Homogeneous Enzyme Immune Assay for Staphylococcal Enterotoxin B†

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A simple homogeneous enzyme immune assay was developed for detection of staphylococcal enterotoxin B by a conjugate of β-amylase coupled with the enterotoxin.

Since 1969, when Avrameas (1) was able to conjugate enzymes to other proteins, there has been a proliferation of clinical applications of enzyme-protein conjugates for detecting specific proteins in serum and urine. This method, known as enzyme-linked immunosorbent assay, is gaining wide acceptance as a substitute for the radioimmunoassay technique. Investigators have applied the enzyme-linked immunosorbent assay for detecting enterotoxin A (6) and B (H. Fey and G. Stöffler-Rosenberg, Experientia 33:1678, 1978). Both radioimmunoassay and enzyme-linked immunosorbent assay have the disadvantage of requiring adsorption of one of the reactants onto a solid surface. In addition, washing steps are required to remove unbound from bound reactants.

A modified method, enzyme-multiplied immunoassay technique (EMIT, Sylva Co., Palo Alto, Calif.) developed by Rubenstein et al. (5), is a simpler technique which requires neither solid surfaces nor separation of unbound from bound reactants. This method has also been called homogeneous enzyme immunoassay as opposed to heterogeneous enzyme immunoassay (enzyme-linked immunosorbent assay). Our goal was to apply this technique to the detection of staphylococcal enterotoxin B (SEB).

In the assay, a known amount of anti-SEB is added to the sample containing an unknown amount of free SEB. Then the conjugate (enzyme-SEB) is added. Free SEB (to be measured) competes with enzyme-linked SEB for antibody binding sites. Because the enzyme linked with SEB is rendered inactive with the attachment of anti-SEB, enzyme activity, measured photometrically, is related directly to the concentration of free SEB.

The search for an appropriate enzyme for the conjugate led to our choice of β-amylase because this enzyme is not produced by Staphylococcus aureus. Thus, a possible complication in assaying samples of culture media would be eliminated.

The coupling method used was essentially that of Engvall et al. (3). The enzyme, β-amylase from potatoes (Sigma Chemical Co., St. Louis, Mo.), was obtained as a 25-mg/ml suspension in ammonium sulfate. SEB was supplied by E. J. Schantz (University of Wisconsin, Madison, Wis.). A 0.1-ml portion of β-amylase suspension was centrifuged in the cold for 10 min at 12,000 rpm. The supernatant was decanted, and the remaining pellet was dissolved in 1.0 ml of 0.05 M phosphate buffer, pH 6.8, containing 500 µg of enterotoxin B. (The same buffer was used in all subsequent steps and in the assay as a diluent unless stated otherwise). After overnight dialysis against buffer, 0.025 ml of 2.5% glutaraldehyde (grade I, 25% aqueous solution, Sigma) was added. The mixture was held at room temperature for 2 h and then redialyzed.

The complex was separated from other fractions on a Bio-Gel P-150 column (1 by 40 cm; Bio-Rad Laboratories, Richmond, Calif.), which had been equilibrated with the buffer, by elution with the same buffer. Absorbance at 280 nm was measured for each of the 1-ml fractions, and those fractions in the first protein peak eluted were pooled. The conjugate was assayed for amylase activity by the Bernfeld method (2). To increase the readings, the time of substrate reaction was increased from 3 to 10 min for most systems.

The appropriate dilution of each conjugate preparation was selected that would give an optical density reading in the 0.6 to 0.8 range, which was obtained with dilutions between 1:15 and 1:20. Anti-enterotoxin B serum titrations were then carried out with antisemur dilutions of 1:100 to 1:800. The dilution selected was in the zone of slight antigen excess, which was 1:400 with the particular lot of antisemur used in this study.

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A standard curve was run with SEB dilutions ranging from 1 to 100 ng/ml in phosphate buffer as follows: step 1, 0.2 ml of SEB dilution plus 0.15 ml of anti-SEB (1:400; 1 h at room temperature, overnight refrigeration); step 2, 0.15 ml of conjugate dilution (1:15 or 1:20; 2 h at room temperature); step 3, 0.5 ml of 1% soluble starch in 0.016 M acetate buffer (10 min at 25°C); step 4, 1.0 ml of color reagent (boiled 5 min, cooled, and absorbance read at 540 nm). Absorbance was read against a blank containing 0.5 ml of phosphate buffer in place of steps 1 and 2. In the antiserum control, SEB was replaced with buffer; in the conjugate control, SEB and anti-SEB were replaced with buffer; and in the SEB control, anti-SEB was replaced with buffer. Data were plotted on semilog paper (Fig. 1).

The assay method has been used for NZ Amine NAK culture medium for S. aureus and food extracts containing known amounts of SEB. S. aureus, strain S-6, grown in 4% NAK supplemented with 0.5 µg of thiamin per ml and 10 µg of niacin per ml under aerated conditions, produced 120 µg of SEB per ml as determined by the Oudin single gel diffusion method (4). Dilutions of 1 to 100 ng of toxin per ml were made with 4% NAK which had been dialyzed against phosphate buffer. Whole milk was acidified to pH 4.5 with 1 N HCl, centrifuged, and adjusted to pH 6.8 with 1 N NaOH. For mayonnaise, 1 ml of phosphate buffer was added per g of mayonnaise, homogenized in a blender, and centrifuged. For weiners, 1 ml of phosphate buffer was added per g of meat, homogenized in a blender, and centrifuged. For cornstarch, a 4% starch paste in 4% NAK (7) was centrifuged. All supernatants were dialyzed overnight before assay. SEB dilutions of 5 to 100 ng/ml were made with the dialyzed food extracts. Antiserum and conjugate controls and blanks all contained food extracts or NAK. SEB controls without antiserum were also done with the 100-ng/ml samples. For unknown samples, similar food extracts were added to the antiserum controls. Because all controls contain food extracts or culture media, it is possible to detect any inhibitory or stimulatory effect of the medium or food extracts upon the assay system itself.

The absorbances obtained for SEB dilutions of 1, 5, 10, 25, 50, 75 and 100 ng/ml made with dialyzed NAK were 0.60, 0.63, 0.69, 0.81, 1.11, 1.56 and 1.77. As shown in Table 1, it is possible to detect quantities of SEB as low as 5 ng/ml in food extracts (lowest amount tested) by means of the homogeneous enzyme immune assay. Samples containing minimal amounts of SEB may be first concentrated with Aquacide (Calbiochem, Los Angeles, Calif.) or by other means. Maximum sensitivity was obtained by using a 10-min substrate reaction time beyond which sensitivity decreased. Incubation times other than 1 h at room temperature and overnight refrigeration for SEB with anti-SEB and 2 h at room temperature after addition of conjugate have been tried, but more consistent results were obtained with the recommended times. Al-

![FIG. 1. Standard curve for SEB in phosphate buffer with substrate reaction time of 10 min (four replications for each concentration).](http://iai.asm.org/)
though Saunders and Bartlett (6) found that organic matter in food extracts did not appear to interfere with the enzyme-linked immunosorbent assay method, we found that the optical density of the antiserum control and blanks were too high with the 10-min substrate reaction time. Rather than decrease the sensitivity by decreasing reaction time, the extracts were dialyzed overnight against buffer.

Work is now in progress to extend the testing of the technique and to apply it to the detection of other staphylococcal enterotoxins.

LITERATURE CITED


