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PERSISTENCE AND DEGRADATION OF MAIZE-EXPRESSED VACCINE PROTEIN, *ESCHERICHIA COLI* HEAT-LABILE ENTEROTOXIN SUBUNIT B, IN SOIL AND WATER

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Abstract—Transgenic plants represent an innovative platform for the cost-effective large-scale production of various pharmaceutical proteins. The eventual open-field production of plant-made pharmaceuticals (PMPs) requires risk assessment to determine the potential for harm to the surrounding ecosystem. In the present study, the environmental persistence of a transgenic maize-expressed antigen, *Escherichia coli* heat-labile enterotoxin subunit B (LTB), was studied under laboratory conditions. To semiquantitatively monitor the persistence of LTB in soil, extraction with a high-salt, high-pH extraction buffer was optimized using the closely homologous *Vibrio cholerae* enterotoxin subunit B (CTB) as a test substance. The time to dissipation of 50% (DT50) of the extractable fraction of maize-expressed LTB was 4 to 15 d in pond water and 35 to 90 d in soils. Both extraction efficacy and persistence were strongly affected by the matrix type and incubation conditions. In contrast with maize-expressed LTB, the DT50 for bacterially produced LTB and CTB was less than 4 d both in pond water and soil. Although maize-expressed LTB was more stable than bacterially produced analogue, its dissipation was governed by an initial lag, which could be attributed to release from the plant material, followed by rapid decline.

Keywords—*Escherichia coli* *Vibrio cholerae* Plant-made vaccine Transgenic maize Environmental fate

INTRODUCTION

Thanks to recent advancements in plant biotechnology, transgenic plants are considered to be an effective platform for the production of pharmaceutically active recombinant proteins as an innovative new strategy for pharmaceutical production [1–3]. The potential advantages of such plant-made pharmaceuticals (PMPs) include low cost of large-scale production, no risk of animal pathogens, high storability and transportability, capacity to express multiple proteins, and easy introduction to food crops for oral administration [4].

Escherichia coli heat-labile enterotoxin subunit B (LTB) is a nontoxic subunit of heat-labile enterotoxin (LT) produced by a toxic strain of *E. coli* that consists of one enzymatically active A subunit ([LTA], 27.0 kDa) and five smaller B subunits ([LTB], 11.6 kDa). Specific binding of pentameric LTB to intestinal cell surface ganglioside GM_1 allows LTA to cross the membrane and cause diarrhea symptoms [5]. Strong immunogenicity of pentameric LTB with no evidence of mammalian toxicity makes it a desirable vaccine to prevent LT-induced diarrhea diseases [6]. In addition to its immunogenicity, LTB also is known as a potent mucosal adjuvant that stimulates the immune response of coadministered antigen proteins [7]. To realize safe, practical, and low-cost application of LTB for both human and animal treatments, plant-based recombinant LTB production has been investigated for several crops [8]. Maize has been transformed successfully to express LTB in its kernels [9], and its oral administration to mice elicited strong mucosal and serum antibody responses even under the nanogram level dosage [10–12]. Thus, large-scale production of plant-made LTB is a feasible means for therapeutic applications.

Although there are many potential advantages of plant-based LTB production, uncertainties regarding risks to both human and environmental health must be addressed. According to Wolt et al. [13], the risks of PMPs arise from intended use by end users, including worker exposure during growing and processing; unintended consumption of adulterated product through inadvertent occurrence of PMPs in foods and feeds; and environmental exposure as a result of PMP open-field production. Because LTB has a relatively long history of research for pharmaceutical purposes, its mammalian bioavailability and toxicity are characterized in previous studies [14,15]. Using such toxicological profiles, Wolt et al. [16] assessed the risk of the unintended occurrence of maize-expressed LTB because it potentially could impact human health. In their risk assessment, the human health concerns from inadvertent occurrence of LTB were minimal due to limited exposure potential from confined production of LTB-maize and lack of mammalian toxicity.

Unlike human health risks, the risk of maize-expressed LTB to the ecosystem still is understood poorly. Quantitative risk assessment that characterizes exposure and effect of stressors through an ecological risk-assessment framework [17] provides a means to understand the implication of plant-made LTB to the environment [16]. In order to evaluate the environmental exposure of biogenic maize products, Wolt et al. [18] conducted quantitative exposure assessment for the harvest loss of maize kernels in the field under various scenarios for confinement management. Such quantitative assessment of exposure aids the estimation of the maximum field exposure level of PMPs to the surrounding ecosystem. Among various potential environmental routes of exposure for maize-expressed LTB, postharvest dropped kernels in the production field are considered the route that causes the greatest exposure

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Table 1. Recovery of *Vibrio cholerae* enterotoxin subunit B (CTB) from soil using various buffers

Buffer[a]	pH	Description	CTB recovery[b]	Published Bt[c] recovery	Reference
Water	7.2	Purified water (Nanopure D4741, Barnstead International, Dubuque, IA, USA)	0%	—	—
PBS	7.1	Phosphate buffer with saline (10 nM Na_2HPO_4, 3 mM KH_2PO_4, 100 mM NaCl)	0%	—	—
PBST	7.2	PBS + 0.05% Tween 20	0%	30.3%	[22]
PALM	9.5	High Salt, high-pH buffer (50 mM $Na_2B_4O_7$-$10H_2O$, 0.75 M KCl, 10 mM $C_6H_8O_6$, 0.075% Tween-20)	18.4%	17–70%	[21]
SHAN	7.3	Artificial marin worm gut fluid mimic buffer (355 mM NaCl, 25 mM Na_2SO_4, 7.9 mM KCl, 2 mM $NaHCO_4$, 11.9 mM, 46 mM $MgCl_2$-$6H_2O$, 13 mM sodium taurocholate, 0.1% BSA)[d]	0%	64–116%	[22]
CALLUS	6.6	Buffer for LTB[e] extraction from maize kernel (25 mM sodium phosphate buffer pH 6.6, 100 mM NaCl, 1 mM EDTA,[f] 0.1% Triton X-100, 10 μg/ml Leupeptin, 0.25 mM Pefablock SC)	0%	—	[10]

[a] PBS = Phosphate buffered saline.
[b] Recovery from the Webster-Nicollet soil (initial concentration: 100 ng CTB/g soil, extracted three times by a bench-top shaker with 3 ml of pH 9.5 PALM buffer).
[c] Bt = *Bacillus thuringiensis* protein.
[d] BSA = bovine serum albumin.
[e] LTB = Heat-labile enterotoxin subunit B.
[f] EDTA = Ethylenediaminetetraacitic acid.

of nontarget organisms to LTB at the highest concentration. A further consideration may be the entry of maize-expressed LTB into an aquatic ecosystem and effects on aquatic organisms in cases where the cropped field is close to waterways.

In order to understand the implications of this exposure, the fate of recombinant proteins in the environment must be understood. If the protein is highly persistent in the environment and retains its bioactivity, it would increase the duration and intensity of environmental exposure to nontarget organisms. High persistence also could pose a concern for bioaccumulation, although proteins, in general, are rapidly degraded by digestion.

In the present study, persistence of maize-expressed LTB in soil and water was evaluated under laboratory settings to provide information relevant to the quantitative ecological risk assessment of future open-field LTB production. Bacterially expressed LTB also was considered as an appropriate comparator for evaluation of the effects of LTB expressed in maize. Because of limited bacterial LTB availability, *Vibrio cholerae* enterotoxin subunit B (CTB), a close analog to LTB and a potent subunit mucosal vaccine against cholera toxin, was investigated as a surrogate compound in addition to LTB. The expression of CTB in several plants as PMP has been conducted [3]. Extraction and quantification methods for both LTB and CTB in soil were developed and may provide insights as to the future approaches for studies of novel transgenic proteins.

MATERIALS AND METHODS

Sample proteins

Three highly immunogenic proteins, *V. cholerae* bacterial CTB, *E. coli* bacterial LTB, and *E. coli* maize-expressed LTB, were used in the present study. Both CTB and LTB are known to be highly similar in function and structure having over 75% amino acid sequence homology [19,20], but LTB contains slightly more acidic residues as compared to CTB [20]. Bacterial CTB was obtained from Sigma-Aldrich (Saint Louis, MO, USA) and stored at 3°C as 500 μg CTB/ml water solution. Bacterial LTB was obtained from John Clements of the Tulane University Medical Center (New Orleans, LA, USA) as 1,000 μg LTB/ml water solution and stored at 3°C. A transgenic maize *Zea mays* event expressing LTB was developed and grown by the Iowa State University Plant Transformation Facility (Ames, IA, USA). As described in Chikwamba et al. [9], this transgenic maize expresses LTB specifically in kernels using the maize endoplasm specific γ-zein promoter. Fourth generation LTB-maize produced in 2005 confined field trails was used in the present study. Naturally dried kernels (moisture contents at 9.2% w/w) were stored in a freezer at −20°C until being used by the end of 2006. Finely ground, dried LTB-maize kernels, passed through 425-μm mesh openings were used directly without further purification. The LTB expression level in ground LTB-maize kernels was determined as 30.17 μg LTB/g dry kernel using the method described in Beyer et al. [10] immediately before starting the experiments.

Enzyme-linked immunosorbent assay

The quantities of LTB and CTB in soil extracts and water samples were determined by ganglioside-dependent enzyme-linked immunosorbent assay (ELISA) as described in Beyer et al. [10]. Briefly, 96-well flat-bottom microplates were coated with 2.5 μg/well of ganglioside GM_1 dissolved in phosphate buffered saline ([PBS], 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). After blocking wells with 5% weight/volume dry milk in PBS (Table 1), samples containing LTB or CTB were spiked to each well. After 1 h of incubation at 37°C, LTB was detected by incubating with rabbit anti-LTB antibody (diluted 1:10,000 in PBS with 1% w/v milk; Immunology Consultants Laboratory, Newberg, OR, USA) and CTB was detected by incubating with rabbit anti-CTB antibody (diluted 1:20,000 in PBS with 1% w/v milk; Sigma). To detect bound rabbit antibody, biotin-conjugated goat anti-rabbit immunoglobulin G (diluted 1:5,000 in PBS with 1% w/v milk; Sigma) was spiked, and this second goat antibody was detected with streptavidin-conjugated horseradish peroxidase (diluted 1:1,000 in PBS with 1% w/v milk PBS w/w; Thermo Fisher). After a 30-min incubation with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate buffer (0.1 M citric acid, 0.55 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), pH 4.25), the absorbance at 405 nm was measured on a THER-

Table 2. Soil characteristics[a]

Series[b]	Type	Texture (%)			OM[c] (%)	pH[d]	CEC[e] (cmol/kg)	Water content[f] (%)	Matric potential[g] (kPa)	Notes
		Sand	Silt	Clay						
Webster-Nicollet	Loam	47	36	17	2.6	6.8	14.9	12.4	−1,500	Surface soil
Canisteo	Clay loam	36	34	30	4.0	6.0	20.1	17.7	−400	Surface soil
Hanlon	Sand	90	6	4	0.5	7.3	6.9	0.4	−20	Subsoil (80-cm depth)

[a] Analysis by Midwest Laboratories (Omaha, NE, USA; http://store.midwestlabs.com/wfdata/frame373-1066/File6.pdf).
[b] Taxonomy classes: Webster-Nicollet is fine-loamy, mixed, mesic Aquic Hapludalf; Canisteo is fine-loamy, mixed, mesic Aquic Hapludalf; Hanlon is coarse-loamy, mixed, superactive, mesic Cumulic Hapludolls.
[c] OM = Organic matter, Walkley Black titration method.
[d] Soil pH: Soil water (1:1) determined by a glass electrode pH meter.
[e] CEC = Cation exchange capacity: Ammonium replacement method.
[f] Gravimetric moisture content of field-sampled soil.
[g] As used.

MOmax™ microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) with quantification by SOFTmax™ software (Molecular Devices, Sunnyvale, CA, USA). A standard curve was prepared using bacterially produced proteins (bacterial LTB or CTB) diluted in the same pH extraction buffer as for sample extracts. The linear range of the standard curve was 1.25 to 12.0 ng/ml and dilutions of sample extracts were made with control extracts, because they have similar soil–water conditions without containing target proteins.

Water description

Two different types of water were used in the present study: Purified water that has minimal suspended materials with no biological activity (Nanopure D4741; Barnstead International, Dubuque, IA, USA) and pond water collected at the Iowa State University Horticulture Research Farm (Ames, IA, USA) immediately before initiating the experiment. Pond water was collected at the depth of 10 cm under the surface and neither filtration nor autoclaving was conducted. The purified water was pH 7.0 and the pond water was pH 8.1.

Soil description

Three soils collected from maize fields near Ames were used in the present study. The freshly collected soils were sieved within 14 d using 2-mm mesh steel sieve and stored moist at 3°C. Soils were maintained at the moisture content as sampled in all subsequent studies. Characterization of sieved soils was conducted by Midwest Laboratories (Omaha, NE, USA; Table 2). Neither LTB nor CTB was detected when the unamended soils were assayed.

Extraction method development

Extraction method development was conducted primarily using bacterial CTB due to limited availability of bacterial LTB. After the establishment of the bacterial CTB extraction method from soil matrices, the method was applied to the extraction of both bacterial and maize-expressed LTB. For the extraction method development, two different shaking methods, an orbital bench-top shaker (Shaker DO-10L; ELMI, Riga, Latvia) and a high-speed shaker (Geno/grinder 2000; Spex CertiPrep, Matuchen, NJ, USA), were compared under different extraction conditions. Three replications of each sample were prepared throughout the present study and error bars in graphs indicate the 95% confidence intervals calculated using the *t*-distribution.

Extraction buffer optimization

Extraction buffer composition was optimized by testing five different buffers and water (Table 1). Both the PALM [21] and SHAN [22] buffers are reported to be effective for the extraction of *Bacillus thuringiensis* (Bt) insecticidal proteins from various soils and, therefore, were used as the start point for method development. Extractability also was compared using PALM buffers of varied pH (pH 5.5–9.5). The pH of extractants was adjusted through treatment with hydrochloric acid or sodium hydroxide. For the comparison of buffers, bacterial CTB diluted in water was spiked to 2 g of soil in 50-ml polypropylene centrifuge tubes at the concentration of 100 ng CTB/g soil. After 30 min of aging at room temperature, 3 ml of extractant was added to each tube and all tubes were shaken for 20 min on a bench-top shaker at 400 strokes/min. After shaking, samples were centrifuged for 3 min at 5,000 *g* and the supernatants were removed to fresh 50-ml tubes. This extraction process was repeated three times in total, and pooled extracts were analyzed by ELISA.

Bench-top shaker-based extraction condition optimization

On the basis of initial determinations, pH 9.5 PALM buffer was selected as the preferred extraction buffer, and further refinements of conditions (i.e., shaking method, buffer quantity, extraction duration and frequency) for bacterial CTB extraction from soil were considered using this buffer. Conditions for the extraction of bacterial CTB using a bench-top shaker were optimized as follows: To determine the optimal buffer quantity, 8 or 15 ml of pH 9.5 PALM buffer were added to 2 g of soil containing 300 ng CTB/g soil. The soil-buffer mixtures in 50-ml polypropylene centrifuge tubes were shaken for 7 or 20 min at 400 strokes/min and centrifuged for 3 min at 5,000 *g*. After removing the supernatants, this extraction process was repeated six times in total, and the recovery of bacterial CTB in the first three extracts and in the combined extracts was determined by ELISA.

Geno/grinder-based extraction condition optimization

For the optimization of Geno/grinder-based extraction conditions, 2 g of soil containing 300 ng CTB/g soil was prepared in 15- or 50-ml polypropylene tubes depending on the extraction buffer quantity. To enhance the extractability, two 4-mm stainless steel balls were placed in each tube and shaken together with soil. For the extraction, 8 or 15 ml of pH 9.5 PALM buffer was added to soil samples, and Geno/grinding

Table 3. Recovery[a] of proteins from soils

Soil series	Bacterial CTB[b] (%)	Bacterial LTB[c] (%)	Maize-expressed LTB[d] (%)
Webster-Nicollet	57.0 (±3.0)	64.9 (±11.2)	38.9 (±2.2)
Canisteo	51.4 (±6.3)	—	17.3 (±0.3)
Hanlon	30.4 (±10.6)	—	81.3 (±12.2)

[a] Extraction buffer: 8 ml of pH 9.5 PALM buffer [21]. Shaking method: Bench-top shaking for 20 min/extraction at 400 strokes/min. Extraction repetitions: Six times for Webster-Nicollet and Canisteo; four times for the Hanlon soil. Initial protein concentrations for spike-recovery test.
[b] Bacterial CTB (*Vibrio cholerae* enterotoxin subunit B): 300 ng CTB/g soil.
[c] Bacterial LTB (*Escherichia coli* heat-labile enterotoxin subunit B): 300 ng LTB/g soil.
[d] Maize-expressed LTB: 20 mg LTB-maize (603 ng LTB)/g soil.

was conducted at 1,100 strokes/min for 1 or 4 min. After Geno/grinding, samples were centrifuged for 3 min at 5,000 *g*, and the supernatants were removed to fresh tubes. This extraction process was repeated six times in total, and the recovery of bacterial CTB in the first three extracts and in the combined extracts was determined.

LTB extraction method development

The optimized bacterial CTB extraction method was applied to the extraction of both bacterial and maize-expressed LTB from soils. For the maize-expressed LTB extraction method development, the spike recovery was determined for 40 mg of ground LTB-maize, which was uniformly mixed into 2 g of soil in 50-ml polypropylene centrifuge tubes. Based on the measured LTB expression level in kernels (30.17 μg LTB/g kernel), the initial concentration of maize-expressed LTB was 603.4 ng LTB/g soil. After adding 8 ml of pH 9.5 PALM buffer, samples were shaken either by a bench-top shaker for 20 min or by a Geno/grinder for 1 min. The extraction was repeated a total of six times, and the amount of maize-expressed LTB in the first three extracts and in the combined extracts was determined.

Soil extraction method for environmental fate study

The most efficient and practical extraction method on the basis of method development was used for the environmental fate study. Eight milliliters of pH 9.5 PALM buffer was added to 2 g of treated soils, and then the soil-buffer mixtures were shaken for 20 min at 400 strokes/min using a bench-top shaker. After 3 min of centrifugation at 5,000 *g*, sample supernatants were removed to fresh tubes. In considering the difference of protein affinity for each soil, this extraction process was conducted a total of six times for the Webster-Nicollet and Canisteo soils, and a total of four times for the Hanlon soil. Pooled extracts were analyzed by ELISA immediately after extraction to quantify the LTB or CTB concentration in the extracts. Spike-recovery rates from different soils under this extraction method are summarized in Table 3.

Bacterial CTB and LTB water dissipation study

To observe the aquatic dissipation of bacterially produced proteins, bacterial CTB and LTB were added to purified and pond water, and the change in protein concentration was monitored over time. For the present study, bacterial CTB was diluted into 22 ml of water in loosely capped 50-ml glass vials and bacterial LTB was diluted into 13 ml of water in 20-ml glass vials. Initial concentrations of both bacterial proteins were 200 ng/ml. After 30 min of stabilization at room temperature, vials were placed in environmental chambers set at 24 or 36°C. Because there was no effect of lighting conditions (dark or a 16:8-h fluorescent light:dark cycle) on the dissipation of CTB and LTB (data not shown), results are for studies where lighting conditions were not considered. At each sampling time, 1-ml samples were collected from well-shaken glass vials, placed in 2-ml microcentrifuge tubes, and stored at −80°C until being analyzed all at once by ELISA. Preliminary measurements showed markedly different dissipation patterns for CTB versus LTB, so sampling times were adjusted accordingly. Sampling times were 0, 6, 12, 24, 36, 48, and 72 h for bacterial CTB, and 0, 4, 7, 15, and 28 d for bacterial LTB. Samples were stored a maximum of 4 d for bacterial CTB and 30 d for bacterial LTB prior to analysis. For ELISA quantification, samples were diluted with an equal volume of pH 7.0 PALM buffer to adjust sample pH, and diluted samples were spiked directly to ganglioside GM_1–coated wells. Bacterial proteins for development of the standard curve were diluted with 50:50 mixture of purified water and pH 7.0 PALM buffer.

Maize-expressed LTB water dissipation study

Ground LTB-maize kernel (150 mg) was spiked into 24 ml of purified or pond water in loosely capped 50-ml polypropylene centrifuge tubes. Based on the measured LTB expression level in maize kernels (30.17 μg LTB/g kernel), the initial concentration of maize-expressed LTB was 188.6 ng LTB/ml water. Sample tubes were placed in a temperature-controlled room at 24°C and gently bubbled using an air-compressor to maintain aerobic conditions. The volume of treatments was maintained by adding purified water every 4 d to account for evaporation loss. Sample tubes were collected at each sampling time (0, 5, 15, 25, and 45 d) and kept in a −80°C freezer for a maximum of 60 d until being analyzed together.

For the quantification of maize-expressed LTB in water, 12 ml of pH 7.0 PALM buffer was added to each water sample, and sample water-buffer mixtures were shaken for 45 min at 400 strokes/min using a bench-top shaker. After centrifugation at 5,000 *g* for 3 min and removing the supernatants, 8 ml of pH 7.0 PALM buffer was added to plant residue to repeat the extraction twice more. Finally, three extracts were pooled and analyzed by ELISA.

Bacterial CTB and LTB soil dissipation study

For the soil dissipation study, bacterial proteins were spiked into 2 g of soil in loosely capped 50-ml polypropylene tubes at the concentration of 1,500 ng LTB (CTB)/g soil. Purified water was added so as to maintain moisture at the levels sampled from the field (Table 2). After 30 min of aging at room temperature, sample tubes were stored in environmental chambers set at 24 or 36°C under complete darkness. Sampling times for bacterial CTB (0, 6, 12, 24, 48, and 72 h) and bacterial LTB (0, 24, 48, and 96 h) differed due to limited availability of bacterial LTB and somewhat differing patterns of dissipation in preliminary measurements. At each sampling time, tubes were stored in a −80°C freezer for a maximum of 7 d prior to analysis. Samples were thawed immediately before the extraction, and extracted samples were analyzed by ELISA.

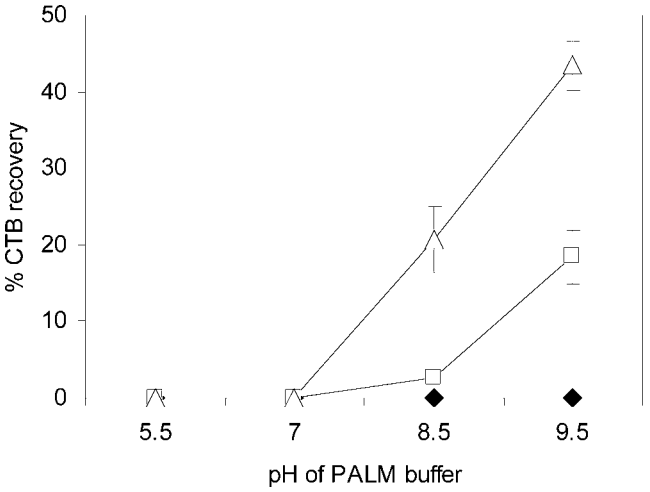

Fig. 1. Extraction buffer pH-dependent difference of *Vibrio cholerae* enterotoxin subunit B (CTB) recovery from soils (□: Webster-Nicollet; ◆: Canisteo; △: Hanlon soil). Extracted three times by a bench-top shaker with 3 ml of different pH PALM buffers [21]. Initially spiked CTB concentration was 100 ng CTB/g soil.

Maize-expressed LTB soil dissipation study

Ground LTB-maize (150 mg) was mixed uniformly into 2 g of soils in loosely capped 50-ml polypropylene centrifuge tubes. Based on the measured LTB expression level in maize kernels (30.17 μg LTB/g kernel), the initial concentration of maize-expressed LTB was 2,263 ng LTB/g soil. This initial concentration represents greater than 1,000-fold the high-end field exposure concentration of maize-expressed LTB (176,667 dropped kernels per hectare at harvest [18], 0.3 g representative kernel weight, uniform incorporation to a 7.5-cm depth in soil with bulk density 1.33 Mg/m^3). After 30 min of aging, sample tubes were placed in environmental chambers set at 24 or 36°C under complete darkness. Purified water was spiked to soils every 4 d to maintain the soil moisture level as previously described for the bacterial CTB and LTB soil dissipation study. Sample tubes were collected at each sampling time (0, 5, 10, 25, 45, and 100 d) and kept in a −80°C freezer for a maximum of 105 d until being extracted and analyzed by ELISA.

RESULTS AND DISCUSSION

Extraction buffer optimization

Among five different buffers and water, only the high-salt, high-pH buffer worked effectively for the extraction of bacterial CTB from the Webster-Nicollet soil (Table 1). This buffer originally was described in Palm et al. [21] for the extraction of bacterial and cotton-expressed Bt Cry1A proteins from soil. Palm et al. [21] reported that Bt protein recovery from soil was greater with alkaline buffer condition, and this pH-dependent effect on protein recovery also was observed for bacterial CTB (Fig. 1). As shown in Figure 1, the extraction of CTB from the Canisteo soil was more difficult than the extraction from the Webster-Nicollet and Hanlon soils. This low recovery of CTB from the Canisteo soil can be explained by the difference of protein affinity for soils. Previous studies [23–25] on the environmental fate of proteins indicated that both bacterially produced and maize-expressed Bt proteins were adsorbed rapidly to clay and humic acid components of soil, and the bound protein would not be desorbed readily. Because the Canisteo soil has higher clay and organic matter contents with higher cation exchange capacity than the other soils in the present study, it is presumed that CTB similarly may be subject to stronger adsorption and irreversible binding to suppress its recovery from this soil.

Although a higher pH buffer worked more effectively for the extraction of bacterial CTB, our preliminary studies indicated that the sensitivity of ganglioside-dependent ELISA is pH-dependent, and the sensitivity is maximized under neutral conditions (data not shown). It also was confirmed that pentameric bacterial CTB is denatured in pH 12.5 or higher PALM buffer (data not shown). Based on these observations, neutral buffer is preferred for sensitive detection of CTB and LTB by ELISA. As an example of the use of the neutral buffer, Shan et al. [22] reported the remarkable improvement of Bt protein recovery from soil using artificial marine invertebrate gut fluid mimetic buffer, and this SHAN buffer was tried for the extraction of bacterial CTB. Although Shan et al. [22] reported over 80% recovery of Bt proteins (Cry1Ab, Cry1Ac, Cry1F) from clay-rich soil, the same buffer did not work effectively for the extraction of bacterial CTB (Table 2). Considering the extractability and detection sensitivity, pH 9.5 PALM buffer was chosen as the preferred buffer for further optimization of extraction conditions.

Extraction condition optimization

Extraction conditions strongly influenced the recovery of bacterial CTB. Fundamentally, a larger quantity of pH 9.5 PALM buffer worked more effectively for both bench-top shaker-based and Geno/grinder-based extraction of CTB from the three soils (Fig. 2). Although Figure 2 shows the recovery of CTB from soils using only 8 or 15 ml of extraction buffer, our preliminary study also indicated the extraction with 8 ml of buffer achieved two- to threefold more recovery of CTB than with 3 to 5 ml of buffer both under bench-top shaking and Geno/grinding (data not shown). This volume effect was especially important for the extraction from the clay-rich Canisteo soil, which has a stronger affinity for CTB.

Optimal shaking duration differed depending on soil types and the shaking method. In the case of bench-top shaking, 20 min of shaking per extraction achieved better recovery of CTB than 7 min of shaking for any of the soils (Fig. 2). On the other hand, the most effective Geno/grinding duration varied among soils; that is, 1 min of Geno/grinding per extraction worked the most effectively for the Hanlon soil, and no large time-dependent difference was observed for the Webster-Nicollet or Canisteo soils (Fig. 2). Varied extraction efficiencies from these soils most likely are due to difference in protein affinity for retention in the soil solid phase. However, it also could relate to rapid loss of activity through degradation, denaturing, or failure to form pentamers, because these factors would be expected to differ among soils.

Because of different protein affinity among the three soils, the number of sequential extracts necessary for optimal CTB and LTB recovery varied among the soils. Although most bacterial CTB (Fig. 2) and maize-expressed LTB (Fig. 3) was extracted in one to three extracts from the Hanlon soil, these proteins still were detected in four to six sequential extractions from the Webster-Nicollet and Canisteo soils. As a result of sequential extraction and the analysis of each extract from the Hanlon soil, the largest quantity of LTB and CTB was extracted in the first extract, and little was detected after four extractions with either bench-top shaking or Geno/grinding (data not shown). On the other hand, LTB and CTB still were quanti-

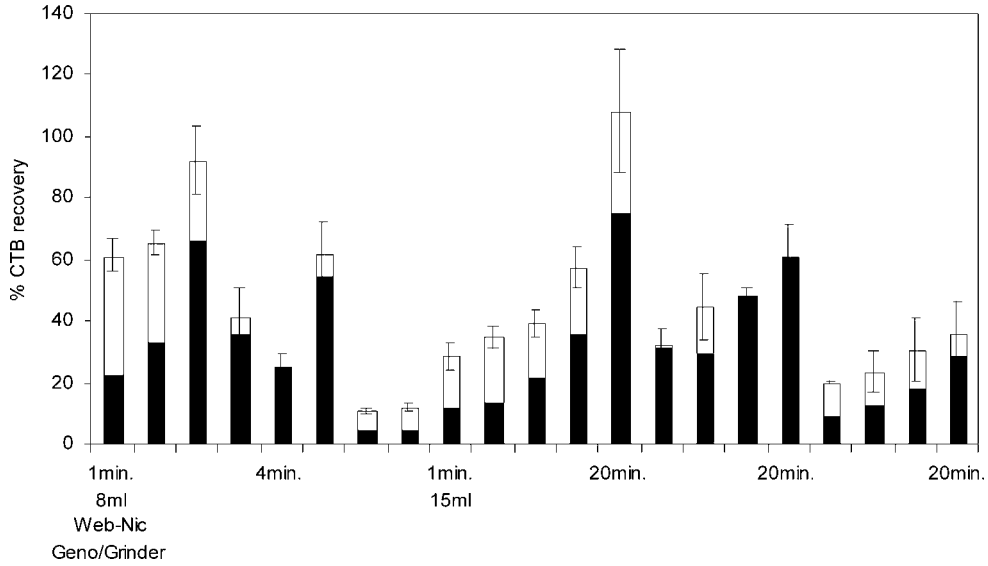

Fig. 2. *Vibrio cholera*e enterotoxin subunit B (CTB) recoveries from soils under different combinations of extraction conditions. Extracted six times with 8 or 15 ml of pH 9.5 PALM buffer [21], and CTB recovery in the first three extracts and entire extracts were measured. Initially spiked CTB concentration was 300 ng CTB/g soil. □: 4–6 extracts; ■: 1–3 extracts. Web-Nic = Webster-Nicollet.

fiable from the Webster-Nicollet soil even in the sixth extract both after bench-top shaking and Geno/grinding (data not shown). Although Shan et al. [22] reported the efficiency of using a Geno/grinder for the extraction of Bt protein from soil, no large difference was observed between the use of a Geno/grinder and a bench-top shaker for the extraction of bacterial CTB (Fig. 2) and maize-expressed LTB (Fig. 3) under the conditions employed here. However, the reduction of extraction time by using a Geno/grinder is advantageous for handling a large number of samples.

Conclusion for extraction method development

On the basis of this method development, the best available soil extraction method for LTB and CTB involved adding 8 to15 ml of pH 9.5, high KCl (PALM) buffer per 2 g of soil; shaking with either a bench-top shaker (20 min) or Geno/grinder (1 min for the Hanlon soil, 4 min for the Webster-Nicollet and Canisteo soils); and sequential extraction (4 times for the Hanlon soil, 6 times for the Webster-Nicollet and Canisteo soils). Spike recoveries ranged from 17.3% (maize-expressed LTB in the Canisteo soil) to 81.3% (maize-expressed LTB in the Hanlon soil) when using the high-pH PALM buffer and the optimized extraction conditions (buffer quantity: 8 ml; Table 3. The overall mean recovery for any soil or protein source was 48.7%. This optimized extraction method was used for the subsequent environmental fate studies.

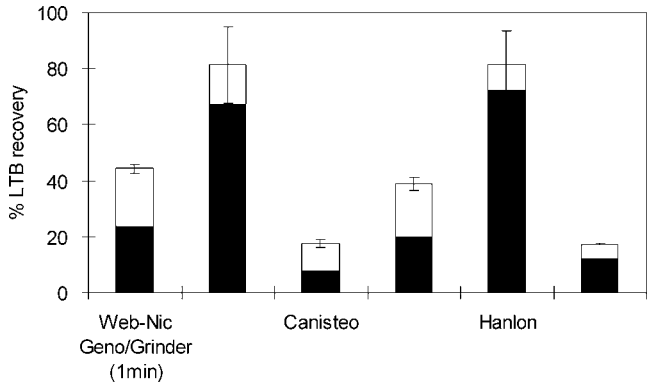

Fig. 3. Maize-expressed *Eschericia coli* heat-labile enterotoxin subunit (LTB) recoveries from soils. Extracted six times with 8 ml of pH 9.5 PALM buffer [21], and LTB recovery in the first three extracts and entire extracts were measured. Initially spiked LTB-maize concentration was 40 mg kernel (603.4 mg LTB)/g soil. ■: 1–3 extracts; □: 4–6 extracts. Web-Nic = Webster-Nicollet.

LTB and CTB dissipation in water

Dissipation of proteins in water was studied for bacterial CTB, bacterial LTB, and maize-expressed LTB. The average spike recovery of protein was 89.3 (±11.0)% for bacterial CTB, 86.1 (±8.2)% for bacterial LTB, and 60.7 (±4.2)% for maize-expressed LTB. These recovery rates were determined by the comparison of the detected concentration in the time-zero samples and the initially spiked nominal concentration. The nominal concentration of maize-expressed LTB was calculated based on the measured expression level of 30.17 μg LTB/g kernel using the method of Beyer et al. [10].

Bacterial CTB rapidly dissipated from water within 2 d regardless of test conditions, and there was no effect of water source or temperature (Fig. 4A). The dissipation of bacterial LTB from water was markedly different from that of bacterial CTB. Although most bacterial LTB dissipated within 5 d in pond water, it persisted in purified water, and could be detected 28 d following addition (Fig. 4B). Incubation temperature had no clear effect on the dissipation of bacterial LTB. Dissipation of maize-expressed LTB also was more rapid in pond water than in purified water (Fig. 5). Maize-expressed LTB in purified water could be detected 45 d after application, although most maize-expressed LTB dissipated within 15 to 30 d in pond water.

Both bacterial and maize-expressed LTB were more persistent in purified water than in pond water. Among several potential factors that influence the fate of LTB, the difference in microbial activities would appear to be the most reasonable cause for the source-dependent difference in LTB dissipation. Because pond water is much more biologically active than purified water, it is expected that microbes in pond water but not in purified water might actively degrade maize materials, including LTB. In addition to the biological activities, the dif-

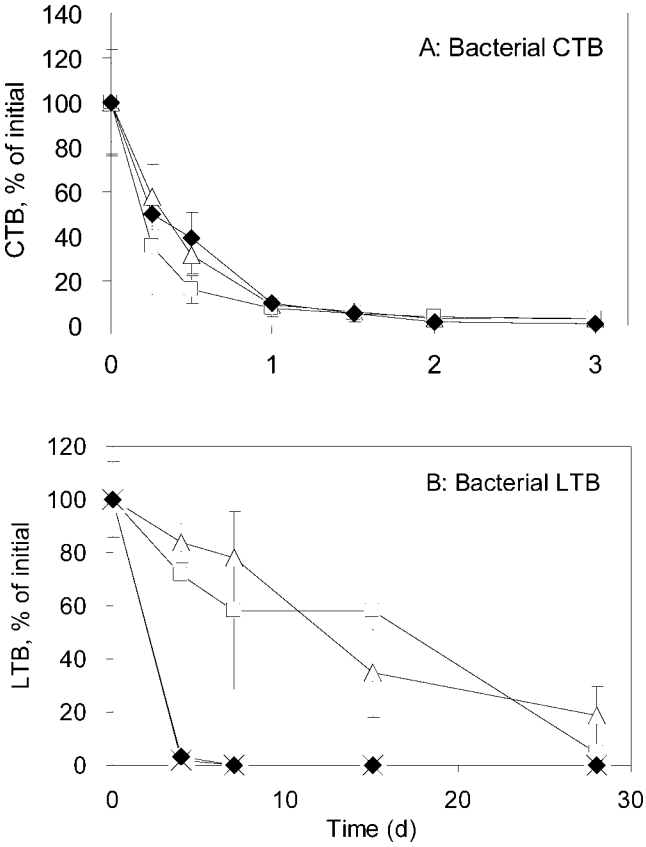

Fig. 4. Bacterially produced protein (**A**) *Vibrio cholera*e enterotoxin subunit B (CTB), (**B**) *Escherichia coli* heat-labile enterotoxin subunit (LTB) dissipation in different conditions of water (△: Purified water at 24°C; □: Purified water at 36°C; ◆: Pond water at 24°C; ×: Pond water at 36°C [only for LTB]). Initial protein concentration was 200 ng/ml. Dissipation is shown as percent of time zero–detected concentration.

ference of water physicochemical properties (i.e., water pH, hardness) needs to be considered, because it might potentially affect the rate of protein denaturing. Pond water contains colloidal organic and inorganic particles, and irreversible binding to those suspending materials is also a possible factor affecting LTB dissipation. Although purified water contained extremely low initial biological activity with no suspending materials, dissipation of LTB in purified water still was observed. This could be due to denaturing of pentameric LTB (the ELISA method used only detects pentamers), loss of sterility over time, or time-dependent irreversible binding of LTB to glass vials.

Although previous studies have shown that LTB and CTB are highly homologous [19,20], the aquatic dissipation of these bacterial proteins showed clear differences. The slightly more acidic nature of LTB relative to CTB [20] may subtly affect reactivity. The most apparent difference in the aquatic fate of these bacterial proteins was the dissipation in purified water. Unlike bacterial LTB, which was relatively persistent in purified water, bacterial CTB dissipated within a day even in nonbiologically active purified water. This observation suggests that abiotic denaturing may be a major cause of bacterial CTB dissipation in water. Because various factors (i.e., protein source, purity, method of preparation) are different between bacterial CTB and LTB used in the present study, and dissipation studies for these proteins were not conducted at the same time, it is difficult to determine the cause for differences

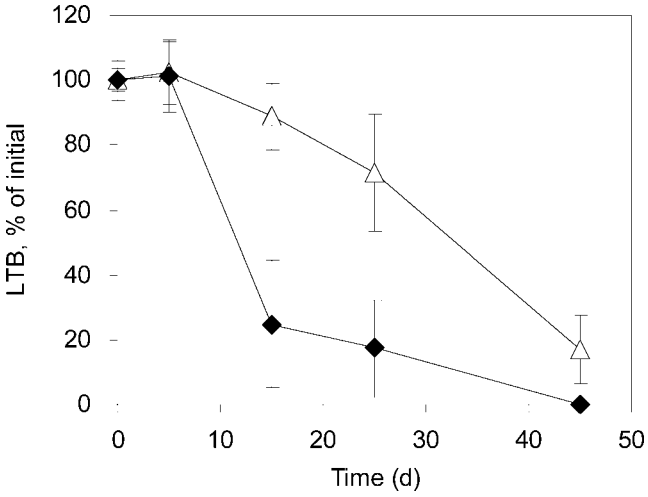

Fig. 5. Maize-expressed *Escherichia coli* heat-labile enterotoxin subunit (LTB) dissipation in different water (△: Purified water; ◆: Pond water) at 24°C. Initial LTB-maize concentration was 6.25 mg kernel (188.6 ng LTB)/ml. Dissipation is shown as percent of time zero–detected concentration.

in dissipation from water. In any case, neither of these bacterial proteins was persistent in pond water, which represents a more realistic field condition.

LTB and CTB dissipation in soils

The extractable pool of bacterial CTB applied to the Webster-Nicollet soil rapidly declined to less than 50% of the initial amount within 1 d (Fig. 6A). Unlike the dissipation in water, the dissipation rate of bacterial CTB in soil was temperature-dependent with dissipation markedly more rapid at 36°C than at 24°C. Although the time to dissipation of 50% of the initial extractable pool (DT50) of bacterial CTB in soil was <1 d under any condition, 10 to 20% of initially extracted bacterial CTB still was detected even after 3 d at 24°C. For soils incubated at 36°C, the level of bacterial CTB extractable after 1 d was negligible. The dissipation of bacterial CTB in the Canisteo and Hanlon soils showed a similar rapid dissipation trend (DT50 < 1 d) with clear enhancement of the dissipation rate at 36°C (data not shown).

Due to limited supplies of test material, the soil dissipation of bacterial LTB was restricted to the Webster-Nicollet soil. As shown in Figure 6B, most of the extractable phase of bacterial LTB was not persistent in soil, and it dissipated within 4 d. The temperature-dependent difference was clear; that is, although most bacterial LTB dissipated within 2 d at 36°C, dissipation was slower at 24°C and 14.5% of extractable bacterial LTB at time zero still was detected 4 d after being added to soil.

Dissipation of maize-expressed LTB varied depending on soil type and incubation temperature. Maize-expressed LTB could be detected up to 100 d after incorporation into soil with interesting differences among the three soils (Fig. 7). For the Webster-Nicollet and Canisteo soils, there was an apparent lag in dissipation for the initial 25 to 45 d after LTB-maize was added to soil, after which, dissipation was relatively rapid (Fig. 7A and B). Furthermore, dissipation was more rapid at 36°C than at 24°C in the Webster-Nicollet and Canisteo soils. On the other hand, no time-lag or temperature-dependent differences were observed in the Hanlon soil (Fig. 7C).

Dissipation of CTB and LTB in soils was affected markedly

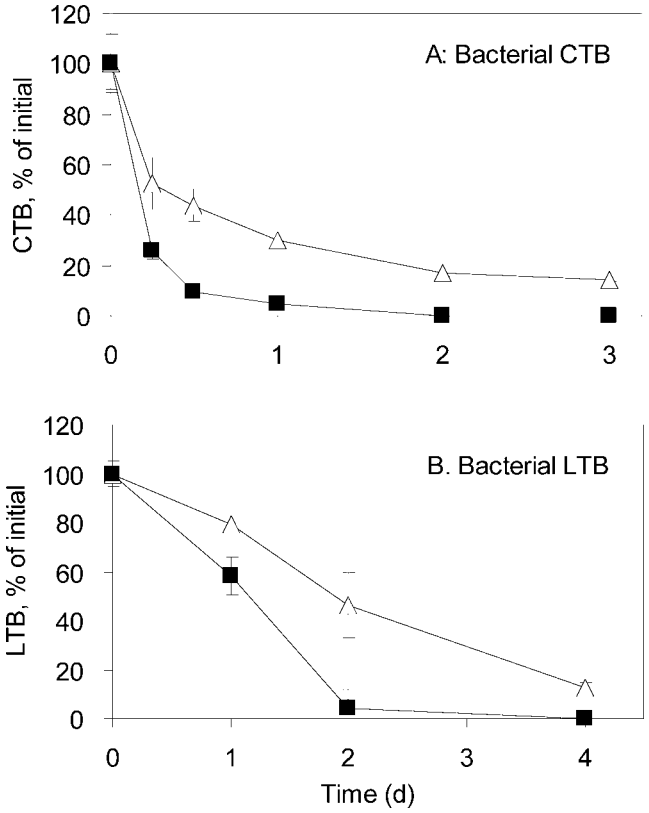

Fig. 6. Bacterially produced protein (**A**) *Vibrio cholera*e enterotoxin subunit B (CTB), (**B**) *Escherichia coli* heat-labile enterotoxin subunit (LTB) dissipation in the Webster-Nicollet soil. Samples were incubated at 24 (△) or 36°C (■) under complete darkness. Initial protein concentration was 1,500 ng/g soil. Dissipation is shown as percent of time zero–detected concentration.

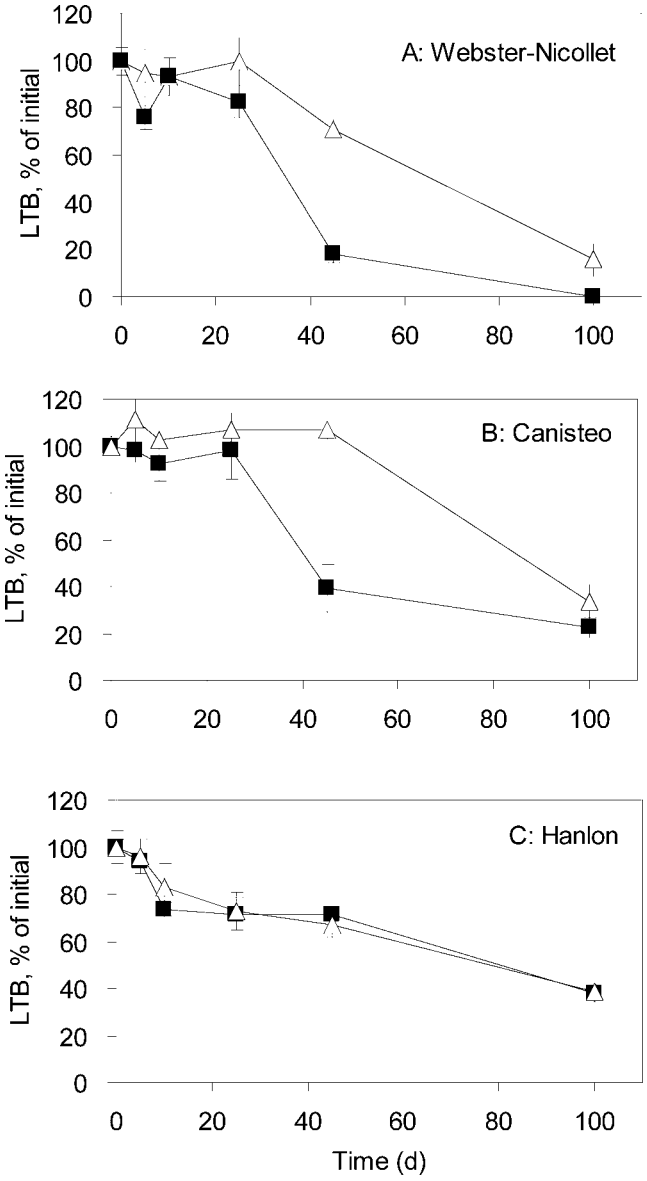

Fig. 7. Maize-expressed *Escherichia coli* heat-labile enterotoxin subunit (LTB) dissipation in different soils: (**A**) Webster-Nicollet, (**B**) Canisteo, (**C**) Hanlon soil. Samples were incubated at different temperature (△: 24°C; ■: 34°C) under complete darkness. Initial LTB-maize concentration was 75 mg kernel (2,263 ng LTB)/g soil. Dissipation is shown as percent of time zero–detected concentration.

by incubation temperature except for the dissipation of maize-expressed LTB in the Hanlon soil. Previous studies indicate that soil microbial activity, in general, is enhanced strongly at higher temperature [26]. Therefore, enhanced soil microbial activities at 36°C might have induced more rapid biological degradation of CTB and LTB in soil. Temperature had no effect on the dissipation of maize-expressed LTB in the Hanlon soil (Fig. 7C). Because the Hanlon soil has low clay and organic matter content and limited available moisture, these factors may be of greater importance to the activity of soil microbes than a change in temperature [27]. Abiotic factors, such as the different rate of protein denaturing or irreversible adsorption to soils, also could have contributed to the temperature-dependent differences in protein dissipation rate.

Soil moisture conditions were maintained at the levels sampled from the field in these studies (Table 1). As a result, moisture was well below field capacity for the Webster-Nicollet and Canisteo soils. Despite these suboptimum conditions for microbial activity, there was relatively rapid dissipation from these soils.

Effect of LTB protein matrix on environmental persistence

The source and purity of LTB may have affected its environmental persistence. In pond water, bacterial LTB dissipated within 5 d (Fig. 4B), but maize-expressed LTB still was detected 25 d after starting the experiment (Fig. 5). As with dissipation in water, maize-expressed LTB was more persistent in soil than bacterially produced LTB. Although most bacterial LTB had dissipated within 4 d at 24°C (Fig. 6B), maize-expressed LTB could be detected even 100 d after incorporation into the Webster-Nicollet soil under the same conditions (Fig. 7A). This clear difference of dissipation rate between bacterial and maize-expressed LTB likely is related to the matrix in which the LTB is present. The source of LTB maize used in the present study expresses LTB only in kernel endoplasm using an endoplasm specific γ-zein promoter. More specifically, transmission electron microscopy observation of immunogold-labeled maize-expressed LTB showed that LTB primarily is localized in starch granules of maize endoplasm [28]. From the standpoint of protein quality, this encapsulation of LTB in starch granules is considered to be advantageous to add resistance against high-temperature, low-pH, peptidase attacks and other factors that inhibit the efficacy of orally administered vaccines [28]. Resistance of maize-expressed vaccines against harsh conditions also is advantageous to reduce

the necessity of a cold-chain transport and storage system, which increases the cost of vaccine distributions, especially in developing countries [8]. From an environmental perspective, however, this encapsulation might add resistance to maize-expressed LTB against both biological and abiotic degradation in the environment and make it more persistent than nonencapsulated bacterial proteins.

Dissipation of maize-expressed LTB shows an initial lag in dissipation in both water and soil. For example, maize-expressed LTB dissipation showed a lag of 5 d after being spiked into pond water (Fig. 5), whereas bacterial LTB started dissipating right after being spiked (Fig. 4B). This delayed dissipation of maize-expressed LTB may be because the LTB must first be released from the seed matrix before it is susceptible to degradation. This lag phase was longer for the dissipation of maize-expressed LTB in the Webster-Nicollet and Canisteo soils. In the Webster-Nicollet soil, there was no apparent dissipation of maize-expressed LTB during the initial 25 d of incubation, after which dissipation occurred at a relatively rapid rate (Fig. 7A). In contrast, bacterial LTB started dissipating right after being spiked into soil (Fig. 6B). Because the biological activity of the Hanlon soil might have been limited, no apparent lag phase was observed for the dissipation of maize-expressed LTB in the Hanlon soil (Fig. 7C).

CONCLUSION

Future study directions

The dissipation of the extractable phase of maize-expressed LTB and related bacterial proteins under relatively conservative laboratory conditions was considered in the present study. Dissipation was strongly influenced by matrix types and incubation temperature. The DT50 of maize-expressed LTB was determined to be 4 to 15 d in pond water and 35 to 90 d in soils. In contrast with maize-expressed LTB, the DT50 for either bacterially produced LTB or CTB was less than 4 d both in pond water and in soil. Maize-expressed LTB was more stable than its bacterially produced analog, and dissipation was governed by an initial lag, which could be attributed to release from the plant material, followed by a rapid decline. The studies reported here give indirect evidence for microbial degradation as the major process governing rapid dissipation of these proteins from soil and water. Definitive studies will be needed, however, to determine the relative importance of biotic versus abiotic processes governing environmental fate of LTB and CTB.

This environmental fate study describes the dissipation of the extractable fraction of protein, whereas the fate of nonextractable (irreversibly bound) fraction of protein still is unclear. Because recovery of both LTB and CTB from fine-textured soil is still low, further improvement in extraction method is needed for more precise understanding of LTB fate in soil. With Bt protein, it is reported that soil adsorbed or irreversible-bound proteins clearly retained insecticidal activity and, in some cases, the toxicity was enhanced as compared to free proteins [29]. It also was observed that soil-bound Bt proteins were less biodegradable than free proteins [29]. These observations emphasize the importance of proper understanding of the fate and bioavailability of soil-bound LTB.

In the present study, only the persistence of the parent protein was observed because ganglioside-dependent ELISA specifically detected pentameric LTB (CTB) and the denatured protein could not be detected. This specific detection of pentameric LTB is important in terms of bioavailability, because pentameric LTB, which binds specifically to intestinal ganglioside GM_1, is more immunogenic than denatured monomers [30]. Further observation of the fate of degradation products, such as LTB monomer, would help to provide more comprehensive understanding of LTB fate in soil. For further observation of degradation products, the use of other analytical methods, such as the Western-blot analysis or high-performance liquid chromatography, may prove useful because these methods may accurately separate degradation products from pentameric parent proteins.

Finally, devitalized seeds (ground maize kernels) were used in the present study to eliminate the negligent spread of viable transgenic seeds. However, in the actual maize field, introduction of LTB to the soil environment will be in the form of intact kernels and this would affect the pattern of dissipation.

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