Detection of Staphylococcal Enterotoxin B in Spiked Food Samples

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ABSTRACT

Contamination of food with infectious agents, intentional or not, is a global concern with far-reaching economic and social impact. Staphylococcal enterotoxins are a major cause of food poisoning, but most methods for the identification of these agents in food require extensive pretreatment or concentration of the sample prior to analysis. The array biosensor was developed as a portable device for the simultaneous analysis of multiple complex samples for targets with minimal sample preparation. In this study, we use an array biosensor to expand and improve on a staphylococcal enterotoxin B (SEB) assay with the ultimate intent of incorporating testing for SEB into a battery of sensitive and convenient assays for food safety validation. In addition to buffer studies, six different types of food samples, including beverages, homogenates of fruit and meat, and carcass washings, were spiked with SEB, incubated for at least 2 h to permit antigen sequestration, and assayed. For all samples, there were differences in fluorescence intensity, but 0.5 ng of SEB per ml could be detected in <20 min with little if any pretreatment and no sample preconcentration.

Furthermore, while the modified ELISA method has been demonstrated with environmental samples and clinical fluids, it has not been used for food samples.

Another method for the detection of SEB employs capillary electrophoresis and laser-induced fluorescence with the fluorophore tetramethyl rhodamine in a competitive immunosensor assay. (10). Lam et al. (10) were able to detect 28 ng of SEB per ml in a 5-μl sample of 5% milk in 10 to 15 min. This method has not been tested for the analysis of other foods of concern, such as fruits and meats, and does not meet the 1-ng/ml goal for solution detection sought by the USDA Food Safety and Inspection Service (FSIS) (11).

Several sensors based on surface plasmon resonance have been developed (8, 13, 14, 17). The two best known commercial instruments are Pharmacia’s Biacore and Texas Instruments’s Spreeta. These instruments also employ antibodies as the recognition element to provide specificity. The level of sensitivity for the detection of SEB in buffer is 10 ng/ml in 10 to 20 min (17, 22). Detection limits as low as 1 ng/ml can be achieved with assay times of >30 min with the use of these instruments (14). Testing of food samples such as milk and potted meat yielded detection limits of 2 to 10 ng/ml. Since surface plasmon resonance is based on a change in the resonance angle from a gold surface (which usually reflects a change in mass), any nonspecific adsorption of components from complex food samples may affect the results, even after the signal from a control surface tested in parallel with the sensing surface has been subtracted.

Immunomagnetic beads have been employed with several sensor systems for the preconcentration of SEB. Yu (25) employed magnetic beads to separate SEB from environmental water samples prior to analysis with a fluorescence...
cent sandwich immunoassay with a 96-well plate. The limit of detection of a 1-h assay was 10 ng/ml for wastewater. The Origen system also uses an immunomagnetic separation step but combines it with electrochemiluminescence. The dynamic range for SEB detection with this system was found to be 0.1 to 100 ng/ml (9) spiked into serum, 5% milk, and kidney homogenates, preconcentrated with the immunomagnetic beads, and analyzed. The sensitivity of the sensor without the preconcentration step was not determined.

For the development of most of the methods available to date, aqueous sources were used as samples. Meat, especially ham, is a prevalent source of SEB in food poisoning. A fiber-optic biosensor was used to detect SEB spiked into aqueous ham extracts (23). This antibody-based system was able to detect 5 to 200 ng of SEB per ml in ham extract, serum, and urine in <15 min with no preconcentration step.

The array biosensor, used in this study, operates on principles similar to those for the fiber-optic biosensor in that a fluorescent complex is formed at the surface of a waveguide via a sandwich immunoassay and excited with evanescent illumination. Unlike the fiber-optic biosensor, however, the array biosensor was developed to detect multiple analytes simultaneously on a single sensing surface. The array biosensor employs a planar waveguide with immobilized capture antibodies in distinct regions, and the identity of the bound target is determined by the location of the fluorescent complex on the waveguide surface. The array biosensor is a small portable device that is appropriate for on-site analysis. In previous studies, SEB was one of six analytes detected in buffer and in the presence of environmental interferents (18–20, 24) at concentrations as low as 5 ng/ml (24). Since the array biosensor was shown to be relatively immune from nonspecific sample matrix effects and since the possibility of testing for a variety of foodborne toxins and pathogens simultaneously was alluring, we explored the capability of the array biosensor in detecting SEB spiked into six types of food samples representing the primary sources of food poisoning.

Spiked samples were used instead of naturally contaminated samples so that the detection signal produced by a known amount of toxin could be determined without reliance on the accuracy of another analytical method. Furthermore, an aliquot of each food sample was thus available without any toxin to serve as a control blank for the identification of false-positive responses, if any. Although toxin in buffer is not exactly analogous to natural contamination, a comparison of the signal from a certain concentration of toxin in buffer with the signal from the same concentration incubated into a food sample should also provide some indication of the tendency of the toxin to bind to the food matrix in such a way as to reduce the signal. On the basis of the success of the assays with spiked samples, naturally contaminated samples can be tested and compared with toxin-containing food standards such as those used in this study.

**MATERIALS AND METHODS**

**Materials.** Two polyclonal antibodies against SEB (sheep and rabbit immunoglobulins G) were obtained from Toxin Technologies (Sarasota, Fla.), and two murine monoclonal antibodies, 2B and 6B, were purchased from IGEN (Gaithersburg, Md.). Two fluorescent dyes with succinimide esters, Alexa-Fluor 647 (AF 647, Molecular Probes, Eugene, Or.,) and Cy5 bifunctional dye (Amersshamb Biosciences, Atlanta, Ga.,) were evaluated for antibody labeling. EZ-Link Biotin-LC-NHS and NeutraAvidin were obtained from Pierce (Rockford, III.). All food was purchased from local grocery stores. Phosphate buffer (PB, pH 7.4), phosphate-buffered saline (PBS, pH 7.4), bovine serum albumin (BSA), and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, Mo.). The assay buffer consisted of PBS, 0.05% Tween 20, and 1 mg of BSA per ml (PBSTB), and the patterning buffer consisted of 10 mM PB, 10 mM NaCl, and 0.05% Tween 20 (PBST). 3-Mercaptopropyltrimethoxysilane and N-[(γ-maleimidobutryl)oxy] succinimide ester were purchased from Fluka Chemical Co. (St. Louis, Mo.). A Waring two-speed commercial blender and minisample container (37 to 110 ml; Fisher Scientific, Pittsburgh, Pa.) was used to prepare some food samples.

**Preparation of labeled antibodies.** On the basis of results from previous studies (18–20, 24), the rabbit anti-SEB polyclonal antibody was biotinylated and the sheep anti-SEB antibody was fluorescently labeled with Cy5. On the basis of information provided by IGEN, the monoclonal antibody 2B was biotinylated while the monoclonal antibody 6B was fluorescently labeled. Biotinylation of the rabbit anti-SEB antibody and the monoclonal antibody 2B was carried out with EZ-link biotin-LC-NHS at a 5:1 (biotin/antibody) molar ratio according to the manufacturer's protocol. Cy5 labeling of the sheep anti-SEB antibody was performed per the manufacturer's instructions. AlexaFluor labeling of the sheep anti-SEB antibody was carried out according to the procedure of Anderson and Nerurkar (1). Modifications were required for antibodies produced in mice. To obtain optimal dye/protein ratios, the mouse antibodies required dialysis against the borate buffer prior to mixing with fluorophore.

**Array biosensor.** The array biosensor has been described in detail elsewhere (6, 18, 19). It consists of a waveguide with immobilized capture antibodies, a diode laser to generate evanescent illumination, and collection optics including a CCD camera to collect the fluorescence signal.

Microscope slides were employed as the optical waveguides, and their surfaces were used for sandwich immunoassays. NeutraAvidin was immobilized on the slides with the use of a thiol silane and a heterobifunctional cross-linker as described by Bhatia et al. (3) and Rowe et al. (18). Biotinylated capture antibodies (10 μg/ml in PBST) were attached to a slide in vertical columns with the use of a poly(dimethylsiloxane) (PDMS) flow chamber and incubated overnight at 4°C (6). In all cases, the channels of the flow chamber were washed with PBSTB, and the slide was removed from the PDMS template and placed in PB containing 10 mg of BSA per ml. After 30 min, the slides were rinsed with deionized water, dried with nitrogen, and stored at 4°C until they were used.

**Food sample preparation.** The methods employed for the preparation of the spiked food samples were based on previously reported methods (4, 5, 7, 12, 17). In all cases, SEB was incubated with the food sample for a minimum of 2 h before the assay in order to allow sufficient time for the interaction of the toxin and the sample matrix. Food samples designated “homogenates” con-
tained large particles when SEB was added and were clarified by centrifugation immediately prior to the assay. Foods were prepared for spiking and testing as follows.

Ten grams of ham or ground beef (20% fat) and 8 ml of PBSTB were combined in a 37- to 110-ml minisample container on a Waring blender and homogenized at high speed for 2 min. The resulting homogenate was placed into a 50-ml centrifuge tube. The blender container was rinsed with 2 ml of PBSTB, and the rinse solution was added to the centrifuge tube to yield a 1:1 (wt/vol) homogenate of meat and buffer. The homogenate was either spiked directly or centrifuged at 3000 rpm for 5 min, after which the supernatant was spiked.

A cantaloupe homogenate was prepared with 5 g of cantaloupe and 5 ml of PBSTB to obtain a 1:1 (wt/vol) homogenate.

Carnation nonfat dried milk (Nestle, Inc., Solon, Ohio) was prepared according to the instructions on the box, and 0.3 ml of 10% PBSTB was added to 2.7 ml of milk to yield a 90% milk sample.

For the carcass wash, a stock solution was produced by combining 100 ml of PBS containing 1 mg of BSA per ml with a 6-lb chicken carcass in a plastic bag. The liquid from the absorbent material in the chicken's wrapping was also added to the bag. The bag was rocked rapidly for 2 h at room temperature. The liquid was removed, aliquoted, and frozen until SEB was added.

Eggs were weighed, combined 1:1 (wt/vol) with PBSTB, and homogenized for 1 min.

SEB was added to the food matrices to obtain final concentrations on the basis of the buffer volume values, e.g., for meat, a 10-ml volume was incubated for 2 h, 4 days, or 7 days, as indicated. Immediately before the analysis, the spiked ham, ground beef, and cantaloupe homogenates were centrifuged at 3000 rpm for 3 min to remove the large particles. The spiked ham supernatant and the milk, egg, and carcass wash samples were analyzed without centrifugation or filtering.

Assays. For the sandwich immunoassay, sample and tracer antibodies were applied to the waveguide surface through PDMS flow cells oriented perpendicular to the columns of capture antibodies (6). The slide with the attached flow cell was connected to a Manostat multichannel pump (Sarah model) with tubing extending from the exit end of each channel. Syringe barrels (1 ml each), serving as reservoirs for the various assay fluids, were attached to the entry end with 25-gauge needles. The channels were first washed with 1 ml of PBSTB at the maximum flow rate. Next, a 0.8-ml sample was applied to each channel over an 8-min period, and this step was followed by another rapid wash with 1 ml of PBSTB. A 0.4-ml portion of the fluorescent tracer antibody (10 ng of fluorescently labeled anti-SEB per ml) was applied to the channels over a 4-min period, and this step was followed by a final rapid wash with PBSTB.

Previous studies of SEB detection with the array biosensor employed Toxin Technologies rabbit anti-SEB as the capture antibody and Toxin Technologies sheep anti-SEB as the tracer antibody (18–20, 24). Monoclonal antibodies from IGEN, as well as the fluorophore AlexaFluor 647 (whose characteristics are similar to those of Cy5), have become available since the previous studies were conducted. Initial studies focused on testing these antibodies and this fluorophore to determine the optimum antibodies and labels. Two antibodies (Toxin Technologies rabbit anti-SEB polyclonal antibody and IGEN’s 2B anti-SEB monoclonal antibody) were tested as potential biotinylated capture antibodies while two antibodies (Toxin Technologies sheep anti-SEB polyclonal antibody and IGEN 6B anti-SEB monoclonal antibody) were labeled with either Cy5 or AlexaFluor 647 to determine their potential as tracer antibodies. For these initial tests, the dye-to-protein (d/p) ratio for the fluorescent antibodies was kept constant. After the antibodies and the fluorophore were selected, a standard buffer curve was generated, and then dose-response analyses of SEB spiked into the various food samples were carried out. The concentrations tested ranged from 0 to 150 ng of SEB per ml.

FIGURE 1. Representative array image with SEB concentrations of 0 to 10 ng/ml shown in the rows.

FIGURE 2. SEB dose-response curve for buffer. The left panel shows the entire curve, and the right panel shows the linear region from 0 to 25 ng of SEB per ml. Values are averages ± standard errors of the mean for eight or more squares combined from several experiments.
FIGURE 3. Time study of the incubation of ham supernatant spiked with SEB. ○, solid line, day 0; ▲, dashed line, day 7. Values are averages ± standard errors of the mean for eight or more squares.

FIGURE 4. SEB dose-response curves for spiked meat matrices. ○, solid line, ham supernatant; ■, dashed line, ham; ▲, dotted line, ground beef; ●, dash-dot line, chicken carcass wash. The right panel shows the linear region from 0 to 25 ng of SEB per ml. Values are averages ± standard errors of the mean for eight or more squares.

Data collection and analysis. The image of the array was captured with SpectraSource software in the Flexible Image Transport System digital format. This intensity image was converted to average fluorescence intensity per square of capture antibody with the use of a program developed at Naval Research Laboratory (21). Basically, the program identifies the location of the square and the background areas next to the square. The background average intensity value is subtracted from the average fluorescence intensity of the assay square and exported as a comma-separated value file, which can be imported into Microsoft Excel. The average mean intensity for each spot after background subtraction was used for the analyses.

RESULTS AND DISCUSSION

With new fluorescent dyes and recently developed antibodies against SEB, our first goal was to determine whether combinations of biotinylated capture and fluorescently labeled tracer antibodies that had not previously been used with the array biosensor would provide increased signal intensity and ultimately lower the limit of detection. Various combinations of biotinylated anti-SEB antibodies and fluorescent (Cy5 or AF 647) anti-SEB antibodies were tested in a sandwich immunoassay format. The d/p ratios for the fluorescent antibodies were approximately 4:1. To reduce variability parameters, this d/p ratio was chosen because at higher ratios Cy5 fluorescence becomes quenched; AlexaFluor 647 can label at higher d/p ratios without quenching (1). Different antibody combinations were used in assays for SEB at three concentrations (5, 25, and 50 ng/ml). The combination of biotinylated IGEN 2B anti-SEB as the capture antibody and AF 647–labeled Toxin Technologies sheep anti-SEB antibody proved to provide the strongest signal intensities at all three concentrations. This combination was employed for the remainder of the study, except that the d/p ratio for the AF 647 sheep anti-SEB was increased to 6.6:1 to obtain stronger fluorescence signals.

A standard curve for the detection of SEB in buffer was generated with the optimized capture-tracer antibody combination. A representative array image of a slide with 0 to 10 ng of SEB per ml is shown in Figure 1. This image was converted into intensity values for the six squares in each row and for the six rows with the NRL program. From these values, a standard dose-response curve for SEB in buffer was generated. Figure 2 shows the combined results for several different slides. A minimum of eight mean values were obtained for each concentration. From these results, the limit of detection, the lowest concentration that was greater than three standard errors of the mean above the mean intensity of the blanks, was determined to be 0.5 ng/ml. This value represents an improvement over the previously reported detection limits of 10 and 1 ng/ml obtained with the array sensor (18-20, 24). The standard curve fits an asymmetric sigmoid form, $y = 1.691 + (36.176 - 1.691) \left[1 + \left(\frac{x}{10.7}\right)^{2.6}\right]$, with the linear region of the sigmoid form being between 0 and 25 ng/ml.

More important than the dose-response curve for buffer is how the system works for the detection of SEB in food. Dose-response curves for SEB spiked into several different types of foods (or food homogenates) were determined. A representative food sample was used; fat and small particles were not removed. For the spiked food homogenate samples with large particles, such as ham, ground beef, and cantaloupe homogenates, a centrifugation step was included just prior to the analysis to remove large particles that would clog the fluidics. A concern associated with the spik-
FIGURE 5. SEB dose-response curves for spiked nonmeat food matrices. , solid line, 100% milk; , dashed line, 95% milk; , dotted line, egg; , dash-dot line, cantaloupe. The right panel shows the linear region from 0 to 25 ng of SEB per ml. Values are averages ± standard errors of the mean for eight or more squares.

ing and analysis of samples on the same day is that the analyte does not have adequate time to nonspecifically bind to components of the sample (protein, lipid, etc.), as it would for natural contamination. To provide at least a cursory evaluation of the effect of incubation time on toxin availability, the ham supernatant was assayed 2 h, 4 days, and 7 days after SEB was added. Figure 3 shows dose-response curves for day 0 and day 7. At the 95% confidence level, there was no significant difference between day 0 and day 7 curves as determined by an analysis of variance and Fisher’s F test (data not shown). Similar results were obtained for day 4. All of the other food samples were tested in the same way. In no case was there any difference between results obtained after 2 h, 4 days, and 7 days. Therefore, the results presented below are means and standard errors of the mean for days 0, 4, and 7 combined.

Figures 4 and 5 show the SEB dose-response curves for meat (ham, ground beef, and carcass wash) and nonmeat (milk, cantaloupe, and egg) samples, respectively. The right panels show the linear region from 0 to 25 ng of SEB per ml for each food type. The limits of detection and the linear regression results for the curves in the insets of the figures are shown in Table 1. There are no differences between the limits of detection for any of the food samples tested; therefore, this system could be used to test for the presence of SEB in multiple types of matrices at concentrations of >0.5 ng/ml. With regard to quantitation, there are significant differences in the slopes of the curves for the different matrices, including buffer, which would affect quantitation for an untested matrix based on a dose-response curve for a different sample matrix. On the basis of a dose-response curve for the same type of food sample, SEB can be quantitated. Carcass wash and ham supernatant have the lowest slope and \( R^2 \) values (<0.95), which suggests that the curves are not linear in that region and that there is some interference from the food sample that affects binding in the sandwich assay format. Further detailed investigation of the food sample components would be needed to determine the exact nature of the interference. For all of the tested foods in Figures 4 and 5, the detection limit is below the USDA-FSIS solution goal (11).

An improvement in the limit of detection of SEB in buffer (0.5 ng/ml) with the use of the array biosensor has been demonstrated. This improvement is due to changes in the fluorophore and capture antibodies employed. In addition, this improved limit of detection was maintained for samples from food matrices spiked with SEB, and thus the system can easily detect the presence of SEB. There are differences in the dose-response curves for the different matrices, including buffer; therefore, it is recommended that quantitation for any unknown sample be performed with the use of a standard curve generated for that sample matrix. This SEB assay can easily be combined with other assays developed for the array biosensor to provide a sensor system capable of on-site, multianalyte, multisample analysis of a variety of matrices.

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<table>
<thead>
<tr>
<th>Matrix</th>
<th>Detection limit (ng of SEB per ml)</th>
<th>Linear regression for 0–25 ng of SEB per ml</th>
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<td>( y = 123x + 714 )</td>
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<td>Ham</td>
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<td>( y = 672x + 1.269 )</td>
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<tr>
<td>Ground beef</td>
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<td>( y = 486x - 5 )</td>
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<td>Carcass wash</td>
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<td>( y = 305x + 2.126 )</td>
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REFERENCES


