CHAPTER 21

BEHAVIOR AND SEROLOGICAL IDENTIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN IN THERMALLY PROCESSED MUSHROOMS

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1. INTRODUCTION

Enterotoxin produced by some strains of *Staphylococcus* spp. is a relatively common cause of bacterial foodborne illness. This preformed microbial toxin is produced in a variety of foods over a wide range of conditions. Bacterial populations proliferate when food is time-temperature abused and the contaminating organism, if enterotoxigenic, subsequently produces the enterotoxin. When this abused food is consumed, foodborne illness may occur. Although most outbreaks are due to the contaminated food that has not received severe heat treatment, evidence shows that foods receiving medium to high heat treatment also may cause staphylococcal intoxication. Examples of such foods include boiled goat's milk (Drysdale, 1950), spray dried milk (Anderson & Stone, 1955; Armijo *et al.*, 1957), cooked sausage (Tatini, 1976), canned lobster bisque (CDC, 1976) and processed infant formula (Bennett, 1982). In 1989, canned mushrooms were purported to be responsible for a number of outbreaks in the United States (CDC, 1989), and staphylococcal toxin was found in product that was untreated or exposed to 6.0-8.0 M urea before serological testing (Bennett, 1991, 1992).

Since the mid-1960's, a number of studies on the thermal stability and behavior of the staphylococcal enterotoxins have been conducted in actual food substrates or in extracts of foods (Read *et al.*, 1965; Denny *et al.*, 1971; Humber *et al.*, 1975; Soo *et al.*, 1973; Tatini, 1976; Lee *et al.*, 1977; Bennett & Berry, 1987). Although it was generally concluded that the heat process normally used in canning destroys any enterotoxin in a food substrate (Denny *et al.*, 1966; Denny *et al.*, 1971; Humber *et al.*, 1974; Bergdoll, 1989), more recent serological evidence with canned mushrooms (Bennett, 1991, 1992) indicates otherwise. Previous studies showed a retention of biological toxin activity in kittens fed heated, serologically inactive enterotoxins A and D in selected food substrates (Bennett & Berry, 1987). In addition, it was suggested that the clinical symptoms of enterotoxemia

were enhanced in humans who ingested enterotoxin type A in milk heated at 100°C for 25.4 min. (Dangerfield, 1973). Monkeys fed heated toxin of serologically measured 5 ng activity appeared to be more severely ill than those fed the corresponding unheated toxin (Tatini, 1976). The serological renaturation of enterotoxin A has been successfully demonstrated in pasteurized milk (Tatini, 1976) and canned mushrooms (Bennett, 1991, 1992) when these foods were treated with 6.0-8.0 M urea.

Similarly, serological reactivation of staphylococcal enterotoxin by high pH treatment has been reported for a variety of foods and other menstrua heated at 80-100°C for 10 min (Schwabe *et al.*, 1990). This paper addresses the thermal inactivation kinetics of enterotoxin A, the denaturation and renaturation behavior of enterotoxins after treatment with urea and the toxin's attendant degradation, or spontaneous changes in toxin serology after thermal and chemical treatment.

2. RECOVERY AND SEROLOGICAL IDENTIFICATION OF ENTEROTOXIN IN CANNED MUSHROOMS

Naturally contaminated mushrooms that purportedly received normal thermal time-temperature processing were used to study the serological fate of enterotoxin and its response to chemical renaturation (serological reactivation).

To simulate the canning process, fresh mushrooms were sliced into "pieces and stems," and staphylococcal enterotoxin type A (SEA) was added at levels of 5, 10, and 20 ng toxin/g of product. The product (4 oz drained wt) was then placed in appropriate cans, sealed, and retorted at $F_0 = 7.5$ min, monitored with thermocouples.

After retorting of the product to which toxin had been added, the cans were chilled in cold water and given an initial toxin assay approximately 4 days after the thermal processing event. Other studies used various temperature/time conditions for mushrooms to which SEA was added in various concentrations before heating at 121.1°C for 15-30 min.

General steps in identifying toxin in thermally processed mushrooms are (1) extraction of toxin from food components; (2) urea treatment of extract to renature toxin that may have been preformed (i.e., formed before thermal processing); and (3) serological identification of enterotoxin by an enzyme-linked immunosorbent assay (ELISA) or other acceptable serological tool to determine whether suspect toxin was in its native or heat-altered form.

2.1. Extraction of Thermally Processed Mushrooms

A 200-ml volume of 0.25 M Tris, pH 8.0, was added to each 200 g of product and homogenized at high speed for 3 min in a Waring blender. The mushroom slurries were transferred to centrifuge bottles (stainless steel or equivalent), and then centrifuged at 27,300 x g for 20 min at 5°C. The resulting extracts were decanted into suitable containers. A 100-ml amount of untreated extract was removed and dialyzed in polyethylene glycol (PEG) as described under "Urea Treatment," below. From each extract, 100 ml was reserved for urea treatment. After dialysis of the 100 ml amounts of each untreated extract, the dialyzed volume was kept at 5.0 ml and clarified by centrifugation. Each dialyzed extract was filtered through a cotton-plugged disposable syringe (the cotton-plugged syringe was prepared by inserting a piece of cotton about 0.5 cm thick into a syringe; it was wetted by passing distilled water through the syringe to ensure tight packing of the cotton). For filtration, the plunger was removed and the extract was placed in the syringe; the plunger was then reinserted, and the extract was carefully pumped through the cotton plug into a plastic vial (preferably

polypropylene or equivalent).

The pH of the 5.0-ml cotton-filtered extracts was adjusted, when necessary, to between pH 7.0 and 8.0 with NaOH or HCl. Then, 50 µl of "sample additive" contained in the ELISA kit was added and the preparation was thoroughly mixed. A 200-µl volume of each extract was tested, as required by the kit's instructions.

2.2. Urea Treatment to Renature Thermally Denatured Toxin

The extracts (100 ml) reserved for urea treatment were placed in 250-ml beakers to which 36.0 g urea (M.W. 60.06) was added to achieve a 6.0 M solution. Where appropriate, other concentrations (5.0, 7.0, and 8 M) of urea were used for treatment of the heat-altered toxins.

The mixtures were stirred on a magnetic stirrer for 4-5 h at 25°C. After urea treatment, the mixtures were placed in dialysis tubing (cellulose casing of 2-3 cm flat width with 12,000 to 14,000 M.W. exclusion). Before the mixture was placed in the dialysis tubing, the tubing was checked for leaks by filling it with distilled water. Dialysis tubing containing the urea-treated extracts was placed in polyethylene glycol flakes (PEG, 20,000 M.W.) and allowed to remain until a volume of approximately 5 ml or less was reached; this is normally an overnight process. After dialysis, the tubing's exterior was washed thoroughly with tap water to remove residual PEG. The tubing was then soaked in 0.25 M Tris, pH 8.0, but only for as long as its total volume did not exceed 5 ml of the urea-treated extract. Extracts were removed from the dialysis tubing as previously described, clarified by centrifugation, and filtered through absorbent cotton. When necessary, the pH of the 5.0 ml extracts was adjusted to between 7.0 and 8.0 with NaOH or HCl; then 50 µl of "sample additive" was mixed with each extract.

3. SEROLOGICAL IDENTIFICATION OF STAPHYLOCOCCAL ENTEROTOXINS

An ELISA using polyvalent antisera (antibody serotypes A, B, C, D, and E) in a double polyclonal antibody "sandwich" configuration was used to screen test portions for the presence of staphylococcal enterotoxin in the untreated and urea-treated mushroom extracts (Bennett *et al.*, 1990).

The commercially available ELISA used was the TECRA, Staphylococcal Enterotoxin Visual Immunoassay (manufactured by TECRA Diagnostics, 28 Barcoo St., Roseville, NSW 2069, Australia and distributed in the USA by International Bioproducts, Redmond, WA, USA). This method was used to screen mushrooms for staphylococcal enterotoxin because of its previous success with a wide variety of foods contaminated with these toxins (Bennett & Atrache, 1989; Bennett *et al.*, 1989).

The protocol consisted of adding 200-µl aliquots of the 5.0-ml additive-treated extracts (pH adjusted to 7.0-8.0) to prewashed (10 min at 25°C) anti-staphylococcal enterotoxin (serotypes A-E) coated microtiter wells, incubated for 2.0 h at 35°C. Wells were washed (3X), treated with 200 µl of polyvalent antisera (A-E) enzyme conjugate, and incubated for 1 h at 25°C; wells were again washed (5X) and substrates (200 µl) were treated for 30-45 min at 25°C, when 20 µl stop solution was added. A positive test was indicated by enzyme action (green) on the colorless substrate. For visual determinations, the tray was placed on a white background and individual test wells were compared to a color standard (color comparator) supplied in the kit. Absorbance (A) of the test solutions was determined with a microtiter tray reader (V Max Kinetics Microplate Reader,

Molecular Devices, Menlo Park, CA 94025-9994, USA) or comparable, using dual wavelengths [405 and 490 (or 492) nm]. Test solutions that gave an absorbance of >0.200 were considered positive for enterotoxin. Those that gave an absorbance of ≤0.200 were considered negative for enterotoxin.

If confirmation by the AOAC method was necessary, the 5-ml (200 µl) urea-treated preparation was extracted with $CHCl_3$, diluted with 40 volumes of 0.005 M phosphate buffer, pH 5.7, chromatographed, and tested by the microslide gel double diffusion test (AOAC, 1990). When necessary, untreated mushroom extracts were processed by the AOAC method (Bennett *et al.*, 1988).

4. HEAT AND CHEMICAL DENATURATION OF TOXIN

The action of heat on globular proteins such as the enterotoxins leads to changes in molecular structure (denaturation) and/or particle size (Jaenicke, 1967). Such changes in the conformation of the protein, as a result of heat stress, may occur in a series of steps, from the native form through a number or intermediate, partially denatured forms to a fully denatured type which lacks a conformational likeness to the structure of the native protein.

Many studies on the heat inactivation of staphylococcal enterotoxins have indicated the thermal stability of the toxins. Nevertheless, high thermal exposure appears to destroy the serological activity of the enterotoxins while retaining some of their biological activity (Bennett & Berry, 1987).

If mushrooms are contaminated with enterotoxigenic *Staphylococcus* spp., followed by time and temperature abuse of the product, various amounts of enterotoxin may be produced. When the product is retorted, the canning conditions ultimately determine whether enterotoxins will be completely or partially inactivated. Studies have shown that the degree of serological inactivation differs with the effects of heat (F_0 = 7.5 min) on 50 and 100 ng SEA/g of product (Fig. 1). The preparation containing 50 ng SEA/g was totally inactivated and, when serologically analyzed by ELISA, produced an absorbance ≤0.200 (0.180). Although the serological inactivation pattern was similar in the preparation containing 100 ng SEA/g of product, total inactivation by heat did not occur and produced an absorbance > 0.200 (0.270).

Urea can also act as a chemical denaturant on certain proteins and has been shown to have a denaturing effect on staphylococcal enterotoxin (Warren *et al.*, 1974; Bennett, 1991, 1992). Figure 2 shows the effect of urea treatment time on the denaturation of 5.0, 10.0, and 20.0 ng staphylococcal enterotoxin/g in its native form in mushroom extracts. While 5 ng SEA/g was denatured in 6 h, producing negative ELISA results, denaturing of the toxin in the preparation containing 10 ng SEA/g required 12 hr. Table 1 presents data on the identification of enterotoxin in its native form in naturally contaminated canned mushrooms and the effect of urea treatment on rendering the toxin serologically inactive.

5. UREA RENATURATION OF TOXIN

The effects of urea and other chemicals as denaturants have been shown in pasteurized milk (Tatini, 1976) and, more recently, in mushrooms that received even more severe heat treatment (Bennett, 1991, 1992). Figure 3 compares the effect of urea concentration and treatment times of 4, 5, and 6 h on the serological renaturation of staphylococcal enterotoxin from its heat-altered form in naturally contaminated canned mushrooms. The data suggest that treatment time is very important, although the urea concentration is also a factor in the protein renaturation process. In this study, 6

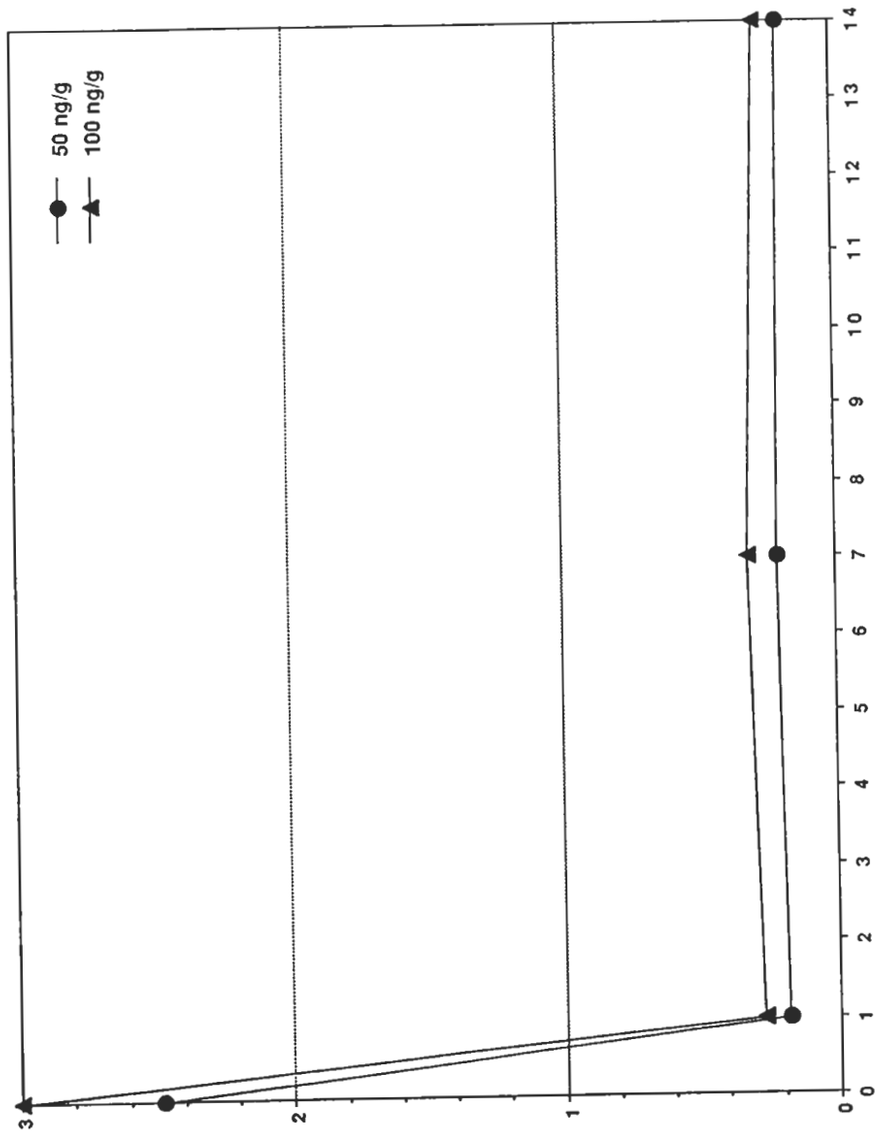

FIGURE 1. Comparative serological inactivation of various concentrations (50 and 100 ng SEA/g product) of staphylococcal enterotoxin in canned mushrooms (F_0 = 7.5 min).

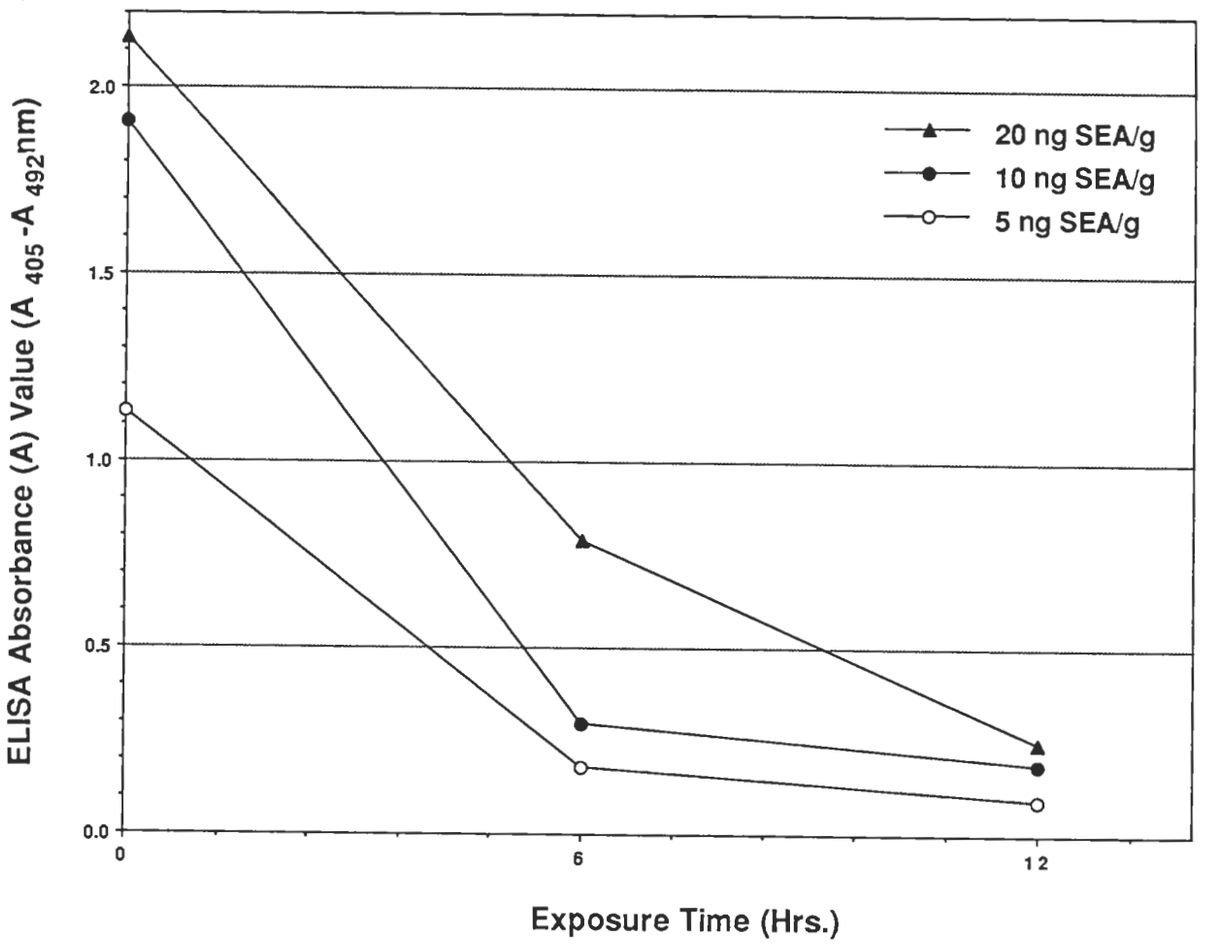

FIGURE 2. Effect of urea (6.0 M) exposure time on the denaturation of various concentrations (5.0, 10.0, and 20.0 ng/g) of staphylococcal enterotoxin in the native form in mushroom extracts. Absorbance 0.200 indicates serological absence of toxin.

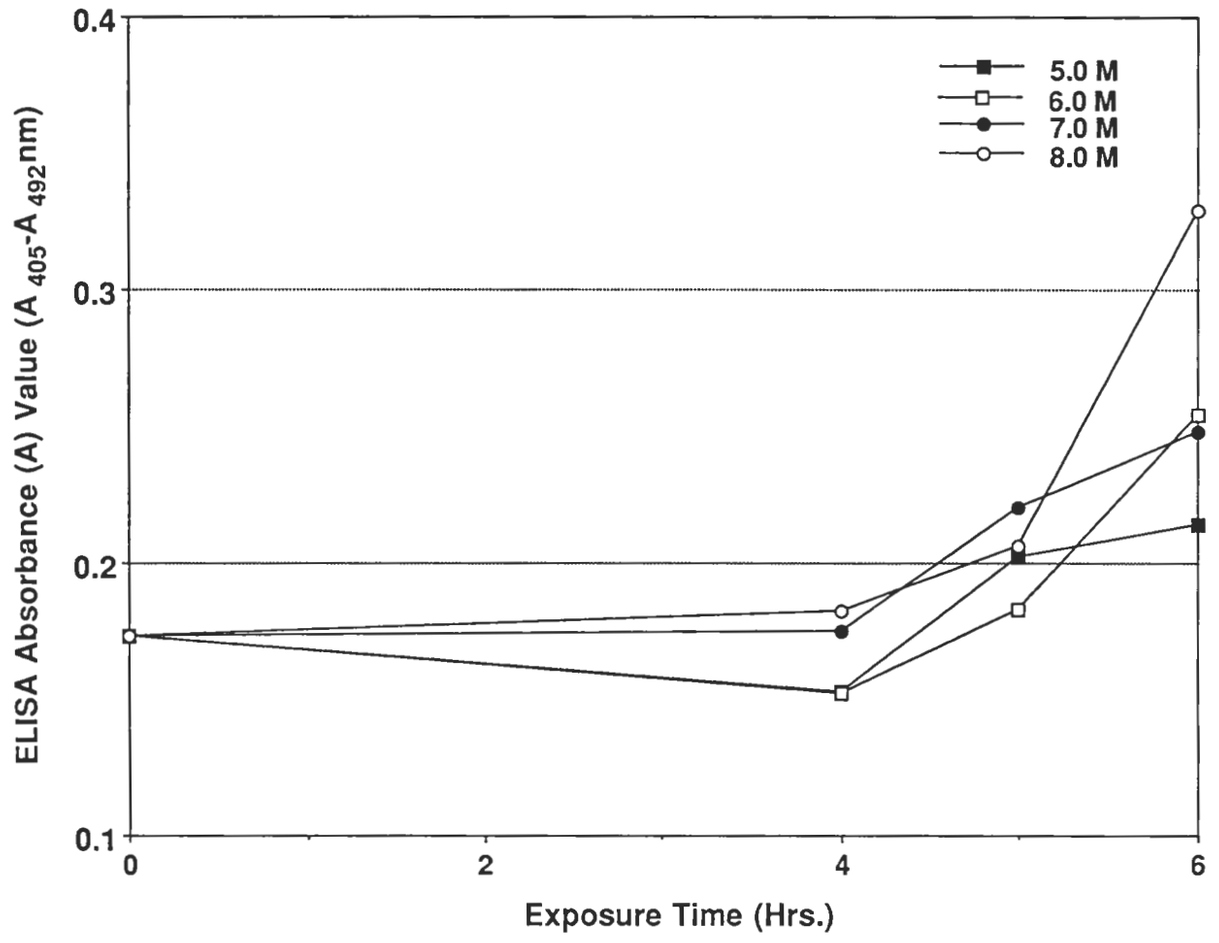

FIGURE 3. Comparative effect of urea concentration (5.0, 6.0, 7.0, and 8.0 M) and exposure time (4, 5, and 6 h) on the serological renaturation of staphylococcal enterotoxin in naturally contaminated canned mushrooms. Absorbance ≤0.200 indicates nonidentifiable toxin; absorbance > 0.200 indicates serological identification of toxin.

TABLE 1. Identification of staphylococcal enterotoxin in the native form and the effect of urea[1] on denaturation (serological inactivation) of the toxin

Product designation	Extract treatment	ELISA response	
		visual	(absorbance)[2]
26a	untreated	+	(0.223)[3]
26a	urea	-	(0.130)[4]
51a	untreated	+	(0.343)
51a	urea	-	(0.139)
779	untreated	+	(0.612)
779	urea	-	(0.149)
784	untreated	+	(0.756)
784	urea	-	(0.099)
182	untreated	+	(0.341)
182	urea	-	(0.063)
191	untreated	+	(0.575)
191	urea	-	(0.058)
198	untreated	+	(0.370)
198	urea	-	(0.069)
656	untreated	+	(0.945)
656	urea	-	(0.106)

1 Preparation treated with urea (6.0 M, 6 h, 25°C).
2 Absorbance determined on a microtiter plate reader at wavelengths of 405 and 490 (492) nm.
3 Absorbance > 0.200 indicated toxin presence.
4 Absorbance 0.200 indicated no serological identifiable toxin.

h of urea treatment was the most effective in restoring serological integrity to the toxin molecule. Table 2 provides data on the serological reactivation of thermally inactivated enterotoxin in commercially processed mushrooms after treatment with 6.0 M urea for 6 h.

The existence of multiple structural forms (native and heat-altered) of enterotoxin in canned mushrooms was previously observed (Bennett, 1991, 1992). This phenomenon may be the result of underprocessing, in which all of the toxin moieties have not been completely denatured. The product may also contain such large amounts of toxin before retorting that the heat or canning specifications are ineffective in denaturing all of the toxin (Table 3).

Recent work with metallic ions has been attempted to develop a more effective toxin renaturation procedure. Zinc acetate (0.354 g/ml) effectively amplified the urea renaturation process. Table 4 provides data on the effect of urea and urea plus zinc acetate as denaturants with small amounts (5.0, 10.0, and 20.0 ng/g) of heated (121.1°C, 30 min) enterotoxin in mushrooms and a commercially processed preparation.

6. SEROLOGICAL DEGRADATION OF HEAT-ALTERED ENTEROTOXIN

Naturally contaminated mushrooms that purportedly received the normal thermal processing time and temperature were tested to determine the serological stability of the enterotoxin under

TABLE 2. Serological reactivation (renaturation) of thermally inactivated (denatured) staphylococcal entertoxin in canned mushrooms after urea treatment[1].

Product designation	Extract treatment	ELISA response	
		visual	(absorbance)[2]
847	untreated	-	(0.176)[3]
847	urea	+	(0.234)[4]
Eden	untreated	-	(0.189)
Eden	urea	+	(0.227)
103	untreated	-	(0.123)
103	urea	+	(0.257)
175	untreated	-	(0.078)
175	urea	+	(0.291)
538	untreated	-	(0.109)
538	urea	+	(0.212)
278	untreated	-	(0.060)
278	urea	+	(0.217)
337	untreated	-	(0.061)
337	urea	+	(0.225)
816	untreated	-	(0.115)
816	urea	+	(0.236)

1 Preparation treated with urea (6.0 M, 6 h, 25°C).
2 Absorbance determined on a microtiter plate reader at wavelengths of 405 and 490 (492) nm.
3 Absorbance ≤ 0.200 indicated toxin absence.
4 Absorbance > 0.200 indicated toxin presence.

TABLE 3. Identification of multiple structural forms of staphylococcal enterotoxin in canned mushrooms

Product designation	Extract treatment	
	No urea	Urea
448	+(1.727)[1]	+(>3.00)
377	+(>3.00)[2]	+(0.618)
344-B.C.	+(>3.00)	+(>3.00)
	+(1:320,[3] 0.234[4])	+(1:1,280,[3]0.220[4])

Source:Modified from Bennett, 1992.
1 Absorbance measurement on microtiter plate reader at wavelengths of 405 and 490 nm.
2 Absorbance limit, 3.00.
3 Dilution of extract in positive (> 0.200) absorbance range.
4 Absorbance reading at specified extract dilution; absorbance reading ≤ 0.200 negative for toxin. Absorbance reading > 0.200 indicated toxin presence.

TABLE 4. Effect of urea[1] and Zn^{++}[2] amplifier on the renaturation of heat-altered staphylococcal enterotoxin A in mushrooms.

Product status	Toxin ng/g	Treatment		ELISA-Serology (absorbance)[3] Renaturation Process, 6h	
		unheated	heated		
				Urea	Urea + Zn
Toxin added	5	+(0.747)[4]	-(0.195)[5]	-(0.186)	+(0.221)
Toxin added	10	+(1.436)	-(0.136)	-(0.127)	+(0.226)
Toxin added	20	+(1.907)	-(0.142)	-(0.131)	+(0.249)
Natural	TPF[6]	NA[7]	-(0.170)	-(0.184)	+(0.426)

1 Treatment with urea (6.0 M, 6 h, 25°C).
2 Zinc acetate (0.354 g/ml).
3 Absorbance on microtiter plate reader at wavelengths of 405 and 492 nm.
4 Absorbance > 0.200 indicated toxin presence.
5 Absorbance ≤ 0.200 indicated no serologically identifiable toxin.
6 Toxin preformed (TPF).
7 NA = not available; product commercially retorted.

selected storage conditions.

Figure 4 shows serological patterns that occurred in thermal processing simulation studies in which 5, 10.0 and 20.0 ng toxin/g was added to mushrooms before retorting that rendered the toxin serologically inactive. However, treatment of the toxin- containing preparations with urea resulted in the serological reactivation of the toxin. Immediately after exposure of the heat-altered toxin to urea, toxin was spontaneously reactivated for approximately 5 days; then subsequent serological degradation began regardless of the concentration of SEA.

Serological degradation of staphylococcal enterotoxin in naturally contaminated canned mushrooms held under conditions of abuse during transport is summarized in Table 5. Initial analysis of the product indicated the presence of toxin; however, after transport under abuse conditions, during a 4-day period, only one of seven of the mushroom extracts remained positive for toxin. The one positive urea sample (367a) in this series contained the greatest amount of toxin and was not completely degraded between the initial and later assays. However, the degree of serological degradation was generally equal to that of the other degraded preparations. These findings prompted further investigation of the effect of storage temperature on the serological behavior of staphylococcal enterotoxin in canned mushrooms. Figure 5 compares the effect of temperature (5 and 25°C) on untreated canned mushrooms. Both preparations showed drastic serological degradation within the first week of storage regardless of storage temperature, although degradation of both preparations was greatest when they were held at 25°C. Generally, degradation of serologically identifiable toxin continued, but at a slower rate, during the remaining weeks of monitoring. Interestingly, one preparation (Q-1-2,/5°C) showed degradation the first week, with intermittent spontaneous serological reactivation, and then showed rather drastic serological degradation. Figure 6 compares the effect of storage temperature on the serological behavior of untreated and urea treated mushroom preparations. The similar serological degradation patterns of these preparations suggest that this phenomenon is not inhibited by urea treatment of the heat-altered toxin.

These studies indicate that assays to determine the presence of toxin should be performed

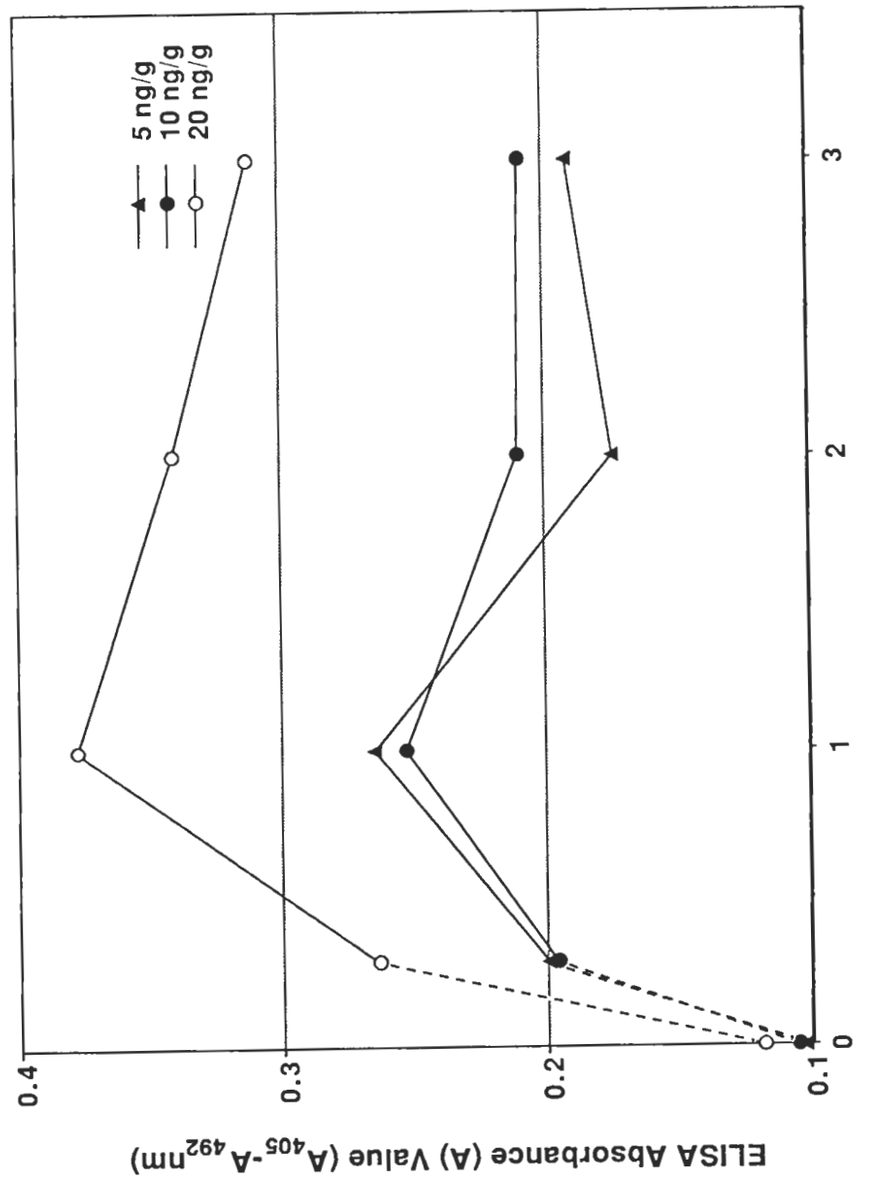

FIGURE 4. Comparative effect of renaturation (----, 6.0 M urea) and storage (5°C, 3 wk) on various concentrations (5.0, 10.0, 20.0 ng/g) of heat-altered (F_0 = 7.5 min) staphylococcal enterotoxin type A (SEA) in canned mushrooms and their spontaneous and/or degradative serological behavior (—). Absorbance > 0.200 indicates serological presence of SEA; absorbance 0.200 indicates absence of serologically identifiable SEA.

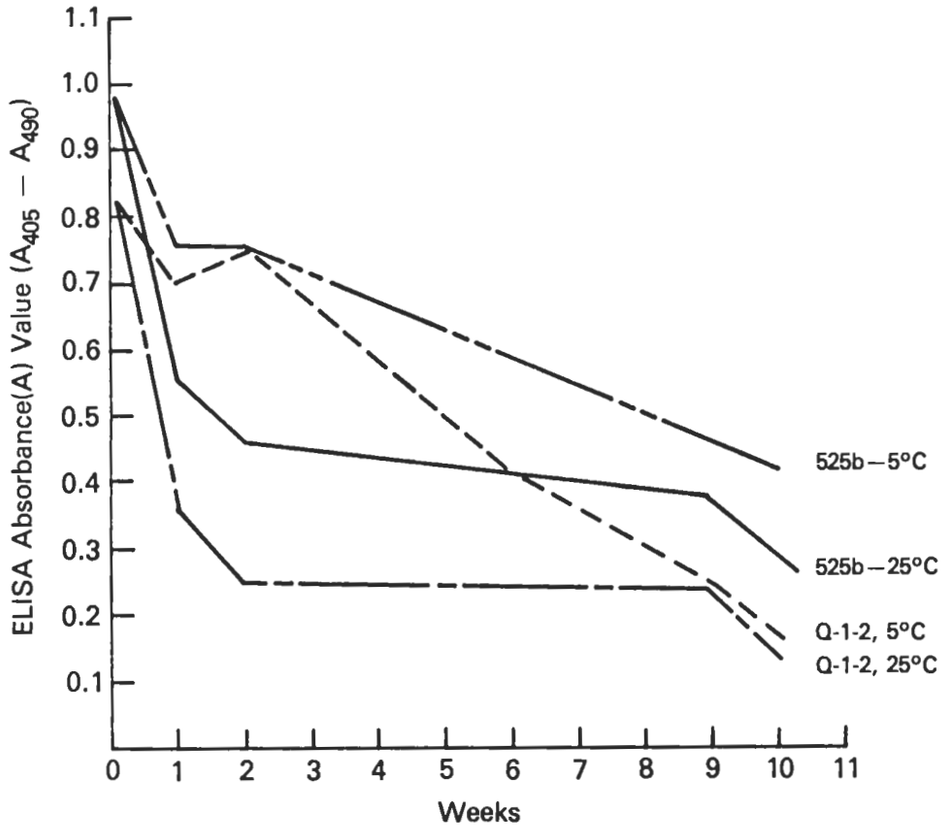

FIGURE 5. Comparative effect of temperature (5, 25°C) on serological degradation of staphylococcal enterotoxin in two (525b, Q-1-2) untreated canned mushroom extracts. Absorbance of ≤0.200 indicates absence of serologically identifiable enterotoxin.

immediately after extraction of food and urea treatment. If tested in this manner, preparations containing serologically identifiable toxin will be identified successfully. Conversely, short-term or prolonged storage may render the enterotoxins serologically unidentifiable, particularly if preparations contain small amounts of enterotoxin. Wu *et al.* (1992) reported that serological reactions, initially reported positive, were negative after a short interval. This kind of erratic ELISA response may perhaps be traced to serological degradation of the toxin during storage. The first order kinetics of heat alteration (denaturation) and the second order kinetics of urea treatment (renaturation) may affect the serological stability of the toxin, thereby degrading the molecule.

7. CONCLUSION

This paper has addressed the thermal inactivation/reactivation kinetics of SEA in contaminated mushrooms which have been simulated in the laboratory. Evidence shows that preformed staphylococcal enterotoxin can be altered serologically in foods and that this alteration, probably because of the conformation of the molecule, prevents recognition of the specific antibody that was

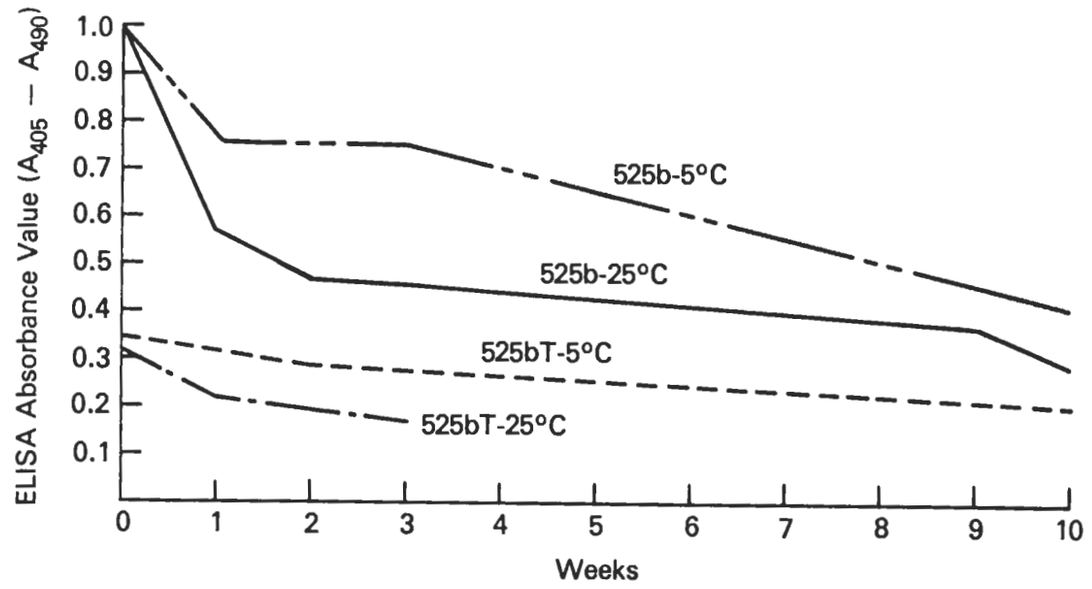

FIGURE 6. Comparative effect of temperature (5, 25°C) on serological degradation of staphylococcal enterotoxin in untreated (525b) and urea-treated (525bT) canned mushroom extracts. Absorbance of ≤0.200 indicates absence of toxin.

generated against the native form of the toxin. Other evidence has shown that these serologically altered molecules can be treated with urea to reverse the conformation to its native position. Thus, the antibody can recognize the heat-altered toxin, making identification possible by *in vitro* serology.

TABLE 5. Serological degradation of staphylococcal enterotoxin A (SEA) from canned mushroom extracts abused during transport (4 days).

Sample designation	Sample treatment	ELISA results	
		1st assay	2nd assay
367a	untreated[1]	+(0.431)[2,3]	-(0.183)[4]
383a	untreated	+(0.228)	-(0.163)
383a	urea[5]	+(0.263)	-(0.174)
383b	untreated	+(0.486)	-(0.153)
383b	urea	+(0.534)	-(0.185)
362a	untreated	+(0.309)	-(0.185)
367a	urea	+(0.610)	+(0.229)

[1] No urea treatment.
[2] Absorbance reading on microtiter plate reader (A_{405}-A_{490}nm).
[3] Readings > 0.200 indicated serologically identifiable SEA.
[4] Absorbance ≤ 0.200 indicated absence of serologically identifiable SEA.
[5] Treatment of the extract with 6.0 M urea for 5 h at 25°C.

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