Recovery of Staphylococcal Enterotoxin from Foods by Affinity Chromatography

CONSTANTIN GENIGEORGIS* AND JASON K. KUO

Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, California 95616

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Extraction, concentration, and serological detection of staphylococcal enterotoxins from foods are laborious and time consuming. By exposing food extracts to an insoluble matrix tagged with specific anti-enterotoxin B, we have been able to recover the toxin from foods in a sensitive and rapid way. After mixing the reagents for 2 h at room temperature, immunoglobulin G antibodies were attached to CNBr-activated Sepharose 4B at pH 8.5 (0.1 M carbonate buffer with 0.5 M NaCl). Sepharose-antibody complex (1 ml) specifically recovered 0.1 to 30 μg of enterotoxin B from 400 ml of food extract (100 g of food) after mixing for 2 h at 4 C. The Sepharose-antibody-toxin complex was washed with 0.02 M phosphate-buffered saline at pH 7.2, and the toxin was dissociated by 2 to 4 ml of 0.2 M HCl-glycine plus 0.5 M NaCl buffer at pH 2.8. The recovered enterotoxin was free of interfering food components and could be detected serologically. Work to couple antibodies A, B, C, D, and E to Sepharose to recover all five toxins in one step is under study.

Staphylococcal food poisoning remains one of the two most common types of food poisoning in this country (5, 12). During the last 10 years, outstanding advances have been made in the elucidation of the environmental conditions under which staphylococci grow and produce their enterotoxins and in the development of diagnostic procedures for the detection of these toxins in foods. Regulatory agencies, the food industry, and academic researchers are always in need of simple, sensitive, reliable, rapid, and inexpensive diagnostic procedures. Such procedures allow regulatory agencies to screen large numbers of food samples, enable the food industry to establish mass screening programs, and permit researchers to plan additional experiments to learn more about the physiology of enterotoxin production.

Detection of enterotoxins present in pure form in solution by the use of a number of different serological methods based on antigen-antibody reactions is highly successful (2).

In the presence of increasing amounts of organic compounds present in the culture media or food or produced by staphylococci, the serological methods become less reliable and require a certain degree of enterotoxin purification. It is this purification that remains the bottleneck of the whole detection effort.

In practice, the procedure for the detection of enterotoxins by serological methods involves four steps: extraction of the enterotoxins from foods, purification, concentration to detectable levels, and detection. The current recommended procedures for the detection of staphylococcal enterotoxins in foods are laborious and time consuming. The most extensively tested method for extraction, purification, and concentration of enterotoxins is the method of Casman and Bennett (6). Depending on the type of food, a number of modifications of the Casman and Bennett method (14, 24, 32) are available that, with the detection step, might take from 1 to 7 days. Most of the methods for the recovery of enterotoxins from foods are based on the use of ion-exchange chromatography. Recently, solid-phase radioimmunoassay methods for the detection of enterotoxins have been reported (9, 18). The methods are fast, simple, and sensitive, but they require special handling of reagents and equipment.

One of the most outstanding advances in biochemical separation methods is the development of affinity chromatography (10). The new method permits the isolation of substances according to their biological function and, thus, differs radically from conventional chromatographic techniques in which separation depends on physical and chemical differences between substances. Because of the unique specificity of antigen-antibody interactions, affinity chromatography allows the separation of either
antigen or antibody in highly purified form (11, 21, 28, 31).

In this paper we report the development of a sensitive and rapid method for the recovery of staphylococcal enterotoxin B from culture media and from foods using affinity chromatography.

MATERIALS AND METHODS

Enterotoxin reagents. Pure enterotoxin B was supplied by M. S. Bergdoll and E. H. Schantz, both of the Food Research Institute, University of Wisconsin, Madison. Specific rabbit antisera and enterotoxin C were prepared in the laboratory (13).

Pure immunoglobulin G (IgG) was prepared by heating 5 ml of antiserum for 30 min at 56 C, centrifuging, and then filtering the supernatant through a Sephadex (Pharmacia, Uppsala, Sweden) column (2.5 by 85 cm). The Sephadex had been previously equilibrated with 0.1 M Tris(hydroxymethyl) aminomethane (Tris) buffer, pH 8.1, with 0.2 M NaCl.

All fractions of 5 ml of the protein peak at an optical density at 280 nm (OD280) with antibody activity as determined by the microslide gel diffusion method (6) against enterotoxin B were pooled. The IgG of the pooled fractions was precipitated with 50% saturated (NH$_4$)$_2$SO$_4$ overnight, and the precipitates were redissolved in 5 ml of Tris buffer and refiltered through the Sephadex column. The main fractions of the antibody peak were pooled and stored in the freezer until needed.

Microbial species. Staphylococcus aureus strain 137 (ATCC 19095) was furnished by M. S. Bergdoll; S. aureus S-6, 196-E, 249 (ATCC 14458), 472, 587, 790, and Wood-46 were originally obtained from the late E. P. Casman and R. Bennett of the Food and Drug Administration, Washington, D.C.; S. epidermidis strains and Micrococcus lysodeikticus (ATCC ISU-1) came from our collection.

Enterotoxin assay. The microslide (6), the single-gel diffusion (29), and the reversed passive hemagglutination (RPHA) (25) methods were used for qualitative and quantitative assay of enterotoxin B. Sensitization of formalized sheep erythrocytes with the antibody and the RPHA test were performed as described by Uemura et al. (27).

Coupling of antibody to Sepharose. The coupling of enterotoxin B IgG to the Sepharose 4B (Pharmacia) matrix was done by the method of Cuartecasas and Anfinsen (10). Ten milliliters Sepharose 4B activated with CNBr was washed with 15 volumes of ice-cold coupling buffer (0.1 M carbonate buffer, pH 8.5, with 0.5 M NaCl) in a Büchner funnel. Three milliliters of antibody solution was then added to the funnel and mixed with the moist Sepharose, and the suspension was transferred to a 25-ml Erlenmeyer flask. The flask was then placed on a reciprocal water bath shaker (140 rpm) and incubated for 2 h at room temperature. At the end of the incubation, the coupled Sepharose was washed with carbonate buffer and with glycine buffer (0.2 M HCl-glycine, pH 2.8, with 0.5 NaCl) alternatively until the OD$_{280}$ of the washings was not measurable. The washed Sepharose was finally equilibrated in buffered saline (0.02 M phosphate buffer, pH 7.2, with 0.15 M NaCl and 1:10,000 merthiolate) and stored in the refrigerator until needed.

Protein determination. Protein measurements were done by the method of Lowry et al. (20).

RESULTS

The recovery of staphylococcal enterotoxin B by affinity chromatography was investigated in a number of experiments, each of which evaluated the various steps of the procedure.

Coupling of antibody. Early studies indicated that more than 75% of the antibody was coupled to Sepharose in the presence of carbonate buffer, pH 8.5, and less than 56% in the presence of phosphate buffer (0.2 M, pH 6.5, with 0.5 M NaCl). The carbonate buffer was used for the rest of the study. The efficiency of the coupling procedure was increased to over 95% antibody attachment when the initial antibody was previously dialyzed against the coupling buffer. This approach was used routinely for the rest of the study. Amounts as high as 7.5 mg of antibody could be coupled to 1 ml of Sepharose, but for reasons of economy of reagents smaller amounts were coupled.

Recovery of enterotoxin from buffers. Amounts of 0.1 µg of pure enterotoxin B were added to 2 ml to 100 ml of buffered saline (0.02 M phosphate, pH 7.2, with 0.15 M NaCl). The toxin solution was filtered through columns (0.6 by 3.5 cm) containing 1 ml of Sepharose-antibody at rates of 1 to 2 ml/min. Using the single-gel diffusion test (29), it was determined that 1 ml of Sepharose containing 500 µg of antibody could bind up to 30 µg of pure toxin.

Elution of enterotoxin from Sepharose-antibody complex. The use of glycine buffer (pH 2.8), urea (4 M), proprionic acid (1 M), guanidine hydrochloride (5 M), and potassium thiocyanate (2 to 4 M) was evaluated for their efficiency in dissociating the antigen-antibody complex to permit recovery of enterotoxin. Urea, guanidine hydrochloride, and potassium thiocyanate failed to show any antigen-antibody dissociation. Propionic acid was also unsatisfactory because of the release of large amounts of protein from the column, possibly due to dissociation of antibody-antigen complex from the Sepharose. Two to four milliliters of glycine buffer per 1 ml of Sepharose was found to be the most efficient buffer in dissociating more than 95% of the enterotoxin attached to Sepharose-antibody complex.

Recovery of enterotoxin B from staphylococcal culture supernatants. Supernatants of toxigenic and nontoxigenic staphylococcal cul-
tures and micrococcal cultures grown in brain heart infusion (Difco) or PHP-NAK broth (11) for 24 to 36 h were lyophilized or kept frozen until use as sources of enterotoxins or controls. In a typical experiment, fresh or reconstituted lyophilized culture supernatant adjusted to pH 7.2 was filtered through a column (0.6 by 3.5 cm) containing 1 ml of Sepharose-anti-enterotoxin complex. Next the column was washed with at least 20 ml of buffered saline, and the enterotoxin was eluted with 9 ml of glycine buffer. The OD_{280} and the amount of enterotoxin in the eluant in one such experiment are shown in Fig. 1. Since the 120 μg of enterotoxin B present in the culture supernatant was much higher than the maximum amount that can be taken up by the 1 ml of Sepharose, most of the toxin passed through the column without reacting with the antibody. Obviously all of the toxin could be recovered if the conjugated Sepharose was more than 4 ml.

Recovery of enterotoxin B from foods. Effort was made to recover enterotoxin B added to Italian style dry salami of various ages, cheddar cheese, and fresh milk by affinity chromatography.

Solid food (100 g) was homogenized in a blender with 400 ml of distilled water and 75 μg of enterotoxin B (1 ml of culture supernatant). The pH of the homogenate or the milk was brought to 4.6 with 1.0 N HCl before it was centrifuged at 20,000 × g for 30 min at 4 C. The supernatant was not treated with chloroform as described by others (24, 32) because the latter had some effect on the ability of the Sepharose-antibody complex to bind enterotoxin. The pH of the supernatant was adjusted to 7.2, centrifuged if there was any turbidity, and then mixed with 3 ml of wet Sepharose-antibody conjugate in a beaker for 2 h at 4 C (batch method). The suspension was then placed in a column (2 cm in diameter) with a sintered-glass filter, drained, and then washed with 50 ml of buffered saline. The enterotoxin was eluted from the column with 12 ml of glycine buffer. Each fraction of 3 ml was adjusted immediately to pH 7.2 with 1 N NaOH and then was tested for the presence of enterotoxin by gel diffusion and RPHA. More than 75% of enterotoxin added to foods was recovered by the affinity chromatography method as determined by the gel diffusion tube test. Nearly 95% of the toxin recovered was present in the first 9 ml of eluent.

Limit of enterotoxin recovery. Amounts of enterotoxin (pure or in culture supernatant) ranging from 0.1 to 200 μg were added to 500 ml of buffer or 100 g of food. By using the batch absorption method, we were able to recover more than 75% of the pure enterotoxin added to food, even at the 0.1 μg level, and more than 95% when the toxin was added to the buffer. Reverse passive hemagglutination was used to determine the pure toxin at levels below 0.3 μg/ml. This method could detect a minimum of 0.001 μg of enterotoxin B/ml when the pure enterotoxin was dissolved in phosphate-buffered saline. Food extracts without added toxin, but treated with conjugated Sepharose and formalinized erythrocytes, served as negative controls when we determined the minimum amounts of enterotoxin recovered by the Sepharose-antibody complex.

When fresh or reconstituted culture supernatants of enterotoxigenic strains other than type B, non-enterotoxigenic *S. aureus*, *S. epidermidis*, micrococcal species, and salami, cheese, and milk extracts were exposed to conjugated Sepharose, no cross-reacting substances to type B enterotoxin were eluted with glycine buffer, as determined by the microslide test (Table 1).

Naturally occurring hemagglutinins in foods were removed completely from the food extracts.

![Figure 1](http://aem.asm.org/) Recovery of enterotoxin B from 4 ml of brain heart infusion broth culture supernatant by 1 ml of conjugated Sepharose. Column size, 0.6 by 3.5 cm; flow rate, 6 drops/min. Column washed with 20 ml of buffered saline and toxin dissociated with 9 ml of glycine buffer and collected in 3-ml fractions. Quantitation of toxin based on tube single-gel diffusion test.
by the affinity chromatography approach, as determined by RPHA. Yet, affinity chromatography did not eliminate completely hemagglutinins present in culture supernatants of all of the *S. aureus* strains and pure enterotoxin C tested by RPHA. Apparently, these substances reacted with the antibodies to pure enterotoxin B and were recovered when the conjugated Sepharose was treated with the glycine buffer. Absorption of the eluates three times with formalinized sheep erythrocytes did not remove the hemagglutinating principle. Further studies indicated that the hemagglutinins were non-dialyzable and not inactivated by trypsin, but their activity was decreased by 50% after heating at 100°C for 35 min (Table 2). Filtration through Sephadex G-10 to G-100 indicated chromatographic behavior very similar to enterotoxins A, B, and C.

**DISCUSSION**

In the present work, affinity chromatography has been shown to be a powerful tool for the recovery of at least 0.1 μg of enterotoxin B from 100 g of food (0.001 μg/g). It has been suggested that as little as 1 μg of enterotoxin can make a person sick (2). Assuming also that a person might eat 100 g of a toxic food, then a food with 0.01 μg of enterotoxin per g can cause food poisoning. Gilbert and Wieneke (14) reported that they detected 0.01 to 0.075 μg of enterotoxin per g in foods involved in food poisoning in England.

It is obvious from the above that a satisfactory method for the recovery of enterotoxins from food should be able to detect at least 0.01 μg/g of food. The current Food and Drug Administration ion-exchange chromatographic method has been claimed to recover 0.0006 μg/g of foods, as determined by the microslide gel diffusion (1). Our approach recovered at least

### Table 1. Reaction of food extracts and culture supernatants of various cocci purified by affinity chromatography and tested for the presence of enterotoxin B by the microslide and RPHA methods

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Type of enterotoxin produced</th>
<th>Microslide reaction</th>
<th>RPHA reaction</th>
<th>RPHA end point well no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure enterotoxins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B, 1 μg/ml</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>C, 5 μg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>C, 500 μg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em> strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-6</td>
<td>A, B</td>
<td>+</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>243</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>16</td>
</tr>
<tr>
<td>137</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>196-E</td>
<td>A</td>
<td>-</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>472</td>
<td>D</td>
<td>-</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>790</td>
<td>E</td>
<td>-</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>587</td>
<td>Toxin type unknown</td>
<td>-</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>Wood-46</td>
<td>Nontoxicogenic</td>
<td></td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>Other species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>(BP5, 15, 23, 47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. lysodeikticus</em></td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Food-media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain heat infusion broth</td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Milk</td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Cheese</td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Various dry salamis</td>
<td>Nontoxicogenic</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> For purification, see references 4 and 11.

<sup>b</sup> For purification, see text and reference 8.

### Table 2. Heat resistance (at 100°C) of a hemagglutinin<sup>*</sup> produced by *S. aureus* strains and reacting with antibodies to pure enterotoxin B after purification by affinity chromatography

<table>
<thead>
<tr>
<th>Min</th>
<th>RPHA end point well no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>*</sup> Prepared from *S. aureus* strain 137 grown in brain heart infusion broth for 24 h.
0.001 μg/g of food. In two experiments we failed to recover 0.0001 μg/g using the RPHA technique. The great advantage of affinity chromatography lies in the fact that it is very rapid and allows, in one step, purification and concentration of enterotoxins to be detected by one of the available serological methods.

If the molecular weight of IgG is considered to be $1.5 \times 10^7$ and enterotoxin B about $3 \times 10^4$, and assuming that one molecule of IgG can bind at the most two molecules of enterotoxin, then 500 μg of pure and specific antibody in 1 ml of Sepharose should bind about 200 μg of enterotoxin instead of the maximum 30 μg observed.

The antibody inefficiency in recovering enterotoxin is understandable since CNBr couples with free amino groups and the IgG molecules are attached in a random fashion. Antigen-binding (Fab) sites are not all available for binding. The presence of other antibodies unrelated to enterotoxin might also contribute to the inefficiency observed.

We are currently trying to increase the efficiency of coupled antibody to bind enterotoxin by using a C₆ to C₁₀ chain molecule as an arm between the Sepharose and antibody molecules (10). Hemagglutinins exhibiting some of the characteristics observed in this study and interfering with the detection of enterotoxin B have been reported previously as being present in staphylococcal cultures (19).

There are three possible explanations for the observed "nonspecific" hemagglutinations. (i) The IgG used in the study has not only specific enterotoxin B antibodies but also minor antibodies to other products produced by various S. aureus strains. These products may be chemically related to enterotoxin B, or they may be different. However, they behave similarly to enterotoxin chromatographically or electrophoretically. (ii) The enterotoxins contain varying amounts of different, but structurally related or similar, antigenic determinants. (iii) Both of the above factors contribute to the problem.

The heterogeneity of staphylococcal enterotoxins B and C has been demonstrated by resolving the pure toxins by hydroxyl apatite chromatography or isoelectric focusing to toxic and nontoxic components immunologically identical for each toxin (7, 22).

A number of workers (3, 15–17) have demonstrated antigenic and chemical similarities among staphylococcal enterotoxins. In the present study, testing of pure enterotoxin C, purified in 1971 (13) according to Borja and Bergdoll (4) by RPHA suggested that enterotoxin C₁ has some similarity to enterotoxin B. Yet, when the C₁ toxin was chromatographed in a hydroxyl apatite column, as described by Chang et al. (7) for enterotoxin B, the major toxic peak obtained was free of any hemagglutinating property (Table 1).

As far as we know, the immunonochemical similarities among the various components obtained by isoelectric focusing or by hydroxyl apatite chromatography of pure enterotoxins have not been evaluated, though components of each serological type have been considered immunologically identical.

The presence of beta-hemolysin as a persistent impurity in preparations of staphylococcal enterotoxin and nuclease has been demonstrated (8). Such impurity induces production of minor antihemolytic antibodies during the immunization of animals for the production of specific antibodies to enterotoxin. Thus, hemolysin in the enterotoxin or antihemolytic antibodies in the anti-enterotoxin serum may cause problems during the application of the highly sensitive hemagglutination method. The accuracy of this method depends wholly on the monovalency of the antisemum and the purity of antigen used for its production (8).

Although beta-hemolysin is heat labile, some activity may survive 10 to 30 min of boiling (8, 26, 30). Beta-hemolysin is also sensitive to trypsin (30). In our studies, trypsin treatment of the interfering hemagglutinin(s) did not reduce its activity. We have also been unable to observe any hemolytic activity at a level of 1 mg of the pure enterotoxin B per ml that was used for the production of the antisemum in this study. If there is, indeed, activity, it should be below the level detectable by the sheep blood agar method (8). The possible role of protein A to the nonspecific hemagglutinations observed is minimized by the fact that this staphylococcal product is sensitive to trypsin (23).

The present study has demonstrated the applicability of affinity chromatography for the recovery of enterotoxin B from culture media and foods in a rapid and simple way. Minute amounts of staphylococcal products possibly related to enterotoxin B, but still enough to cause cross-reactions in the RPHA test, demonstrated the need for highly purified enterotoxins to be used for the production of specific antibodies.

Work is now in progress for the production of such specific antibodies. Work is also in progress for the attachment of type B and other types of enterotoxin antibodies to the same preparation of Sepharose. This would allow the recovery of all known enterotoxins from foods in one simple step. For the time being, the use of gel diffusion methods offers the best ap-
proach for the specific detection of enterotoxin B recovered from foods by affinity chromatography.

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LITERATURE CITED


