (250 g/L) and incubated the mixture at room temperature for 3 h. We then added 50 μL of NaBH4 (5 g/L in 10 mmol/L NaOH), followed 30 min later by an additional 150 μL. We then dialyzed this mixture against a 50 mmol/L solution of Tris HCl (pH 8.0) containing 1 mmol of MgCl2 per liter at 4°C for 4 h. The complete conjugation procedure was carried out in darkness. The conjugate was separated from uncoupled IgG and enzyme by gel-filtration chromatography on a 1 × 48 cm column packed with Ultrogel AcA 34 (LKB, Gaithersburg, MD 20877) that previously had been equilibrated in the Tris/HCl/MgCl2 buffer. Fractions containing enzymic activity and eluting between the void volume of the column and the elution volume of free IgG were pooled and brought to a final concentration of 2 g/L NaN3 and 5 g/L bovine gamma-globulin (Calbiochem, La Jolla, CA 92037). The conjugate could be effectively stored at 4°C for at least two months.

Procedures

Alkaline phosphatase assay. Alkaline phosphatase was assayed with p-nitrophenyl phosphate substrate (0.33 g/L in 50 mmol/L carbonate buffer, pH 10.0, containing 1 mmol of MgCl2 per liter). The progress of the reaction was monitored by the change in absorbance at 405 nm (p-nitrophenol, ε405 nm = 18.5 × 10³). Each reaction (1-mL reaction mixture) was stopped by the addition of 100 μL of 1 mol/L NaOH.

Sandwich enzyme immunoassay. The solid-phase sandwich-type enzyme immunoassay for urinary albumin was carried out as schematically diagrammed in Figure 1. Standard solutions of HSA were prepared from a stock solution containing 1 g of HSA, 5 g of BSA, and 0.2 g of NaN3 per liter of 10 mmol/L PBS, pH 7.6. Immediately before use, antibody-coated bacteria were washed once (batch-wise) with 2 mL of 10 mmol/L PBS, pH 7.6, containing 0.5 g of Tween 20 per liter. We added 5 μL of washed antibody-coated S. aureus (50 g/L) to tubes containing 0.5 mL of HSA standards (25, 50, 100, 200, 500, and 1000 μg/L) or urine samples (diluted 50- or 1000-fold in PBS containing 5 g of BSA and 0.2 g of NaN3 per liter), vortex-mixed, and incubated at room temperature for 1 h. The samples were centrifuged for 5 min at room temperature and the pellets were washed once with 2 mL each of PBS-Tween 20. Each bacterial pellet was then resuspended in 1 mL of alkaline phosphatase anti-HSA conjugate in PBS containing 5 g of bovine gamma-globulin per liter and incubated for 1 h at room temperature. After one wash with PBS-Tween as described earlier, the pellets were resuspended in 0.5 mL of 50 mmol/L carbonate buffer, pH 10.0, containing 1 mmol of MgCl2 per liter. The suspension was then assayed for alkaline phosphatase activity as follows: At 15-s intervals, add 0.5 mL of p-nitrophenyl phosphate (0.66 g/L in carbonate buffer, pH 10.0, containing MgCl2, 1 mmol/L) to each tube; stop the reactions after suitable periods of color development by adding 100 μL of 1 mol/L NaOH. The extent of reaction is monitored by measuring the change in absorbance at 405 nm. A typical saturation curve is shown in Figure 2. The bound enzymic activity is expressed as a percentage of the maximum possible bound activity obtained with an HSA solution of 10 mg/L, normalized by probit transformation (15).

Radial immunodiffusion. Antibody-impregnated agarose gels were prepared as follows: Dissolve agarose (1 g/L) in 50 mmol/L Tris-barbital buffer, pH 8.6, at 50°C and add goat anti-HSA serum to a final concentration of 10 g/L. Pour 4 mL of the fluid agarose–antibody solution onto a 50 × 75 mm glass microscope slide and allow to solidify. Cut eight wells (3-mm diameter) in each plate. Put 30 μL of HSA standard or undiluted urine in each well, then incubate at 4°C for 24 h in a humid chamber. Then cover the plates with a wet disc of Whatman 3 MM filter paper and an absorbant pad of paper towels, and press for 15 min with a 1-kg weight. Wash the flattened plates for 10 min in 0.15 mol/L NaCl, then repeat the pressing process. After being pressed and saline-rinsed three times, the plates are washed in distilled H2O for 15 min, pressed again for 15 min, and finally air dried.

The dried plates were stained in 10 g/L Coomassie blue dissolved in a solution of 25 mL of ethanol and 10 mL of acetic acid per liter and destained for 10 min in the stain solvent.

Results

Effect of incubation time on the enzyme immunoassay. To determine the time necessary for maximal binding of albumin to the immobilized antibodies, we varied the time of incubation from 0.5 to 2 h. The time of exposure to the enzyme–antibody conjugate, which takes place in a subsequent step, was maintained constant at 4 h. The bound activity reached a plateau after 1 h, regardless of the albumin concentration.

The time necessary for the conjugate to bind maximally to the immobilized antigen was established in a separate experiment. The first incubation was held constant at 1 h, while the exposure to the conjugate was varied between 0.5 and 2 h. The maximum amount of conjugate was bound after 1 h.

![Fig. 1. Schematic diagram of S. aureus enzyme immunoassay](image1)

S. substrate, P. product

![Fig. 2. Saturation curve for purified HSA](image2)

Five microliters of antibody-coated S. aureus, 50 g/L, was added to 0.5 mL of sample containing HSA at various concentrations. First and second incubations were 1 h each; 100 μL of conjugate was used in second step.
Effect of conjugate concentration on dose–response curve. Varying the concentration of enzyme–antibody conjugate in the second step affects the steepness of the standard curve as well as the breadth of the working range of the assay (Figure 3). Although 250 mU of conjugate (in terms of the enzyme activity of the conjugate) per milliliter gave a higher, steeper curve, we used 100 mU/mL for the assay because this amount, when normalized by probit transformation, gave the most suitable balance between sensitivity and breadth of working range; moreover, the nonspecific binding of conjugate to S. aureus was minimal at this conjugate concentration.

Range and linearity. The log-probit dose–response curve was linear between 25 and 1000 μg/L (Figure 4). This range was useful for determining urinary albumin concentrations of 1.25–50 mg/L for samples diluted 50-fold and 25–1000 mg/L for those diluted 1000-fold.

Analytical recovery of albumin from human urine. Normal human urine containing a known amount of albumin was brought to final albumin concentrations of 25, 50, 100, 200, 500, and 1000 mg/L by adding pure HSA. The samples were diluted 50- and 1000-fold with PBS/BSA diluent and assayed in duplicate for HSA. The recovery in this range was from 95 to 107%. We performed a similar study, to determine the effect of Bence Jones proteins and hemoglobin on the analytical recovery. A urine containing 4 g of protein per liter, 71% of which was Bence Jones proteins, and a gross amount of hemoglobin was centrifuged and supplemented with pure HSA to various concentrations. The results (Figure 5) indicate that neither of these components significantly affects the ability of the new assay to measure albumin.

Precision. The between-run variability (CV) was found to be 2.5% by performing consecutive determinations (n = 6) on a urine sample included in each day’s assays as a control. The within-run CVs were 3.7, 3.7, and 2.4% for urine samples containing 1.9, 38.1, and 63.8 mg/L of HSA, respectively (n = 12 each).

Reference interval and patients’ data. Twelve healthy volunteers were evaluated for urinary albumin excretion over a 24-h period. The range was 1.25–4.93 mg/24 h (mean 3.05 mg/24 h). Urinary albumin was determined for 49 patients suspected of having renal dysfunction, and these values are compared with those of normal subjects in Figure 6.

Assay validation. To determine if the new enzyme immunoassay contained any systematic variables that would affect the values obtained for HSA in urine, values obtained by the new assay were compared with those obtained by radial immunodiffusion. Concentrations of albumin in 20 urine samples as determined by radial immunodiffusion were plotted vs those obtained by the new enzyme immunoassay (Figure 7).

Another experiment was carried out to demonstrate further that urine constituents did not interfere with the ability of this assay to specifically determine minute amounts of albumin. Hyperalbuminemic urine was serially diluted in PBS/BSA, and 0.5 mL was assayed for albumin by the new assay. As shown in Figure 8, when the log-probit data are plotted with the standard curve obtained for pure HSA, the serially diluted urine curve is superimposable (p <0.001, Student’s t-test).
bumin and found them insignificant. The albumin values obtained by the new assay were validated by comparing them with values obtained by radial immunodiffusion, a commonly used method for measuring low concentrations of albumin.

Sensitive enzyme immunoassay technology, such as the assay described here for quantifying albumin in urine, will be of particular interest in the future for detecting specific molecules in human serum, e.g., tumor-associated antigens. The combined use of protein A-bearing *S. aureus* and goat antibodies in an enzyme immunoassay for measuring urinary components is quite feasible as demonstrated here, but could present certain problems for quantifying an antigen in undiluted serum. For example, the affinity of goat immunoglobulins for protein A is less than that of human immunoglobulins (2, 17); hence, IgG in human serum samples could displace the specific goat IgG from the *S. aureus* membrane and interfere with the assay. Moreover, human serum antibodies against ruminant immunoglobulins (18) could bind to the solid-phase, and in turn bind the goat antibody–enzyme conjugate, causing falsely increased values.

Sensitive sandwich-type enzyme immunoassays like this one are usually developed to use antibodies produced in rabbits or guinea pigs, which have relatively high affinities for protein A; goat or sheep antibodies are more economical, however, because of their abundance.

Certain precautions can be taken to avoid the problems associated with the use of goat antibodies and *S. aureus* in an enzyme immunoassay for antigens in undiluted serum. First, after coating the bacteria with antibodies, the immunosorbant can be treated with glutaraldehyde (8), to bind the antibodies covalently so they cannot be displaced. Second, to prevent anti-ruminant IgG antibodies in human serum from binding the goat antibody–enzyme conjugate, excess nonimmunolnure goat serum can be included in the conjugate solution (19).

The use of protein A-bearing *S. aureus* as the solid-phase matrix in enzyme immunoassays should have noticeable impact on the immunodiagnosis of cancer and other diseases. By using the treated bacteria as an inexpensive, high-capacity absorbant surface for IgG from certain mammalian species, clinical as well as research laboratories can develop convenient, sensitive immunoassays that circumvent the use of radioisotopes.

J. R. H. is a Predoctoral Fellow of the Rosalie B. Hite Foundation and recipient of an Outstanding Young Investigator Award from Boehringer Mannheim Diagnostics. This work was submitted as partial fulfillment of the requirements for his Doctor of Philosophy degree at the University of Texas Graduate School of Biomedical Science at Houston.

N. M. is a recipient of a Junior Science Training Fellowship from the King Foundation.

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