Persistence of Serological and Biological Activities of Staphylococcal Enterotoxin A in Canned Mushrooms

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ABSTRACT

Outbreaks in 1989 of staphylococcal food poisoning linked to the consumption of imported canned mushrooms indicated that staphylococcal enterotoxins (SE) may survive a commercial retort process. To examine this possibility, fresh mushrooms were blanched in boiling water for 5 min and cooled 5 min in sterile water inoculated with enterotoxigenic type A Staphylococcus aureus strain 743, to yield approximately 1.3 × 10⁶ staphylococci per g. Inoculated mushrooms were incubated 20 h at 30°C to simulate time-temperature abuse prior to canning. Mushrooms were sealed in 211 × 212 cans and thermally processed in a still retort to F values of 7, 12, and 18 min at 121 and 127°C. Pre- and post-thermal process staphylococcal enterotoxin A (SEA) serological activity was estimated from a standard curve with purified SEA using a commercial enzyme-linked immunosorbent assay (ELISA) kit. SEA was chromatographically separated from 4-can composite extracts of each F value and temperature. A feline emetic assay was used to determine the biological activity. The dose, administered on a body-weight basis, was equivalent to approximately 0.5 servings of mushrooms and brine for humans. The presence of SEA in the samples was confirmed by Western blotting using anti-SEA immunoglobulin G (IgG). The pre-thermal-process concentration of SEA was about 58 ng/g of mushrooms. Serological and biological activities were detected after all sterilizing values tested at 121 and 127°C. The inactivation of serological activity occurred in two phases, with a rapid initial rate and a distinctly slower rate at higher F values. Attenuation of biological activity, noted by a reduction in the number of emetic episodes and an increase in time to an emetic response, was observed with increasing F values of the processes.

Key words: Heat stability, mushrooms, staphylococcal enterotoxin

Staphylococcal food poisoning is estimated to be the second most frequent bacterial foodborne illness in the US, responsible for up to 20% of foodborne disease (10). It results from the consumption of foods containing enterotoxins produced by staphylococci in foods, usually after contamination by a food handler, followed by subjection of the food to inappropriate conditions. Foods implicated most often in outbreaks include proteinaceous foods that do not necessarily receive a heat treatment prior to consumption such as salads with meat or poultry, and those foods containing custards or cream fillings.

The heat stability of staphylococcal enterotoxins (SE) has been well documented by many researchers (13, 18, 19, 25, 31, 33, 41, 45, 46). Postheating recovery of enterotoxin in these studies was affected by the serological type of SE used, the initial concentration and state of SE (crude or purified), the sensitivity of the assay for SE detection, the heating medium, and the nature of the recovery procedure (serological or biological basis). Z values (the temperature in °C required for the thermal destruction curve to traverse one log cycle) for the SEs are reported to range from 25 to 33°C, D₁₀₀°C values (time in minutes at 121°C for 90% destruction of SE) from 8.3 to 34 min, and F₁₂₀°C values (equivalent time in minutes at 120°C for destruction of SE) up to 30 min. In addition, the biological effects of SE type A (SEA) in humans may be potentiated by application of heat (100°C for 25.4 min) to the SEA prior to consumption (17). In spite of this evidence, the long-standing belief in the food industry has been that commercial retort processes for low-acid canned foods would be adequate to destroy even high concentrations of enterotoxins in foods (19).

Between February and April 1989, there were four reported outbreaks of staphylococcal food poisoning affecting 102 persons in the US associated with consumption of canned mushrooms produced in the People's Republic of China (PRC) (15). In each incident, SE was recovered from the remaining food product and from unopened cans with the same production codes. Thermal processing records indicated that the food products had been adequately processed (15). The evidence suggested that the staphylococcal outbreaks were the result of preprocess formation of enterotoxin in fresh, blanched, or salt-brined mushrooms and subsequent survival of the enterotoxin in the thermal process (24). Several Class II recalls and product seizures of imported canned mushrooms initiated in 1991, 1992, 1994, and 1995 because of product contamination with SEs (5, 6, 7, 22), indicate that the problem is still occurring. Canned mushrooms imported from the PRC are now under automatic detention by the U.S. Food and Drug Administration.
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(FDA) (40) and entry is permitted only on a lot-by-lot basis, once the product is demonstrated to be free from SEs.

This study was undertaken to evaluate the heat stability of low levels of SEA produced in situ in blanched mushrooms subjected to simulated commercial low-acid canned-food retort processes over a range of sterilizing values at two temperatures. The SEA postthermal process serological and biological activities were measured and compared.

MATERIALS AND METHODS

Thermal processes

Thermal processes for whole mushrooms were determined in 211 × 212 cans at 121 and 127°C in a vertical still retort, using the standard heat-penetration test (2) at 113, 116, 121, and 127°C.

Fresh mushrooms, Agaricus bisporus, were obtained from the Pennsylvania State University Mushroom Test Demonstration Facility (MTDF) and were held overnight at 4°C. Only intact whole mushrooms with tightly closed caps were used. The mushroom stipes were trimmed by hand just prior to use. Mushrooms were blanched for 5 min at 100°C in tap water in a steam-jacketed kettle, cooled in tap water for 5 min, and drained 5 min, before filling into 211 × 212 cans (120 g per can) fitted with copper-constantin thermocouples (Ecklund Custom Thermocouples, Cape Coral, FL). A 40-g (ca. 2.59 g) sodium chloride (NaCl) tablet was added to each can and the cans were topped with boiling tap water prior to closure. The cans were sealed under vacuum in a flowing steam at 40 lb/in² in a Canco 423-IES-00 closing machine (American Can Co., Greenwich, CT), and immediately processed in a vertical still retort (Model BRD-962B, Berlin Chapman Co., W. Berlin, WI). Heating data were collected with a Kaye Digistrip III programmable data-logger (Kaye Instruments Inc., Bedford, MA). Four process runs were conducted at each temperature. The heat distribution within the retort was monitored by placement of additional thermocouples in the retort at specified locations during each retort run. The food product heating factors were calculated with the Calsoft II software program (TechniCAL, Inc., Berlin, MI). For inoculum preparation, two frozen beads were subcultured in 10 ml of tryptic soy broth (TSB) (Difco) at 37°C. After 18 h, 0.1 ml of the subculture was transferred to 10 ml of fresh broth for an additional 18 h. One-half milliliter of the second subculture was used to inoculate 100 ml of TSB for incubation at 37°C for 18 h. The third subculture was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was discarded and the cell pellet was resuspended and washed twice in 100 ml 0.1 M potassium phosphate buffer (pH 7). The washed cells were subsequently used to inoculate 38 liters of chilled (4°C) sterile deionized water to yield approximately 3 × 10^7 CFU/ml as determined by total plate count in tryptic soy agar (TSA). The inoculated water was used for subsequently cooling and inoculating blanched mushrooms.

Inoculation of mushrooms

Fresh mushrooms (54.4 kg), Agaricus bisporus, were obtained from a single crop at MTDF and held overnight at 4°C. The mushroom stipes were hand-trimmed by gloved workers. Only intact whole mushrooms with tightly closed caps were used. Trimmed mushrooms were blanched as described previously, cooled for 5 min in the 38 liters of cooling water (4°C) inoculated with S. aureus strain 743, and drained for 5 min. The cooling water temperature was maintained during the inoculation step by holding the cooling water vessel in an ice bath. The drained, inoculated mushrooms were transferred to a sterile stainless-steel container for incubation. Uninoculated control mushrooms from the same MTDF crop were similarly prepared and blanched, but were cooled in sterile uninoculated deionized water, drained, and transferred to a separate sterile polyethylene container for incubation. The uninoculated and inoculated mushrooms were each incubated at 30°C for 20 h to stimulate abusive handling conditions prior to canning.

Enumeration of S. aureus

The staphylococci and background mushroom flora were enumerated after inoculation and after the 20-h incubation. The inoculated and uninoculated control mushrooms were each stirred, and 100 g of each were blended separately in a Waring blender (high speed, 2 min) with 100 g of 0.25 M Tris buffer (pH 8). Serial dilutions of the blends were prepared in 0.1% peptone water and plated in TSA (Difco) for a total plate count and TSA containing 7% NaCl for preferential enumeration of the staphylococci. Plates were incubated at 35°C for 48 h. The remaining blended mushrooms were centrifuged at 10,000 × g for 20 min at 4°C. The supernatants from each were evaluated for the presence of SEA using a serological assay.

Serological assay for SEA

The presence of SEA in mushrooms was determined using the Tegra® Staphylococcal Enterotoxin (SET) Visual Immunoassay (TECRA Diagnostics, Roseville, Australia). This procedure is recommended by the U.S. Food and Drug Administration (12). The manufacturer’s instructions for the assay were followed, with the exception that extracts from thermally processed mushrooms were concentrated by placement in 12,000 to 14,000 molecular weight exclusion tubing (Spectrum®, Houston, TX) with dialysis against 20% polyethylene glycol (12); no urea pretreatment was performed. Absorbance values were read with a Microplate Autoreader (Bio-Tek Instruments, Wooniski, VT) at a dual wavelength of 405 and 490 nm. Levels of SEA present were estimated in a standard curve prepared with purified SEA (Toxin Technology Inc., Sarasota, FL) in a canned mushroom extract. The sensitivity of the assay was determined to be 0.5 ng/ml.

Processing of inoculated mushrooms

After the 20 h incubation period at 30°C, 120 g of uninoculated or inoculated mushrooms were filled into 211 × 212 cans and the cans were chilled for 2 h at 4°C prior to processing. The individual thermal process treatments for F values of 7, 12, and 18 min at 121 and 127°C were assigned in random order. Just prior to processing, 20 filled cans were removed from the cooler, a 40-g NaCl tablet was added to each can, the cans were topped with boiling tap water, sealed as described previously, and immediately retorted for the time required in the scheduled process. For estimation of the preprocess SEA level in the mushrooms (F value of 0), 2 filled cans were removed from each of the 6 retort loads prior to processing and were chilled rapidly in an ice slurry. The contents of each can were blended with an equal weight of Tris buffer, as described previously, centrifuged, and the supernatants

Staphylococcus aureus and inoculum preparation

Enterotoxigenic Staphylococcus aureus strain 743, a type A enterotoxin (SEA) producer implicated in a food-poisoning outbreak, was obtained from R. Bennett of the Division of Microbiological Studies of the FDA. It was presumed that primarily SEA was produced by S. aureus strain 743. The test organism was maintained on sterile glass beads in nutrient broth (Difco Laboratories, Detroit, MI) with 15% glycerol at −70°C. For inoculum preparation, two frozen beads were subcultured in 10 ml of tryptic soy broth (TSB) (Difco) at 37°C. After 18 h, 0.1 ml of the subculture was transferred to 10 ml of fresh broth for an additional 18 h. One-half milliliter of the second subculture was used to inoculate 100 ml of TSB for incubation at 37°C for 18 h. The third subculture was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was discarded and the cell pellet was resuspended and washed twice in 100 ml 0.1 M potassium phosphate buffer (pH 7). The washed cells were subsequently used to inoculate 38 liters of chilled (4°C) sterile deionized water to yield approximately 3 × 10^7 CFU/ml as determined by total plate count in tryptic soy agar (TSA). The inoculated water was used for subsequently cooling and inoculating blanched mushrooms.
 tested with the Tecra@) SET assay. The individual SEA levels determined from these unprocessed cans were used to calculate an average initial SEA level for all of the heat treatments. Unincubated control mushrooms were processed only at an F value of 7 min at each temperature.

The postthermal process SEA recovered for each F value and temperature was estimated by evaluation of the can contents of 4 individual cans per sterilizing value, using the Tecra@) SET assay as described previously. The average SEA recovered was calculated for each F value.

Additional experiments were performed using mushrooms from other MTDF crops following the inoculation and incubation procedures described previously. Mushrooms were thermally processed over a range of F values from 4.5 to 60 min at 121°C and 2 to 45 min at 127°C. Postprocess SEA was determined with the Tecra@) SET assay.

Separation of SEA from canned mushrooms for biological assay

SEA was separated by ion-exchange chromatography from 4-can composite extracts prepared from each of the six thermal process treatments using the method of Bennett (12), with the following modifications: all proportions of the materials needed in the procedure for extraction were increased fourfold to accommodate the 4-can composite samples; and the chloroform extraction steps were omitted from the procedure. As a result of sample limitation, uninoculated control extracts were prepared from 2-can composites and extraction materials were similarly increased twofold. After lyophilization of the samples, they were stored at −20°C in a desiccator until they were evaluated for SEA activity or analyzed by polyacrylamide gel electrophoresis and Western blotting procedures.

Evaluation of the biological activity of thermally processed SEA

The biological activities of the composite extracts were evaluated using a feline emetic assay (23) with female domestic cats, 25 to 28 weeks of age and weighing approximately 2.3 to 3.3 kg (Harlan Sprague-Dawley, Indianapolis, IN). The lyophilized composite mushroom extracts were rehydrated in 20 ml of sterile 0.9% saline solution (Phoenix Pharmaceutical, Inc., St. Joseph, MO) and filter-sterilized through 0.22-μm pore size low-protein-binding disposable filters (Millipore Products Div., Bedford, MA). Uninoculated control extracts were rehydrated in 10 ml of saline and filter-sterilized. The volume of sample given per animal was calculated on a body-weight basis to deliver 1,000 ng of pre thermal process SEA per kg of body weight and was equivalent to one-half or less of a serving size of canned mushrooms and brine for humans (65 g). Animals receiving control composite samples were given a comparable volume on the weight basis. The samples were administered intravenously by a Penn State University veterinarian with a sterile 25-gauge 0.75-in. (ca. 1.9-cm) single-use winged infusion set (Terumo Medical Corp., Elkton, MD) inserted in the right or left cephalic vein. The tubing in the infusion sets were flushed with 1 ml of sterile 0.9% saline prior to removal, to ensure that the entire volume of each sample was delivered. Each sample was given to 4 animals and the animals were continuously observed over a 4-h period for emesis and other symptoms. An emetic response by an animal during the 4 h was considered a positive reaction. The number of emetic episodes and time period between sample administration and emesis were noted. The animals were allowed to recover for 7 days between tests and each was challenged with SEA-containing samples on only two occasions. A separate portion of each sample was evaluated for SEA serological activity with the Tecra@) SET assay immediately after the injection took place. In addition, biological assay samples were screened for the presence of residual staphylococcal hemolysins. Hemolytic activity was determined by the addition of 0.15 ml of each sample divided between three 4-mm wells cut in 5% sheep blood agar plates (Remel, Lenexa, KS). Plates were incubated up to 5 days at 30 to 35°C. Hemolysis was indicated by the clearing of the blood agar around the perimeter of the wells. A filter-sterilized broth culture extract from S. aureus strain 743 was used as a positive control for hemolytic activity.

**Polyacrylamide gel electrophoresis (PAGE) and Western blotting**

Native discontinuous PAGE and Western blotting techniques were used to confirm the presence of SEA in the composite chromatographically separated sample extracts. A Mini PROTEAN II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA) and programmable 2,000-V power source (Series 700, Fisher Scientific, Pittsburgh, PA) were used in both the PAGE and protein blotting procedures. Precast 15% acrylamide, 0.375 M Tris-hydrochloride (HCl) gels (pH 8.8; Bio-Rad), premixed 25 mM Tris-192 mM Tris-HCl (pH 6.8; 12.5%), glycerol (10%), and 0.5% bromophenol blue (5%) in deionized water. Twenty- to 30-μl sample volumes were loaded onto the gels. Duplicate gels were electrophoresed at a constant voltage of 200 V at room temperature. At the completion of the runs, one gel was silver stained (34) and the second gel was used for electrophoretic transfer to a 0.2-μm nitrocellulose membrane (Bio-Rad at 4°C and at a constant voltage of 30 V overnight in the same premixed buffer system. A 1:500 dilution of rabbit-generated anti-SEA IgG (immunoglobulin G) (Toxin Technology Inc.) and an amplified alkaline phosphatase goat anti-rabbit immuno-blot® assay (Bio-Rad) were used for SEA detection on the nitrocellulose. The Western blot was scanned using an Imaging Densitometer (Model GS-670, Bio-Rad) at a resolution of 400 μm with a red filter to quantitate the observed differences in band intensities for each of the composite extracts. Relative band intensities were calculated by subtracting a global background using the Molecular Analyst®/PC image analysis software (version 1.1.1, Bio-Rad).

**RESULTS**

**Thermal processes**

Whole mushrooms in brine, in 211 × 212 cans in the still retort, exhibited characteristics of a simple heating curve at each temperature. To achieve F values of 7, 12, and 18 min at 121°C, the total process times calculated were 16.5, 22, and 28 min, respectively. Similarly, for equivalent F values at 127°C, the total process times calculated were 11, 13, and 15 min, respectively. The heat distribution within the retort was found to be uniform and no “cold” spots in the retort were detected.

**Enumeration of S. aureus and background flora**

As determined in preliminary experiments, an inoculum of approximately 3 × 10⁵ CFU/ml S. aureus strain 743 in the cooling water was needed for consistent inoculation of blanched mushrooms, and subsequent growth of the test strain in the presence of the competitive background flora. The total plate count and staphylococcal count for the cooling water were close to this target inoculum (Table 1).
TABLE 1. Enumeration of S. aureus strain 743 and background flora in cooling water and blanched mushrooms

<table>
<thead>
<tr>
<th>Cooling water or mushrooms</th>
<th>Aerobic plate count (CFU/ml or g)</th>
<th>TSA + 7% NaCl&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SEA serological activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooling water</td>
<td>3.6 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.1 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Postblanch inoculated mushrooms</td>
<td>1.4 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.3 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Negative</td>
</tr>
<tr>
<td>30°C-20 h abused inoculated mushrooms</td>
<td>2.1 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2.3 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Positive</td>
</tr>
<tr>
<td>Postblanch uninoculated mushrooms</td>
<td>est. 7</td>
<td>est. 7</td>
<td>Negative</td>
</tr>
<tr>
<td>30°C-20 h abused uninoculated mushrooms</td>
<td>1.6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.4 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Negative</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tryptic soy agar.
<sup>b</sup> Tryptic soy agar with 7% sodium chloride.
<sup>c</sup> SEA serological activity determined with Tecra™ SET assay.
<sup>d</sup> Assay absorbance value >0.200 is considered a positive result.

All of the colonies observed and counted on the two media from the cooling water were typical S. aureus strain 743. This number of S. aureus in the cooling water permitted the inoculation of blanched mushrooms with approximately 1.3 × 10<sup>3</sup> staphylococci per g. The colonies observed on TSA and TSA with 7% NaCl from inoculated mushrooms appeared to be only S. aureus, with an occasional colony characteristic of an aerobic sporeformer. There was no detectable SEA serological activity in the mushrooms after blanching. After the 20-h incubation, the number of staphylococci in the inoculated mushrooms increased by approximately 6.2 log units, to 2.3 × 10<sup>9</sup> CFU/g (Table 1), and SEA serological activity was detected.

The native flora of the uninoculated blanched mushrooms were primarily aerobic sporeformers that survived the blanch treatment, estimated at 7 CFU/g after the blanch (Table 1). Their numbers increased approximately 7.4 log units to 1.6 × 10<sup>8</sup> CFU/g of mushrooms during the 20-h incubation. Growth of the native flora on the TSA with 7% NaCl was inhibited, as shown by reduced colony size and number. Typical S. aureus colonies were not found in either enumeration medium with the uninoculated mushrooms and there was no evidence of SEA, by serological assay post-blanching or after incubation.

Recovery of SEA serological activity after thermal processing

The average initial SEA concentration in inoculated mushrooms prior to thermal processing (F value of 0) was 57.8 ng/g of mushrooms. The thermal destruction of SEA serological activity in canned mushrooms occurred in two phases at 121° and 127°C, with a very sharp decrease in recoverable activity at the lowest F value of 7 min at each temperature, followed by a slower rate of inactivation as sterilizing values increased (Fig. 1).

At 121°C the decreases in serological activity with thermal processing were 2.2, 2.5, and 2.8 log units, or 99.4, 99.7, and 99.8% destruction of activity, as the F value increased from 7, 12, to 18 min, respectively (Fig. 1). Likewise, the losses of serological activity at equivalent sterilizing values at 127°C were 2.3, 2.7, and 2.6 log units, or 99.5, 99.8, and 99.8% destruction of activity, respectively (Fig. 1). These data indicate the degree of SEA thermal destruction was similar at both temperatures, but serological activity was still evident even after severe thermal processes equivalent to F values of 18 min at either temperature.

Evaluation of thermally processed SEA biological activity

In preliminary experiments, it was determined that the feline emetic dose for unheated, purified SEA suspended in 0.9% saline was between 100 and 300 ng per animal. Administration of saline alone did not elicit any response or symptoms. The biological activities as determined by a feline emetic assay of the composite extracts prepared from uninoculated control mushrooms and inoculated mushrooms, processed at each of the F values at 121° and 127°C, are shown in Table 2. There was no biological response or evidence of other typical staphylococcal enterotoxin-induced symptoms observed in the 8 animals tested (4 animals per sample) with the two extracts from uninoculated mushrooms processed to F values of 7 min. The extracts from uninoculated mushrooms were also negative for SEA serological activity at the time of sample injection and no
TABLE 2. Biological activity of SEA in canned mushrooms thermally processed at 121°C and at 127°C

<table>
<thead>
<tr>
<th>Thermal process temp.</th>
<th>Total process time (min)a</th>
<th>F-value delivered</th>
<th>Emetic responseb (no. positive/no. tested)</th>
<th>Total no. emetic episodes/F value</th>
<th>No. emetic episodes/animal</th>
<th>Time to 1st emetic response (avg. time)</th>
<th>Serological activityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>121°C</td>
<td>16.5</td>
<td>7</td>
<td>0/4</td>
<td>None</td>
<td>None</td>
<td>Not applicable</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>7</td>
<td>4/4</td>
<td>25</td>
<td>1-11</td>
<td>0.3-3.3 h (1.3 h)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>12</td>
<td>3/4</td>
<td>14</td>
<td>4-6</td>
<td>0.9-2.5 h (1.7 h)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>18</td>
<td>1/4</td>
<td>2</td>
<td>2</td>
<td>3.2 h</td>
<td>Positive</td>
</tr>
<tr>
<td>127°C</td>
<td>11</td>
<td>7</td>
<td>0/4</td>
<td>None</td>
<td>None</td>
<td>Not applicable</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7</td>
<td>4/4</td>
<td>28</td>
<td>1-10</td>
<td>0.3-2.4 h (1.0 h)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>12</td>
<td>3/4</td>
<td>4</td>
<td>1-2</td>
<td>0.9-2.9 h (2.1 h)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>18</td>
<td>2/4</td>
<td>9</td>
<td>4-5</td>
<td>1.1-2.7 h (1.9 h)</td>
<td>Positive</td>
</tr>
</tbody>
</table>

a Total thermal process time in minutes to achieve equivalent F value at specified temperature.
b Feline emetic assay by intravenous injection of approx. 1000 ng prethermal process SEA/kg of body weight.
c Serological activity determined immediately after sample injection with Tecra™ SET. Absorbance >0.200 is considered positive.
d Uninoculated mushrooms.

Hemolytic activity was detected. Biological activity, as determined by the emetic response within the 4-h test period, was however detected in the composite extracts from inoculated mushrooms processed to sterilizing values of 7, 12, and 18 min at 121°C and at 127°C. Immediately prior to emesis, symptoms of increased salivation and swallowing were noted. Other symptoms typical of staphylococcal intoxication such as diarrhea were not observed.

Positive responses by 4 of 4 animals tested were each observed for extracts prepared from mushrooms processed to equivalent F values of 7 min at 121 and at 127°C. The animals given the samples processed to an F value of 7 experienced the highest total number of emetic episodes, with 25 for the 121°C process extract, and 28 for the 4 animals given the 127°C process sample (Table 2). Likewise, the number of emetic episodes observed per animal was highest for those given the extracts processed to an F value of 7, and they showed the shortest incubation period between the time of sample administration and time when the first emetic response was observed. A thermal process equivalent to an F value of 7 min is the recommended minimum safe thermal process for whole mushrooms in 211 × 212 cans (36).

Extracts prepared from inoculated mushrooms processed to equivalent F values of 12 min at 121 and at 127°C were also biologically active, with 3 of the 4 animals tested with each extract experiencing emesis within the test period. The total number of emetic episodes experienced by animals given the 121°C extract was approximately 3-fold higher than those given the extract from mushrooms processed at 127°C. In addition, the number of emetic episodes observed per animal was three to four times higher with those given the extract from the 127°C process, which may reflect differences in sensitivity to SEA between individual animals. The time period between sample administration and the appearance of first emetic response was similar for the two extracts. Both extracts were also positive by serological assay at the time of injection (Table 2).

SEA biological activity and serological activity were still evident in extracts prepared from inoculated mushrooms subjected to a severe thermal process equivalent to an F value of 18 min at 121 and at 127°C. The mushrooms processed at 127°C, with 2 of the 4 animals having a positive response, showed a greater total number of emetic episodes (9), and increased number of episodes per animal (4 to 5).

No staphylococcal hemolytic activity was detected in any of these samples used for biological assay.

**Polyacrylamide gel electrophoresis (PAGE) and Western blotting**

The presence of SEA in the composite, chromatographically separated samples used for biological assay (Table 2) was confirmed with PAGE and Western blotting techniques. The calculated relative band intensities for the samples, derived from the scanned Western blot, are shown in Fig. 2. It is apparent by the decreasing band image calculated that the serological activity of the SEA was reduced as the F values increased at 121°C (Fig. 2A). Similarly, at 127°C the extract from mushrooms processed to F value 7 had the highest band intensity in the scan (Fig. 2B). However, the extract from the F value 18 process at 127°C had a larger calculated image intensity than the F value 12 extract, suggesting more survival or serological activity after the higher thermal process at this temperature (Fig. 2B). These results are in agreement with those obtained with the Tecra™ SET assay (Fig. 1), as well as with the biological assay (Table 2).

**DISCUSSION**

Low populations of *Staphylococcus aureus* strain 743 grew sufficiently in blanched mushrooms with the competi-
production in the PRC, there is considerable human contact with the mushrooms during harvest and postharvest prior to thermal processing, which provides ample opportunities for contamination of mushrooms with staphylococci (11, 24).

The thermal destruction of SEA in mushrooms occurred in two phases at 121°C and 127°C. The ELISA procedure used in this study was capable of detecting low levels of residual SEA serological activity surviving even the severe thermal processes given equivalent to F values of 18 min. Detection of SEA serological activity after thermal processing to F values of 7, 12, or 18 in a food system may not be unexpected, since thermal destruction endpoints of 19 to 30 min have been reported for higher concentrations of SEA in buffer or beef bouillon at 121°C (18, 27, 46). On the basis of the high z values determined for SEA, it might be anticipated that its persistence would be greater after heating to equivalent sterilizing values at 127°C than at 121°C, since total process times were 33 to 46% less at 127°C (Table 2). A difference in destruction of serological activity between the two temperatures, however, was not detected.

Biphasic thermal destruction patterns over a temperature range from 80°C to 120°C have also been observed with crude SEA, SEB, and SEC in saline or culture broth by other researchers (28, 42, 43, 46). The biphasic nature of the thermal destruction of SEA serological activity is more evident in Figure 3, which summarizes the results of a series of additional experiments conducted in our laboratory with SEA produced in situ in mushrooms, thermally processed in 211 × 212 cans over a wide range of sterilizing values at 121 and 127°C, respectively (3). Endpoints for destruction of SEA serological activity were not reached at either temperature, even with F values as severe as 60 min at 121°C or 45 min at 127°C, confirming the extreme thermal stability of low concentrations of SEA in a food system. Two-phase thermal destruction of other bacterial proteins at 55 to 160°C has also been widely observed with heat-resistant extracellular proteases and lipases in milk and in various buffers (4, 9, 20, 39). These enzymes, although not generally stabilized by disulfide bonds like SEs, typically contain divalent cations which contribute to stability and heat resistance (4, 38). They are slightly larger proteins than SEs with molecular
masses of 37,000 to 50,000 D, but exhibit similar thermal stability to SEs with reported $z$ values of 32 to 39°C (1, 4, 44). Each of the phases of enzyme inactivation also follows first-order reaction kinetics. Andersson et al. (4) concluded that the two-stage inactivation of *Pseudomonas fluorescens* lipase in milk may be the result of complex formation between the enzyme and food components. Driessen and Stadhouders (20), however, suggested that at higher temperatures, the native conformation of lipase is modified to a temperature-stable form, which is still enzymatically active.

Either or both of these hypotheses may partially account for the extreme thermal stability of SEA in mushrooms observed in this study. First, it has been widely reported that crude preparations of SE are more resistant to heat than purified SE (41, 43, 45), as with SEA produced in situ in blanched mushrooms. The presence of food components in the heating menstrua has been shown to increase the thermal stability of SEs approximately two- to fivefold (18, 31). It was suggested that the food components provide some unknown protective effect against SE heat inactivation, possibly through protein-protein interactions. There is evidence that chitin, the primary structural component of mushroom tissue (32) binds proteins in stable but disso-ciable complexes, between N-acetylglucosamine and $\alpha$-amino acids, particularly tyrosine (35). SEA contains approximately 18 tyrosine residues (26). It is readily acknowledged that proteins can be stabilized to heat through immobilization or attachment to supports by increasing the rigidity of the protein (29). Formation of a complex between the chitin in mushrooms and SEA may occur to stabilize the toxin during thermal processing.

Secondly, thermal denaturation or unfolding of proteins is a first-order sequential process that represents a shifting probability distribution of molecules from the native conformation to the denatured state (30). The formation of intermediates, particularly stable forms during this process, resulting in biphasic protein denaturation, may not be unexpected (16, 30). The relative biological activity of a stable intermediate form would be dependent on how closely the conformation resembles the native protein.

In contrast, Denny and coworkers (18), Hilker et al. (25), and Humber and others (27) all observed single-phase, first-order inactivation of approximately 100 to 1,000-fold higher concentrations of crude SEA in beef bouillon, with a $z$ values of 27.8 to 30.6°C, using the Oudin gel diffusion assay to determine surviving serological activity after heating at 98 to 126.7°C. The sensitivity of this gel assay is about 1 $\mu$g/ml, whereas the ELISA technique used in this study is about 2,000-fold more sensitive. The single inactivation phase reported by these researchers is probably representa-tive of our first, more rapid inactivation phase, since the Oudin gel assay is not capable of detecting low levels of persisting SEA activity (less than 1 $\mu$g) in the second, slower destruction phase observed here.

SEA heated in canned mushrooms was still biologically active and induced emetic responses after all of the thermal process treatments tested. The processes for F values of 12 and 18 are approximately 1.7-fold and 2.5-fold higher than the recommended minimum safe thermal process for whole mushrooms in 211 × 212 cans (36), respectively. They are also more severe than most adequate thermal processes given to brine-packed, convection-heating foods. In prelimi-nary experiments, SEA containing mushrooms processed to an $F_{127C}$ of 25 min also exhibited biological activity in cats (unpublished data). The ratio of positive responses to the number tested and the number of emetic episodes observed decreased, and the incubation period between sample administration and emetic response increased, as thermal process severity increased. These trends suggest that biological activity was attenuated as F value increased. It may be possible then to inactivate SEA or decrease the biological activity below biologically effective levels at sterilizing values higher than 25 min. The quality and palatability of foods processed to such high F values, however, would be notably compromised. The attenuation effect is not as obvious at $F_{127C}$ values of 12 and 18 min, where the number of emetic episodes was higher and average incubation period was slightly shorter, at an F value of 18 than at 12. At 127°C, the total process time difference for F values of 12 and 18 is only 2 min (Table 2). It may be that the additional 2 min at 127°C to achieve an F value of 18 does not contribute dramatically to additional SEA destruction. This also sug-gests that at 127°C, higher equivalent sterilizing values may be less effective in destroying SEA biological activity because of the shorter process time required to achieve equivalent lethality.

The thermal stability of SE biological activity has been shown by others. Read and Bradshaw (41) found the endpoint for destruction of biological activity of purified SEB in buffer at 115.6°C was 32.5 min in a cat intravenous test. This was also the endpoint for SEB serological activity. Low concentrations of crude SEA andSED were still biologically active via intravenous injection in kittens after thermal processing to $F_{127C}$ values of 3 and 8 min in cream of celery soup and in infant formula (13). The same samples, however, were found to be negative for SE serological activity. Using intraperitoneal injection of cats, Denny and coworkers (19) found the endpoint for thermal destruction of a higher concentration of crude SEA in culture medium was 11 min at 121°C. The same material had a destruction endpoint of 8 min when tested intragastrically in monkeys. The difference in endpoints observed may be due to differences in animal species sensitivity, since monkeys are less sensitive to SE effects than cats or kittens (14). A higher endpoint value of 15 min at 121°C, using monkey feeding, was reported by Humber et al. (27) with crude SEA heated in beef bouillon. Biological activity was inactivated before serological activity in that study.

The results of this study show that, contrary to the long-standing belief in the food industry, low concentrations of SEA in a food system may not be serologically or biologically inactivated by the adequate or even severe thermal processes given to most canned foods. The staphy-lococcal food poisoning outbreaks from PRC canned mush-rooms support our findings. The only practical way to eliminate future staphylococcal outbreaks in thermally processed foods is to prevent the contamination and proliferation of enterotoxigenic staphylococci in foods before processing.
through improved worker hygiene, proper food handling, and compliance with good manufacturing practices.

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