APPLIED MICROBIOLOGY, Sept. 1973, p. 309-313

Vol. 26, No. 3
Printed in U.S.A.

Staphylococcal Enterotoxins A and B: Solid-Phase Radioimmunoassay in Food

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Received for publication 14 May 1973

An immunoassay employing ^{125}I labeled enterotoxins A and B and polystyrene tubes coated with specific antibodies was used for detection and quantitation of enterotoxin in food. Ham salad, cheddar cheese, custard, condensed milk, and salami were studied. Enterotoxin was successfully determined in all the foods by simple extraction procedures. The assay was sensitive to 1 to 10 ng of toxin per g of food; nonspecific inhibitions were 15% or less.

We recently developed a solid-phase radioimmunoassay test for assaying staphylococcal enterotoxins types A and B in purified form and in culture form (9). The test has also been used to study the antigenic relationships among enterotoxins types A, B, and C (8). The data presented here indicate that the solid-phase radioimmunoassay test is suitable for the detection and quantitation of enterotoxins A and B in several foods. The method is particularly attractive because of high sensitivity and minimal preparation of food extracts for analysis. Current methods for detection of enterotoxins in food either lack sensitivity, require cumbersome extraction and concentration of food extracts, or present problems of nonspecific reactions (1).

MATERIALS AND METHODS

Purified enterotoxins. Purified staphylococcal enterotoxins A and B were supplied by M. S. Bergdoll, University of Wisconsin, Madison. The purified toxins contained less than 5% impurities (1). The toxins were used for the radiolabeling and spiking of foods.

Enterotoxin antisera. Antisera to enterotoxin B were produced in rabbits as previously described (6). Antisera to enterotoxin A were obtained from R. W. Bennett, Food and Drug Administration, Washington, D.C. Pools of anti-A and anti-B contained approximately 1 and 2 mg of antibody protein per ml, respectively, as determined by quantitative precipitation (2).

Iodination of enterotoxins. Enterotoxins A and B were radiolabeled with ^{125}I by the chloramine-T procedure (5, 7), modified as previously described (9). Labeled enterotoxin contained approximately 40 μCi activity per μg of protein.

Preparation of antibody-coated polystyrene tubes. Polystyrene tubes (10 by 75 mm) were sensitized with antibody to enterotoxin as previously described (9). Briefly, 1-ml amounts of sodium sulfate-precipitated antibody in phosphate buffered saline (PBS), pH 7.2 (0.07 M NaCl, 0.07 M phosphate), containing approximately 10 to 20 μg of protein per ml, were added to the polystyrene tubes with a volumetric pipette. After 2 h at room temperature, the antibody was removed and the tubes were washed once with 2.0-ml amounts of 1.0% bovine serum albumin (BSA) in PBS to which sodium azide (0.1% final concentration) had been added as preservative. The tubes were then filled with 1.0% BSA and left overnight at room temperature. The BSA was removed, and the tubes were washed once with PBS and stored inverted at 4 C until used. The preparation of anti-enterotoxin-coated polystyrene tubes is illustrated in Fig. 1.

Solid-phase radioimmunoassay. Portions (1 ml) of extracts from foods spiked with or without enterotoxin were added to the antibody-sensitized tubes. The tubes were shaken 10 times and incubated at 37 C for 18 h. The extracts were then removed, and 1 ml of 1% BSA and 0.001 μg of ^{125}I-enterotoxin A or B (in 0.1 ml of 1% BSA) were added to each tube. The tubes were shaken 10 times, incubated at 37 C for 4 h, washed once with 2 ml of PBS, and counted. The inhibition of binding of ^{125}I-enterotoxin was determined by comparing the radioactivity of tubes containing extracts with that of tubes containing only 1% BSA in the 18-h incubation.

Counting equipment. Radioactivity was measured with a Packard Auto-Gamma Counter (Model 5320). This system has a counting efficiency of approximately 62% for ^{125}I in a 1-ml geometry and a background count rate of approximately 50 counts/min.

Preparation of food extracts. Various amounts of purified enterotoxin were added to 10-g amounts of ham salad contained in Waring Blenders, followed by the addition of 10 ml of 1% BSA in PBS (with sodium azide to 0.1%). The ham salad was blended at high speed for 3 min at room temperature and then centrifuged for 15 min at 20,000 rpm in an International BD-2 centrifuge at 25 C. The supernatant was collected, and the pH was adjusted to 7.4 to 7.5. The

supernatant was recentrifuged at 20,000 rpm for 30 min, removed, passed through a Kimwipe tissue, and stored frozen until used.

Cheddar cheese extracts were prepared as described for ham salad, except that 20 ml rather than 10 ml of 1% BSA was added to 10 g of cheese.

Extracts of a packaged dry custard mix (Jello) were prepared as described for ham salad, except that the mixture was blended for 1.5 min instead of 3 min.

Various amounts of enterotoxin were added to 10-ml samples of condensed milk. The pH was adjusted to 4.5 with 4 N HCl to precipitate the milk proteins. The milk was centrifuged for 30 min at 15,000 rpm at 25 C. The supernatant was removed, and the pH was adjusted to 7.4 to 7.5 with NaOH, followed by recentrifugation at 15,000 rpm for 15 min. The supernatant was collected, passed through a Kimwipe tissue, and stored frozen until used.

Extracts from pork and beef salami were prepared as described for cheddar cheese, except that the extract was dialyzed against PBS at 4 C until the dialysate showed no absorption at 215 nm in a Spectronic 600 spectrophotometer. Aromatic substances have a high absorptivity at this wavelength. The salami extracts were not removed from the antibody-coated tubes after 18 h of incubation, as were the other food extracts.

Determination of enterotoxin in food extracts. ^{125}I labeled enterotoxin (0.001 μg/g of food) was added to the various foods and extracted as described above. The ratio of the radioactivity of 1 ml of supernatant extract to the radioactivity of 1 ml of food homogenate before centrifugation was used to determine the concentration of toxin "recovered."

RESULTS

Ham salad from three sources was studied. For enterotoxin A, recovery varied from 54 to 60% for the different sources of ham salad; for enterotoxin B, it varied from 88 to 97%. Table 1 presents data on the inhibition of binding of ^{125}I labeled enterotoxins A and B to their corresponding antibody-coated tubes by extracts of enterotoxin obtained from one source of ham salad. For the enterotoxin A system, inhibition varied from 23.1 to 55.7%, corresponding to 0.001 to 0.01 μg of enterotoxin A per g of ham salad. Enterotoxin B (0.01 μg/g of ham salad) and ham salad alone gave 7.4 and 14.4% inhibition of the A system, respectively, indicating nonspecific effects. Similar data were obtained for the enterotoxin B system, with 0.001 to 0.01 μg of B per g of ham salad producing 31.3 to 69.3% inhibition of the binding of labeled enterotoxin B. Inhibitions by extracts of ham salad alone and enterotoxin A in ham salad were 16.7 and 20.4%, respectively. If nonspecific inhibition is taken into consideration, the solid-phase radioimmunoassay test for enterotoxin in ham salad has a lower level of sensitivity of 0.001 to 0.0025 μg of enterotoxin per g of ham salad. The data obtained for the two other samples of ham salad are not shown but were similar to that presented in Table 1.

Cheddar cheese from two sources was studied. In contrast to the 1:1 ratio for ham salad, cheese extraction was performed, using one part cheese to two parts diluent (wt/vol) to reduce the viscosity of the cheese homogenate. Recovery rates ranged from 77 to 98% for enterotoxin A and from 111 to 126% for enterotoxin B. Inhibition data on extracts from one of the cheeses are presented in Table 2. Sensitivity of the assay was approximately 0.0025 μg/g of cheese. Nonspecific effects, cheese alone and heterologous enterotoxin in cheese, were responsible for about 10 to 15% of the inhibition. Data similar to those presented in Table 2 were obtained with the other cheese source.

Inhibition data on custard and enterotoxins A and B are presented in Table 3. The results are similar to those obtained with ham salad in terms of the sensitivity of the assay, and nonspecific inhibitions were approximately 4 to 10%. Recovery values of enterotoxins A and B for the single custard sample examined were 97 and 94%, respectively.

Inhibition data on condensed milk are presented in Table 4. Nonspecific and heterologous enterotoxin inhibitions were less than 10%. As little as 0.001 μg of enterotoxin A or B per ml gave good, specific inhibition (21.4% for A and 24.5% for B). The simplified procedure of extraction by acid precipitation of milk proteins resulted in 68.9 and 74.4% recovery of enterotoxins A and B, respectively.

A blind study was conducted for the detection and quantitation of enterotoxins A and B in condensed milk. The unknown samples were prepared by an individual not involved in the assay. Standard curves were constructed, using

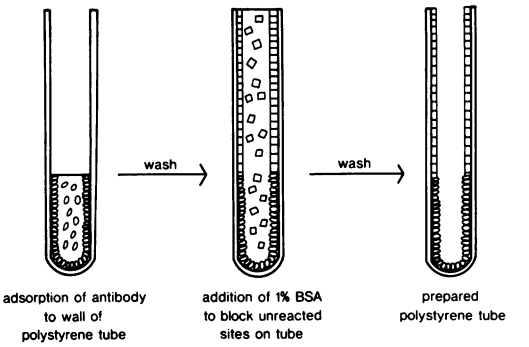

FIG. 1. *Diagrammatic representation of the preparation of enterotoxin antibody-coated polystyrene tubes.*

TABLE 1. *Inhibition of binding of ^{125}I labeled enterotoxin to anti-enterotoxin-coated tubes by extracts of enterotoxin in ham salad*

Enterotoxin system	Inhibitor (extract)	Inhibitor concn (μg added/g of ham salad)	Inhibition (%)	
			Duplicates	Average
^{125}I labeled enterotoxin A and anti-A-coated tubes	Ham salad		13.9, 14.9	14.4
	Enterotoxin A in ham salad	0.001	25.2, 21.1	23.1
		0.0025	34.4, 34.5	34.4
		0.005	44.6, 44.4	44.5
		0.01	56.7, 54.8	55.7
	Enterotoxin B in ham salad	0.01	7.9, 7.0	7.4
^{125}I labeled enterotoxin B and anti-B-coated tubes	Ham salad		21.7, 11.7	16.7
	Enterotoxin B in ham salad	0.001	33.6, 29.1	31.3
		0.0025	43.3, 43.1	43.2
		0.005	60.5, 57.2	58.8
		0.01	72.1, 66.5	69.3
	Enterotoxin A in ham salad	0.01	25.1, 15.8	20.4

TABLE 2. *Inhibition of binding of ^{125}I labeled enterotoxin to anti-enterotoxin-coated tubes by extracts of enterotoxin in cheddar cheese*

Enterotoxin system	Inhibitor (extract)	Inhibitor concn (μg added/g of cheese)	Inhibition (%)	
			Duplicates	Average
^{125}I labeled enterotoxin A and anti-A-coated tubes	Cheese		13.1, 11.5	12.3
	Enterotoxin A in cheese	0.001	11.1, 0.9	6.0
		0.0025	29.0, 32.1	30.5
		0.005	31.6, 34.4	33.0
		0.01	41.9, 41.8	41.9
		0.02	62.7, 67.9	65.3
	Enterotoxin B in cheese	0.02	11.4, 15.7	13.5
^{125}I labeled enterotoxin B and anti-B-coated tubes	Cheese		13.1, 11.5	12.3
	Enterotoxin B in cheese	0.001	30.5, 23.0	26.7
		0.0025	39.6, 41.5	40.5
		0.005	56.3, 57.6	56.9
		0.01	68.1, 70.7	69.4
		0.02	71.5, 70.6	71.1
	Enterotoxin A in cheese	0.02	14.0, 9.1	11.5

the inhibition data of Table 4 in a manner described previously (9). The results (mean values from triplicate determinations) are presented in Table 5. The enterotoxin type was properly identified for each of 11 samples studied. Furthermore, remarkably good agreement was obtained between the values for the amounts of enterotoxins added to the samples and the amounts determined by radioimmunoassay. Only two determinations showed less than 70% recovery, while one showed 140% recovery. Nine samples showed recoveries that ranged from 75 to 116%. The data establish the solid-phase radioimmunoassay procedure as both a qualitative and quantitative test for enterotoxins A and B in food.

The most difficult food encountered thus far in the application of the radioimmunoassay

TABLE 3. *Inhibition of binding of ^{125}I labeled enterotoxin to anti-enterotoxin-coated tubes by extracts of enterotoxin in custard*

Enterotoxin system	Inhibitor (extract)	Inhibitor concn (μg added/g of custard)	Inhibition (%)	
			Duplicates	Average
^{125}I labeled enterotoxin A and anti-A-coated tubes	Custard		10.0, 11.6	10.8
	Enterotoxin A in custard	0.001	18.8, 20.4	19.6
		0.0025	36.8, 35.8	36.3
		0.005	46.2, 44.5	45.3
		0.01	55.8, 58.2	57.0
		0.02	68.5, 72.1	70.3
	Enterotoxin B in custard	0.02	11.8, 9.6	10.7
^{125}I labeled enterotoxin B and anti-B-coated tubes	Custard		6.8, 0.4	3.6
	Enterotoxin B in custard	0.001	36.2, 20.3	28.2
		0.0025	49.1, 38.6	43.8
		0.005	56.0, 49.1	52.5
		0.01	64.9, 67.7	66.3
		0.02	72.4, 73.5	72.9
	Enterotoxin A in custard	0.02	3.8, 10.8	7.3

TABLE 4. *Inhibition of binding of ^{125}I labeled enterotoxin to anti-enterotoxin-coated tubes by extracts of enterotoxin in condensed milk*

Enterotoxin system	Inhibitor (extract)	Inhibitor concn (μg added/ml of milk)	Inhibition (%)	
			Replicates	Average
^{125}I labeled enterotoxin A and anti-A-coated tubes	Milk		6.1, 10.2, 7.5	7.9
	Enterotoxin A in milk	0.001	24.5, 21.4, 22.7	21.4
		0.0025	39.7, 38.3, 36.4	38.1
		0.005	48.3, 49.7, 46.4	48.5
		0.01	59.7, 58.9, 58.1	58.9
		0.05	73.5, 72.8, 74.5	73.6
		0.1	79.0, 78.9, 78.1	78.6
	Enterotoxin B in milk	0.1	8.2, 7.8	8.0
^{125}I labeled enterotoxin B and anti-B-coated tubes	Milk		6.1, 10.2, 7.5	7.9
	Enterotoxin B in milk	0.001	26.8, 23.6, 23.2	24.5
		0.0025	47.7, 49.2, 46.1	47.7
		0.005	60.2, 60.2, 61.1	60.5
		0.01	68.2, 67.7, 66.7	67.5
		0.05	71.3, 72.4, 73.9	72.8
		0.1	74.7, 75.7, 74.2	74.9

procedure to food has been salami. Extracts from salami, prepared as described for cheddar cheese, have resulted in as much as 50% or greater nonspecific inhibition. We have been able to eliminate most of this nonspecific inhibition by dialyzing the salami extracts against changes of PBS at 4 C until no absorbance has been detected in dialysates at 215 nm in a Spectronic 600 spectrophotometer. Preliminary data on inhibitions with enterotoxin A extracts are presented in Table 6. Nonspecific inhibition was approximately 15%. The lower limit of sensitivity appears to be between 0.005 and 0.01 μg of enterotoxin A/g of salami. The extraction procedure recovered about 52% of the enterotoxin added to the salami.

DISCUSSION

The data presented here demonstrated that the solid-phase radioimmunoassay procedure is applicable for the sensitive and specific detection of staphylococcal enterotoxins A and B in food. The sensitivity of the assay is in the range of 1 to 10 ng/g of food. It seems that a sufficient number and variety of foods have been examined here to suggest that the procedure is suitable for general application and is not restricted to a few types of foods. The toxin extraction procedures, for example, were quite simple and required minimal manipulation. This is in marked contrast to current techniques for extraction of enterotoxins from food for determination by immunoassay procedures (1).

Enterotoxin A is bound by food particles more extensively than is enterotoxin B, as indicated by the extraction studies with ^{125}I labeled enterotoxins. This phenomenon could possibly be related to the much higher incidence of food poisoning involving enterotoxin A as opposed to enterotoxin B (1). It has been shown, for example, that enterotoxin is intimately associated with the bacterial cell wall (3, 4). Enterotoxin A receptors in food could possibly react with toxin at the surface of the bacterial cell and thus stimulate the cell to produce more toxin. One would except enterotoxin B to be the most prevalent toxin in staphylococcal enterotoxin food poisoning, since, when compared to enterotoxin A, it is relatively easy to produce in large quantities under laboratory conditions (1). The concept presented above may explain this paradox.

It has been shown previously that staphylococcal enterotoxins are antigenically related (8). It has also been demonstrated that this antigenic relatedness is of no consequence from the practical and applied point of view because of the extremely high homologous-to-heterologous inhibition ratios in solid-phase radioimmunoassay. Thus, inhibitions observed with extracts of food treated with 0.01, 0.02, or 0.1 μg of heterologous enterotoxin per g of food were no different from those of extracts from foods not treated with enterotoxin. Sensitivity values presented for the assay take into account the slight, nonspecific inhibitory effects, which are usually about 15% or less. Preliminary data (unpublished) indicate that the specificity of the radioimmunoassay is maintained with extracts of food treated with crude enterotoxin preparations or with cultures of nontoxigenic *Staphylococcus* organisms.

TABLE 5. *Summary of blind assay of condensed milk for enterotoxin*

Sample no	Enterotoxin added		Enterotoxin found by radioimmunoassay		Recovery of toxin (%)
	Type	μg/ml	Type	μg/ml	
1	A	0.075	A	0.057	76
2	B	0.05	B	0.042	84
3	A	0.05	A	0.054	108
4	A	0.002	A	0.0018	90
5	B	0.01	B	0.0049	49
6	A	0.05	A	0.058	116
	B	0.05	B	0.07	140
7	B	0.075	B	0.062	83
8	A	0.01	A	0.006	60
9	A	0.001	A	0.00094	94
10	B	0.002	B	0.0015	75
11	B	0.001	B	0.00092	92

TABLE 6. *Inhibition of binding of ^{125}I labeled enterotoxin A to anti-A-coated tubes by extracts of enterotoxin in salami*

Inhibitor (extract)	Inhibitor concn (μg added/g of salami)	Inhibition (%)	
		Duplicates	Average
Salami		11.8, 17.6	14.7
Enterotoxin A in salami	0.002	11.7, 13.1	12.6
	0.005	25.9, 15.1	21.5
	0.01	47.2, 38.1	42.6
	0.02	65.8, 63.8	64.8

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