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Determination of Staphylococcal Enterotoxin A in Cheddar Cheese Produced Without Starter Activity

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Three variants of the chloramine-T radioiodination method were used to iodinate staphylococcal enterotoxin A with ^{125}I. Only one method consistently produced usable labels for radioimmunoassay. The iodine incorporation was 55 to 76%; the specific activity was 3.5 to 5.5 μCi/μg of enterotoxin, and the label was extremely stable on storage at −20°C. Determinations of the enterotoxin in extracts of cheddar cheese produced without starter activity were carried out with the radioimmunoassay system and protein A as antibody immunoadsorbent. The assay buffer used in this system significantly influenced the detected levels of enterotoxin in the cheese extracts. Phosphate buffer, but not tris(hydroxymethyl)aminomethane (Tris) buffer, caused gelling of cheese extract proteins, thus resulting in an incomplete separation of free from antibody-bound ^{125}I enterotoxin. When Tris buffer was used, the results indicated a high degree of accuracy and precision for this radioimmunoassay. The lowest detectable enterotoxin concentration in cheese extract was 0.5 ng/ml.

A number of radioimmunoassay methods for the detection of staphylococcal enterotoxins in food have been reported (1, 4, 5, 7, 9-11). However, only the method of Miller, Reiser, and Bergdoll (7) appears to have the reliability, sensitivity, and rapidity required for routine tests of food extracts. The present communication reports on our experience with this method, using different methods of radioiodination of antigen and different assay buffers, to determine the concentration of enterotoxin A in extracts of cheddar cheese produced with induced starter failure.

MATERIALS AND METHODS

Enterotoxin A and antiserum. Highly purified staphylococcal enterotoxin A (SEA), batch 82B, and its antiserum, batch AS-13, were provided by M. S. Bergdoll, University of Wisconsin, Madison.

Radioiodination of enterotoxin. Three variants of the chloramine-T method of iodination of Greenwood et al. (3) were used. (i) This was essentially as described by Miller et al. (7). A 15-μg amount of SEA, dissolved in 15 μl of solution containing 0.3% brain heart infusion broth, 0.9% NaCl, and 1:10,000 thimerosal were added to 2.5 ml of 0.05 M sodium phosphate buffer (pH 7.5) (PB), containing 1 mCi of carrier-free $Na^{125}I$ in NaOH (Radiochemical Centre, Amersham, England). While stirring continuously in an ice bath, we added, dropwise, 0.5 ml of a freshly prepared chloramine-T solution (20 mg/ml) over a period of about 1 min. After 10 min, the reaction was terminated by the dropwise addition of 0.7 ml of a freshly prepared solution of sodium meta-bisulfite (20 mg/ml). After 10 min of stirring, the reaction mixture was transferred to a 1.2- by 25-cm Sephadex G-25 column, which had been previously equilibrated with 0.05 M PB containing 0.25% bovine serum albumin. (ii) A 10-μg amount of SEA dissolved in 10 μl of 0.25 M PB was added to 10 μl (1 mCi) of carrier-free $Na^{125}I$ in NaOH in a 50- by 10-mm glass tube. Chloramine-T (10 μg in 10 μl of 0.05 M PB) was then added, and the reaction tube was shaken gently by hand for 30 s when 4 μg of sodium meta-bisulfite in 10 μl of 0.05 M PB was added. This was followed immediately by the addition of 150 μg of potassium iodide in 150 μl of 0.05 M PB containing 0.25% bovine serum albumin, and the contents of the reaction tube were transferred by a disposable 1-ml syringe to a 1.2- by 25-cm Sephadex G-100 column, which had previously been equilibrated with 0.05 M PB containing 0.25% bovine serum albumin. The reaction tube was rinsed with a further 150 μg of potassium iodide in 150 μl of 0.05 M PB containing 0.25% bovine serum albumin, and this rinse was also transferred to the column. (iii) This was identical procedurally with method (ii), but the masses and volumes of reactants were changed as follows: SEA, 50 μg in 50 μl; ^{125}I, 0.25 mCi in 2.5 μl; chloramine-T, 5 μg in 10 μl; sodium meta-bisulfite, 2 μg in 20 μl.

In all three methods, the reaction mixtures were eluted from the columns with 0.05 M PB containing 0.25% bovine serum albumin. The radioactivity of the eluate was monitored with a Geiger-Müller tube, and the fractions of the plateau region of the ^{125}I SEA peak were collected and stored at −21°C until diluted for testing with 0.05 M tris(hydroxymethyl)aminomethane or 0.15 M PB, each containing 2% bovine serum albumin.

Preparation of immunoadsorbent. *Staphylococcus aureus* strain Cowan-I, obtained from M. S. Bergdoll, was used to prepare staphylococcal cells containing protein A as described previously (7), except that brain heart infusion (BBL Microbiology Systems, Cockeysville, Md.) was used, because the medium

recommended for this purpose (7) was not available in Australia. A 2-liter, cotton-plugged, Erlenmeyer flask containing 600 ml of brain heart infusion was inoculated and maintained at 37°C in a shaker bath (160 reciprocations/min) for 24 h. The cells were centrifuged at 6,895 × *g* for 15 min, washed twice with buffer (0.04 M PB–0.15 M NaCl–0.05% NaN_3 [pH 7.2]), suspended in 4 to 5 volumes of the same buffer but containing 2% formaldehyde, and stirred overnight at 4°C. The cells were centrifuged, washed in buffer without formaldehyde, then suspended in buffer (3× volume of cells). The cells were heated to 80°C for 5 min in a water bath, cooled, centrifuged, washed twice with buffer, and suspended in buffer to a final concentration of 10% (vol/vol).

Radioimmunoassay. Radioimmunoassay was performed exactly as described by Miller et al. (7) with the lyophilized antiserum at a final concentration (≈1:40,000 [wt/vol]), which bound with approximately 50% of ^{125}I SEA. The radioactivities were determined with a Packard Selectronic Autogamma Spectrometer.

Cheese manufacture and preparation of cheese extracts. Cheddar cheese was manufactured with commercial pasteurized milk in a miniaturized plant of 45-liter capacity, which was constructed specifically for the production of experimental cheese. The milk was inoculated, separately, with two enterotoxin A-producing strains of *S. aureus* (ATCC 13565 and NCTC 5655). Starter streptococci were added to the milk, together with an adequate inoculum of its specific bacteriophage to arrest the starter activity. After pressing the curd, the cheese was aseptically subsampled, and the subsamples were maintained at 11°C and 4°C for a period of up to 6 weeks. The cheese subsamples were extracted (7) with 2.0 ml of water per g of cheese.

RESULTS AND DISCUSSION

Iodination procedures. Method (i) failed to produce a consistently usable ^{125}I SEA. Although the first iodination attempt, where an estimated 37% incorporation of iodine was obtained and the preparation had a specific activity of 37 μCi/μg of SEA, was successful, we were unable to iodinate the enterotoxin successfully on the following two occasions. Subsequent iodination attempts of enterotoxin dissolved in 0.25 M PB, without brain heart infusion, also produced poor results. This may well have been due to the very high concentration of chloramine-T used as the oxidizing agent, causing damage to the immunoreactivity of the enterotoxin (1).

The use of method (ii) yielded an iodinated preparation with an estimated 79 to 96% incorporation of iodine and with a specific activity in the range of 79 to 107 μCi/μg of SEA. Such figures are similar to those obtained in many iodinations of pituitary protein hormones carried out in the laboratory of H.M.R., where such preparations, which store well for 2 months at −10°C, are routinely used for hormone assay. The iodinated SEA, however, did not function well in this radioimmunoassay. The standard curve was almost flat, indicating significant loss of immunoreactivity. This was most likely due to the relatively high specific activity of the preparation. Instability and poor performance in assay systems of similar heavily iodinated SEA have previously been reported (1, 6, 8).

Method (iii) consistently produced labels which were usable in the assay systems and were extremely stable, even after storage for 3 months at −21°C. The iodine incorporation by this method was approximately 55 to 76%, and the specific activity of the preparations was in the range 3.3 to 5.5 μCi/μg of SEA. The low specific activity of enterotoxin iodinated by this method appears to be crucial to the stability and performance of ^{125}I SEA. This is in agreement with the results of other workers (1, 6).

Effect of different buffers. At first, our radioimmunoassays for SEA in cheddar cheese extracts as described by Miller et al. (7) produced obvious discrepancies in the amount of enterotoxin present in the cheese. As the age of the cheese, held at 11 or 4°C, increased, SEA levels decreased in some samples. This decrease could not be attributed to proteolytic activities in the cheese because SEA is known to withstand these activities for extended periods (2). Further, reduction of SEA levels was observed, in some samples, after a storage period of only two weeks.

These results were obtained with the use of 0.15 M PB in the assay system and were associated with varying degrees of gelling of the contents in the assay tubes containing the cheese extracts. Such gelling occurred before the addition of the protein A suspension and, in some instances, was so marked that it was possible to completely invert the assay tubes without loss of contents. The variable gelling clearly was a factor in the obvious variation observed in the size of the pellets after centrifugation. These variously sized pellets differed greatly in their moisture content (Table 1), and this would obviously contribute significantly to incomplete separation of free from antibody-bound ^{125}I SEA.

TABLE 1. *Moisture content (g) of pellets after aspiration and drying at 65°C for 22 h*

Pellet size	Moisture content (g)	
	Mean	± Standard deviation
Normal[a]	0.0077	±0.0008
Medium[b]	0.0572	±0.0179
Large[b]	0.1736	±0.0245
Very large[b]	0.3470	±0.2060

[a] Pellets formed in 0.15 M PB.
[b] Pellets formed in cheese extracts.

A reduction in molarity, from 0.15 M to 0.05 M of the PB did lead to a reduction in the degree of gelling, but did not eliminate it. However, gelling no longer occurred when 0.05 M Tris (pH 7.5) was used as the buffer in the assay system. Substitution with the Tris buffer had no effect on the shape or slope of the standard curve (Fig. 1), but in the assay of cheese extracts, no gelling occurred, and the levels of SEA detected were significantly higher ($P < 0.005$) than when PB was used. When 22 samples of cheese extracts, which produced a high degree of gelling with 0.15 M PB, were assayed with phosphate and Tris buffers, the mean values of SEA detected were 1.84 ± 1.61 and 6.08 ± 4.92 ng/ml, respectively.

Statistical parameters. Known quantities of SEA were added to enterotoxin-free cheese extract to yield concentrations in the range 0.2 to 10 ng/ml, and replicate determinations (n = 5) were made, with Tris buffer, at each added level. The recoveries, regression line, and coefficients of variation at the different concentrations for one such assay are illustrated in Fig. 2. The regression line obtained fitted the equation $y = 1.0243x + 0.0677$. The same figure shows that the coefficient of variation for the replicate assays was 10% or less over the range 1.5 to 10 ng of SEA/ml. The lowest SEA concentration which could be estimated with a coefficient of variation of less than 50% was 0.5 ng/ml, a value in close agreement with the results of Miller et

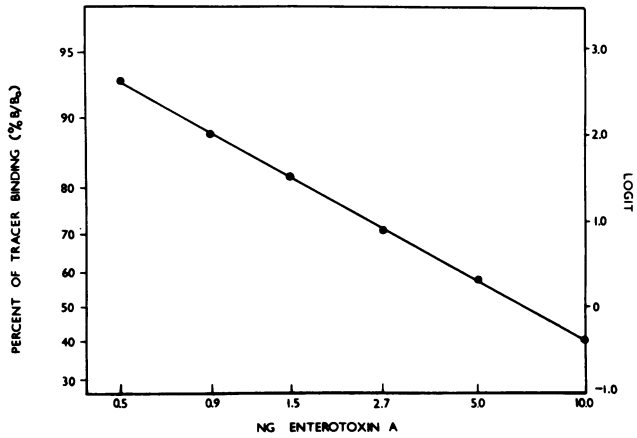

FIG. 1. *Logit-log plot for the standard curve of enterotoxin A.*

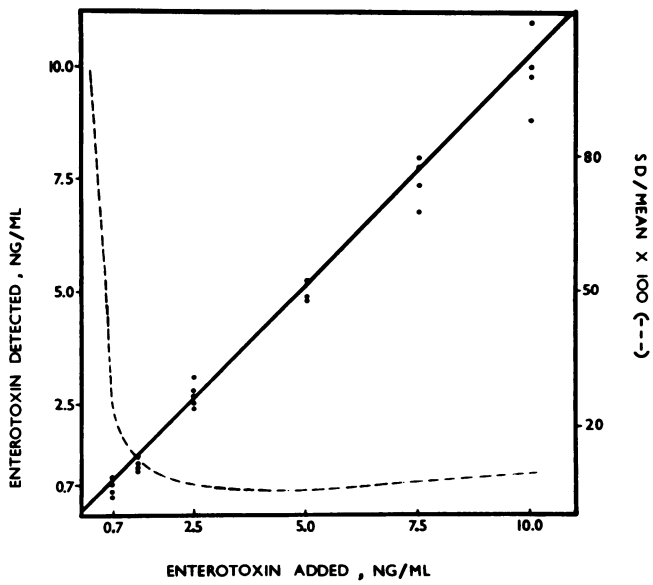

FIG. 2. *Accuracy and precision of enterotoxin A assay as shown by recovery of added enterotoxin to cheddar cheese extract. The coefficient of variation at different concentrations is shown by the dashed line. SD, Standard deviation.*

al. (7).

When six samples of enterotoxin-free cheese extract containing variable amounts of added SEA were assayed on five separate occasions, the enterotoxin concentration was found to be 0.51 ± 0.2, 1.0 ± 0.3, 2.25 ± 0.3, 4.45 ± 0.3, 7.4 ± 0.2, and 10.0 ± 0.1 ng/ml (standard deviation), i.e., the between-assay coefficient of variation was 47, 28, 13, 7, 3, and 1%, respectively, at each of these concentrations.

The results of our study indicate the high degree of accuracy and precision of the method described by Miller et al. However, it is possible that extracts of other foods may produce gelling or protein precipitation in the assay tubes when 0.15 M PB is used in the assay system. Consequently, care must be exercised in the selection of the assay buffer.

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