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Clostridium botulinum Neurotoxin Type B Is Heat-Stable in Milk and Not Inactivated by Pasteurization

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Foodborne botulism is caused by the ingestion of foods containing botulinum neurotoxins (BoNTs). To study the heat stability of *Clostridium botulinum* neurotoxins, we needed to measure and compare the activity of botulinum neurotoxins, serotypes A and B, under various pasteurization conditions. Currently, the only accepted assay to detect active *C. botulinum* neurotoxin is an *in vivo* mouse bioassay, which raises ethical concerns with regard to the use of experimental animals. In this study, noninvasive methods were used to simultaneously detect and distinguish between active BoNT serotypes A and B in one reaction and sample. We developed an enzymatic activity assay employing internally quenched fluorogenic peptides corresponding to SNAP-25, for BoNT-A, and VAMP2, for BoNT-B, as an alternative method to the mouse bioassay. Because each peptide is labeled with different fluorophores, we were able to distinguish between these two toxins. We used this method to analyze the heat stability of BoNT-A and BoNT-B. This study reports that conventional milk pasteurization (63 °C, 30 min) inactivated BoNT serotype A; however, serotype B is heat-stable in milk and not inactivated by pasteurization. Using this activity assay, we also showed that the commonly used food processes such as acidity and pasteurization, which are known to inhibit *C. botulinum* growth and toxin production, are more effective in inactivating BoNT serotype A than serotype B when conventional pasteurization (63 °C, 30 min) is used.

KEYWORDS: *Clostridium botulinum*; fluorescence resonance energy transfer (FRET); pasteurization; SNAP-25; synaptobrevin 2; immunomagnetic beads

INTRODUCTION

Clostridium botulinum is an anaerobic, Gram-positive, spore-forming bacterium that contaminates food and causes food poisoning. Heat treatment at 70 °C for 2 min kills the *Clostridium* vegetative stage but does not kill *Clostridium* spores (*1*). The spores germinate under anaerobic environment conditions and release toxin mostly at the end of the growth phase, coinciding with cell lysis (*2*, *3*). Seven distinct botulinum neurotoxin serotypes, designated as BoNT-A to BoNT-G, are presently recognized; however, only types A, B, E, and F cause disease in humans (*4*). Each one of these neurotoxins cleaves a specific sequence in synaptic proteins at the neuromuscular junction, blocking the release of the neurotransmitter acetylcholine (*5*). This causes clinical signs such as respiratory muscular paralysis and death.

Structurally, BoNT-A and BoNT-B translate as a single polypeptide chain of 150 kDa, later cleaved to form a 100 kDa heavy chain and 50 kDa light chain. BoNT toxins are protected by accessory toxic and nontoxic-hemagglutinin proteins yielding a uniform complex of 900 kDa for BoNT-A and 700 kDa complex for BoNT-B (*6*). The heavy chain binds to the membrane receptor and enters the cell by endocytosis. Inside the cell, the accessory proteins disassociate from the (150 kDa) core toxin, and the light chain is then released in the nerve end. BoNT-A cleaves the Synaptosonal-Associated Protein (SNAP-25), and BoNT-B cleaves the synaptobrevin 2 (VAMP 2) (*5*).

The gold standard method for determining botulinum toxin activity is the mouse bioassay. This procedure involves intraperitoneal injection of suspected contaminated food and observation for clinical symptoms. To identify the toxin serotype, neutralizing antibodies against each botulinum toxin serotype need to be used. However, this *in vivo* methodology raises ethical concerns with regard to the use of experimental animals.

In previous work, we detected active BoNT-A in food using in vitro fluorescence resonance energy transfer (FRET) assay based on the cleavage of the natural substrate SNAP-25 (*7*, *8*). The purpose of this study is to (1) determine whether the activity of *C. botulinum* toxin serotype A and B can be detected simultaneously in the same reaction using an *in vitro* FRET-based assay, (2) determine if the use of a thermal cycler is effective for high-temperature and short-time (HTST) pasteurization, and (3) determine whether commonly used processes of low pH that inhibit *C. botulinum* growth or pasteurization that kills the vegetative stage will inactivate *C. botulinum* secreted toxins.

MATERIALS AND METHODS

Chemicals and Reagents. BoNT-A and BoNT-B complexes were obtained from Metabiologics (Madison, WI). The dual-labeled fluorogenic peptide corresponding to synaptobrevin 2 was labeled with the

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fluorogenic dye o-Abz/Dnp (VAMPtide). The dual-labeled fluorogenic peptides corresponding to SNAP-25 substrate (SNAPtide) were N terminally linked to fluorescein-isothiocyanate (FITC), and the carboxyl terminal Lys was labeled with a quencher DABCYL (List Biological Laboratories, Campbell, CA); shown below is the sequence.

(FITC)HN− Thr− (D)Arg− Ile− Asp− Gln− Ala− Asn− Gln− Arg − Ala− Thr− Lys(DABCYL)− Nle− $CONH_2$

The 12 amino acid synthetic peptide corresponds to the natural sequence (190−201) of SNAP-25 (*9*). The recombinant light chain (Lc), corresponding to serotypes A and B (LcA and LcB), and chicken IgY antiheavy chain BoNT-B were also obtained from List Biological Laboratories. Sheep antibody targeting LcB was obtained from R&D systems (Minneapolis, MN). Monoclonal antibodies targeting the BoNT-A heavy chains and light chains were kindly provided by Larry Stanker from the U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Foodborne Contaminants Research Unit (Albany, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Nonfat dry milk (Nestlé Carnation, Vevey, Switzerland) was reconstituted in water according to manufacturer's instructions (6.67%).

Assays of BoNT Serotype A and B, LcA, or LcB Protease Activities. Assays were performed as described by the manufacturer's instructions (List Biological Laboratories). Briefly, recombinant LcA (250 nM) was reconstituted in 20 mM HEPES and 0.2% Tween-20 (pH 8.2), and LcB was reconstituted in H_2O with 0.05% Tween-20. BoNT-A complex was reconstituted to 250 nM in 1 mg/mL bovine serum album (BSA) and 20 mM HEPES buffer (pH 8.0), and BoNT-B was reconstituted in 20 mM HEPES, 0.2% Tween-20, pH 7.4.

The BoNT-A complex was diluted in reduction buffer [20 mM HEPES, pH 8.0, 5 mM dithiothreitol (DTT), and 0.3 mM $ZnCl_2$], and BoNT-B was diluted in 20 mM HEPES, containing 0.05 mM $ZnSO_4$ and 5 mM DTT. LcA was diluted in reaction buffer, 20 mM HEPES, and 0.2% Tween-20 (pH 8.2), and LcB was diluted in 50 mM HEPES and 0.05% Tween-20 (pH 6.3). Substrates were added to samples with various concentrations of BoNT complex or light chain, at a final concentration of 8 μM for SNAPtide and 10 μM for VAMPtide. The sample and substrate were added to a black 96-well plate (Costar-Corning Inc., NY) at a final volume of 200 μL. After incubation at 37 °C, fluorescent emission at 523 nm was measured with excitation at 490 nm and a cutoff filter of 495 nm, for FITC (SNAPtide), and fluorescent emission at 418 nm with excitation at 321 nm for o-Abz/Dnp (VAMPtide), using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Conventional HTST Pasteurization. Buffer and milk were heated with a dry thermomixer; after they reached a temperature of 72 °C and equilibrated for 5 min, then, the toxin light chain was added and incubated for 15 s. The test tube was rapidly transferred to ice.

Unconventional HTST Pasteurization. Increasing concentrations of BoNT-A were added to milk and HEPES buffer. The samples were heated with a thermal cycler to 72 °C with a slope of 0.5 K s^{-1} and held at 72 °C for 15 s. Subsequently, the samples were cooled to 4 °C with a slope of 2.5 K s^{-1}.

Simulating Long-Time Low-Temperature (LTLT) Pasteurization Conditions. Samples were heated with a dry thermomixer; after they reached a temperature of 63 or 72 °C and equilibrated for 5 min, then, the toxin was added and incubated for 30 m at 63 °C or 15 s at 72 °C. The test tube was rapidly transferred to ice.

Adjusted pH. The pH of each buffer was adjusted by the addition of hydrochloric acid. Buffers were heated with a dry heat block, and when the buffer reached the corresponding temperature, light chain was added and incubated for 30 m. After heat treatment, the buffers were brought back to the optimal pH (8.2 for LcA and 6.3 for LcB) with potassium hydroxide.

Coating Beads with IgG. Coating immunomagnetic beads with antibodies was previously described (*7*). Briefly, 100 μL of Dynabeads M-280 tosyl-activated (Invitrogen, Carlsbad, CA) was washed twice with 600 μL of 0.1 M sodium borate buffer, pH 9.5 (Sigma #B-0394), and diluted in the same buffer to 2×10^9 beads/mL. Purified antibody (30 μg) was added to 1×10^8 beads (50 μL). The antibody and beads were incubated for 24 h at 37 °C on a slow shaker to facilitate covalent binding. The coated beads were washed twice for 5 min at 4 °C with 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA, washed once for 4 h at 37 °C with 0.2 M Tris−HCl, pH 8.5, containing 0.1% BSA, and washed once more for 5 min at 4 °C with PBS, pH 7.4, containing 0.1% BSA. The beads were resuspended in 50 μL of the Tris−BSA buffer and stored in 4 °C for later use.

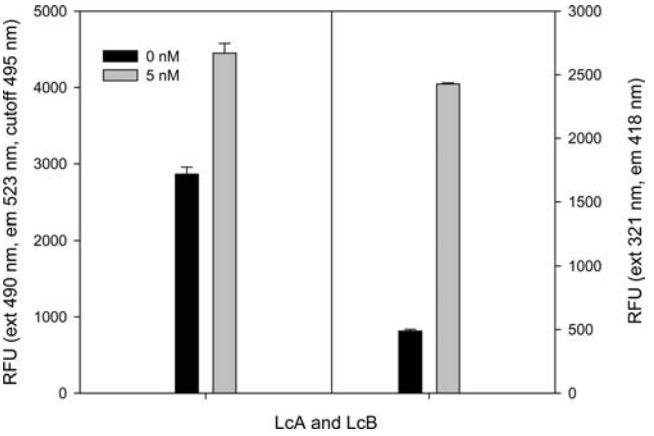

Figure 1. Simultaneous distinguishment between BoNT serotypes A and B. BoNT types A and B were incubated together with both substrates: SNAP-25 labeled with the fluorogenic dye FITC and VAMP 2 labeled with the fluorogenic dye o-Abz. Fluorescence emissions at 523 and 418 nm were measured with excitations at 490 and 321 nm, respectively.

Sample Binding. Toxin binds to beads were previously described (*7*). Briefly, 5 μL of the immunomagnetic beads was incubated with a tilting motion at 4 °C with 1 mL of spiked milk (BoNT-A/B or LcA/B). After 24 h, the milk with toxin-bound immunomagnetic beads was placed on a magnet for 2 min to collect the beads. The beads were washed twice with PBS, pH 7.4, containing 0.1% BSA and resuspended with 1 mL of reduction buffer for BoNT complex or reaction buffer for light chain. The sample (200 μL) was transferred to a black 96-well plate, and substrate was added, followed by incubation at 37 °C.

Statistical Analysis. Statistical analysis was performed using SigmaStat 3.5 for Windows (Systat Software, San Jose, CA). One-way analysis of variance (ANOVA) was used to compare control unspiked buffer or milk with buffer or milk containing increasing concentrations of BoNT complex or light chain. All experiments were repeated at least three times, and results with $p < 0.05$ were considered statistically significant.

RESULTS

Simultaneously Detection and Distinguishment of BoNT Serotypes A and B in One Reaction and Sample. To simultaneously detect BoNT and differentiate between serotypes A and B in one FRET-based assay in the same sample, we added and incubated both BoNT light chain serotypes A and B together with both substrates; the fluorogenic peptides SNAP-25 labeled with the fluorogenic dye FITC and VAMP-2 labeled with the fluorogenic dye o-Abz. The assays were performed in 50 mM HEPES and 0.05% Tween-20 at pH 6.3. Fluorescence was restored as cleavage occured and differentiated between serotypes A and B in the same sample. As shown in **Figure 1**, each BoNT light chain recognizes and cleaves a unique site on one of the substrates, SNAP-25 or VAMP-2. When we assayed one light chain at a time, only one fluorescence signal was observed, 490 nm for LcA and 321 nm for LcB. We were able to detect both toxins and distinguish between toxin serotype A or B simultaneously in the same sample. This result suggests that a multitoxin activity assay is practical.

Conditions That Simulated HTST Pasteurization Using the Thermal Cycler Did Not Reduce the Biological Activity of BoNT-A in Milk and Buffer. The thermal cycler has been used to simulate HTST pasteurization at 72 °C and 15 s (*10*). To evaluate if this method would be effective for conventional HTST pasteurization in reducing BoNT-A activity, milk and buffer were spiked with increasing concentrations of BoNT-A prior to HTST pasteurization

either by a thermomixer or in a thermal cycler. To reduce interference from the milk matrix, we utilized immunomagnetic beads coated with antibody to capture the spiked toxins from the milk. Our data from this comparison show that thermal inactivation is dependent on the pasteurization methods (**Figure 2**). HTST pasteurization using a thermal cycler is less effective than conventional HTST pasteurization given that BoNT-A did not lose their activity after HTST pasteurization using a thermal cycler. However, BoNT-A lost its activity when conventional HTST pasteurization was used.

Effect of Low pH and LTLT Pasteurization at Low pH on Inactivation of LcA and LcB in Buffer. Pasteurization and low pH are commonly used to limit *C. botulinum* growth and toxin production in food. An interesting question is whether these treatments affect BoNT toxin activity. In this experiment, to study the effect of low pH and pasteurization on toxin activity, we spiked HEPES buffer (pH 1.5, 4.0, 5.0, 7.0, or 8.3) with 0.25 or 5 nM LcA or LcB and with internally quenched fluorescence peptide, SNAP-25 for LcA or VAMP-2 for LcB, and treated it at 63 °C for 30 min, simulating LTLT pasteurization conditions. As shown in **Figure 3**, low pH inhibited the catalytic activity of both BoNT serotypes A and B. At strong acid (pH 1.5), the treatment entirely eliminates the activity of each toxin at both concentrations. LcA maximizes its enzymatic activity at pH 8.3, shown by the high fluorescence at pH 8.3. However, the enzymatic activity of LcB was maximized at pH 5. The peptide cleavage assay demonstrated significant dose-dependent increases in cleavage activity. Pasteurization of LcB at pH 5 or LcA at pH 8.3 dramatically reduced but did not eliminate activity. In contrast, pasteurization of either toxins at pH 7 entirely eliminated their cleavage activity.

Milk Protects BoNT-B Activity from Pasteurization. Heat pasteurization of milk is widely used to kill pathogenic microorganisms. Pasteurization also changes the conformation of milk protein, solubility, and other characteristics. To determine whether milk influences thermal inactivation of the toxin, milk was spiked with four concentrations (5, 2.5, 1, or 0.25 nM) of BoNT-A/B or LcA/B and then pasteurized (63 °C, 30 min) and incubated with internally quenched fluorescence peptide SNAP-25 or VAMP-2. To reduce interference from the milk matrix, we utilized immunomagnetic beads to capture and remove the toxin

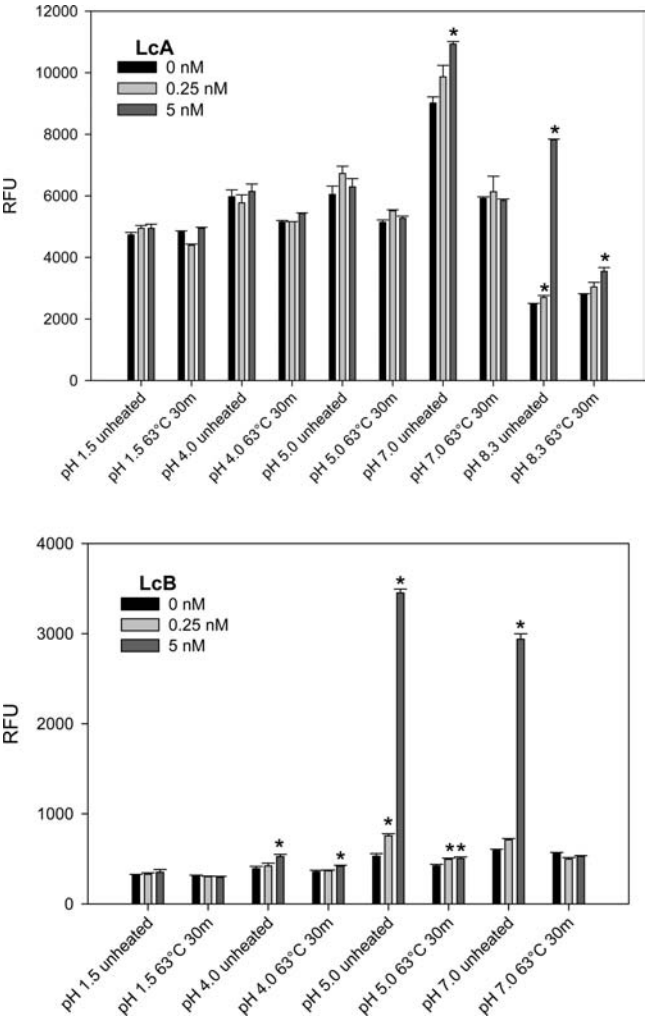

Figure 3. Effect of pasteurization and pH on cleavage activity of LcA or LcB. Buffers were spiked with 0.25 or 5 nm of LcA or LcB, pasteurized, and incubated with internally quenched fluorescence peptide, SNAPtide for LcA, and VAMPtide for LcB. Error bars represent standard errors, and an asterisk indicates significant differences ($p < 0.05$) between buffer containing toxin and without toxin.

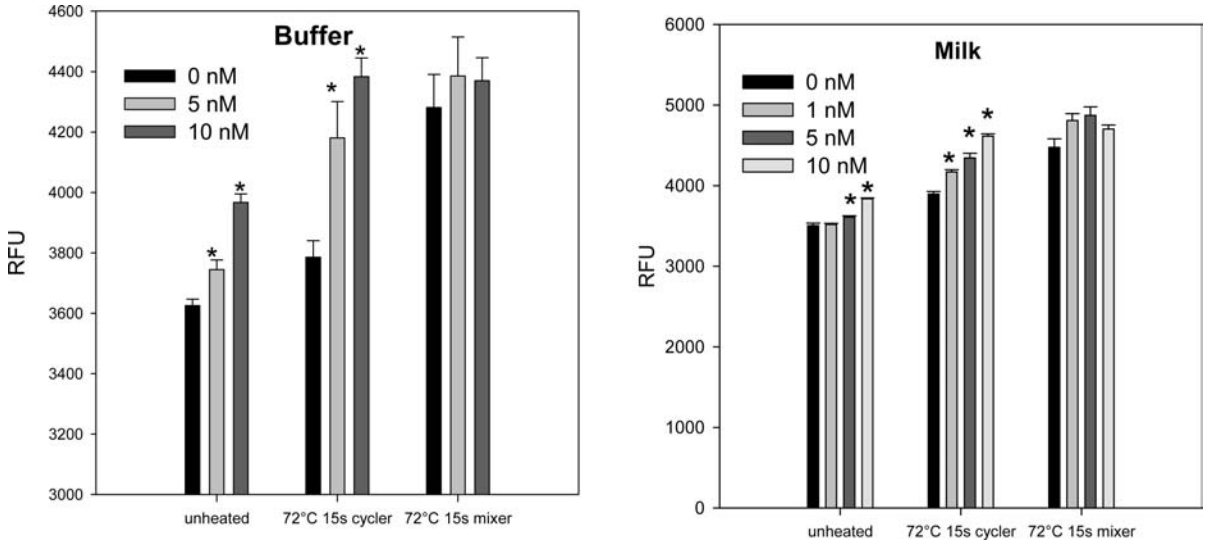

Figure 2. Pasteurization using the thermal cycler did not reduce the biological activity of BoNT-A. Milk and buffer were spiked with increasing concentrations of BoNT-A prior to HTST pasteurization either by a thermomixer or in a thermal cycler. Error bars represent standard errors, and an asterisk (*) indicates significant differences ($p < 0.05$) between spiked and unspiked buffer or spiked and unspiked milk.

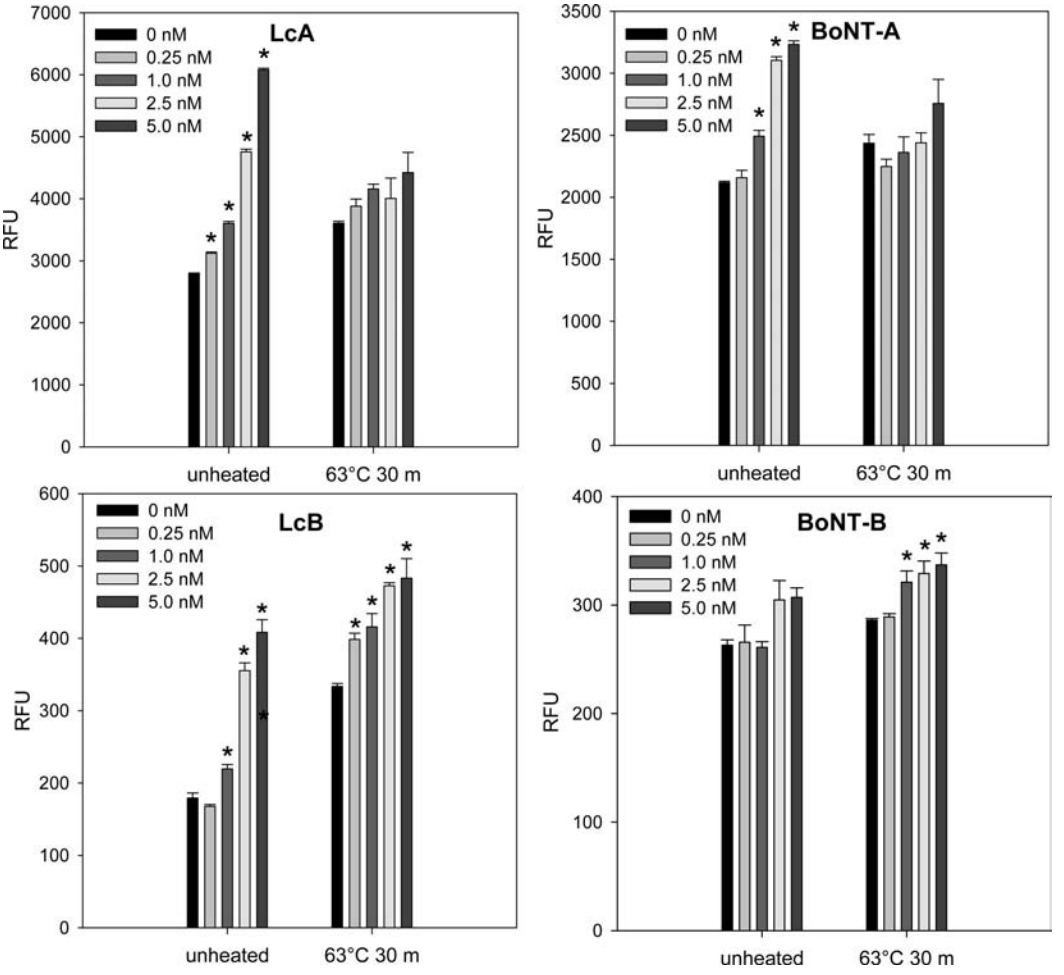

Figure 4. Milk retained BoNT-B and LcB cleavage activity after pasteurization. Milk was spiked with increasing concentration of BoNT-A/B or LcA/B and incubated with internally quenched fluorescence peptide. Error bars represent standard errors, and an asterisk indicates significant differences ($p < 0.05$) between buffer containing toxin and without toxin.

from the milk. As shown in **Figure 4**, pasteurization at 63 °C for 30 min is effective against BoNT-A in milk. LcA and BoNT-A activity was eliminated after heat treatment, but LcB and BoNT-B are still active after pasteurization. Activity levels for BoNT-B are slightly lower than those for LcB. This may be because the reduction buffer used for BoNT-B may not have been entirely efficient, leaving some light chains still attached to the heavy chains, thus, not enabling full cleavage of the dual-labeled fluorescein substrate, resulting in a lower level of detection of 1 nM instead of 0.25 nM. Milk protects BoNT-B and LcB from heat (unlike at pH 7.0 in buffer). The toxin is heat resistant, retains its cleavage activity, and can be detected in a dose-dependent manner. These results demonstrate that BoNT-B in milk is more heat resistant than BoNT-A and thermal inactivation is highly dependent on the matrix in which the toxin is present.

DISCUSSION

In the present study, we evaluated a potential alternative method to the mouse bioassay, the currently accepted method for measuring the biological activity of BoNTs that cannot be performed under most environmental conditions such as field laboratories. Here, we used activity assays, based on the toxins' ability to cleave specific sequences within soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) specific for each toxin (*5*). To simultaneously detect BoNT and differentiate between serotypes A and B in one FRET-based assay, we added BoNT serotypes A and B with the two fluorogenic peptides. SNAP-25 labeled with the fluorogenic dye FITC/DABCYL for BoNT-A, and VAMP 2 labeled with the fluorogenic dye o-Abz/Dnp for BoNT-B. Before cleavage, the fluorescence is quenched by FRET. Fluorescence is restored as cleavage occurs, and enzymatic activity can be monitored continuously. Because each target peptide is labeled with a different fluorophore, peptidase activity specific for both BoNT A and B is simultaneously detected in the same assay. BoNT A activity is detected at light emission at 490 nm and BoNT B at 321 nm. In this assay, we used the light chains of the toxins (LcA and LcB), which were previously shown to simulate the holoenzymes activities but are not toxic (*7*, *8*).

The present results show that inactivation of BoNTs is dependent on the pasteurization methods and matrix components in which the toxin is present. HTST pasteurization using a thermal cycler is less effective than conventional HTST pasteurization (**Figure 2**). Pasteurization of milk with BoNT-B toxin at 63 °C for 30 min did not decrease its activity (**Figure 4**). This result is somehow different than the previous study that demonstrated that pasteurization of milk at 72 °C for 15 s inactivates at least 99.5% of BoNT-B (*11*). Our result also shows that pasteurization of BoNT-B toxin in HEPES buffer at pH 7 entirely eliminates its activity (**Figure 3**). This suggests that milk has a protective effect on the toxin. We also demonstrated that pasteurization of BoNT-A in milk is effective in reducing its activity but not completely eliminating its activity. In addition, we showed that LcB and

BoNT-B are more heat resistant than LcA and BoNT-A (**Figure 4**). This enzymatic activity assay supports previous research that used the mouse bioassay, which shows that gelatin protein added to buffer increases toxin heat stability (*12*) and that BoNT-B is more heat resistant than BoNT-A in salmon paste (*13*). BoNTs are usually produced at pH 4.8−8.5, and the *C. botulinum* spore hardly grows and forms toxins in acidic foods at pH levels of 4.6 or below. However, even acidic foods such as canned tomatoes have been responsible for several cases of botulism food poisoning (*14*). Because some of the active toxin will still be present even in acidic food after pasteurization, this demonstrates a need to prevent early contamination with *C. botulinum*. Once the toxin has forms, it is impractical to eliminate it by thermal treatment without compromising the quality of the food.

The results presented in this study have shown that the pH appears to be an important factor affecting the stability of both toxins. At pH 1.5, similar to the lowest pH of the secreted gastric acid in the human stomach lumen, both toxins lose activity, but at pH 4.0, BoNT-B retains some activity. At pH 5.0, the enzymatic activity of LcB is maximal. These results suggest that some BoNT could possibly overcome the defense barrier of gastric acid, retaining its activity. After exposure to low pH, residual BoNT that enters the intestine would be exposed to digestive enzymes and to the protective mechanisms of the intestine such as protein defenses, mucins, trefoil peptides, filamentous brush border glycocalyx, and secretory IgA. This enzyme as well as the low physiological pH of the stomach lead to a large reduction in toxin activity. These observations suggest that to cause lethality by oral consumption of toxin, there must be larger doses than the corresponding amount administered by ip injection. These results are in agreement with results from oral ingestion of BoNT to achieve toxicity. Oral administration of BoNT needed to be 10^6 times higher than ip injection (*15*, *16*). In previous work (*7*), we demonstrated that by using immunomagnetic beads that captured and concentrate the toxin from food, we were able to overcome food matrix effects that interfere with the assay, and we were able to increase the sensitivity of this assay to 10 pg/mL of BoNT-A, which is more sensitive than the mouse bioassay. The assay is also very specific as shown in the simultaneous detection of BoNT-A/B. We think that using four different peptides containing a unique cleavage site for BoNT types A, B, E, and F labeled with different fluorogenic dyes could be useful for simultaneous detection and subtyping of multi BoNT toxin. Detecting these toxins in the same reaction and sample will make this activity method more useful for food safety.

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