Biomimetic Extraction of Bacillus thuringiensis Insecticidal Crystal Proteins from Soil Based on Invertebrate Gut Fluid Chemistry

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · SEPTEMBER 2005

Impact Factor: 2.91 · DOI: 10.1021/jf0511493 · Source: PubMed

6 AUTHORS, INCLUDING:

Shawna K Embrey
Dow Chemical Company
7 PUBLICATIONS **64** CITATIONS

SEE PROFILE

Jeffrey D Wolt
Iowa State University
80 PUBLICATIONS **1,338** CITATIONS

SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biomimetic Extraction of *Bacillus thuringiensis* Insecticidal Crystal Proteins from Soil Based on Invertebrate Gut Fluid Chemistry

GUOMIN SHAN,*,† SHAWNA K. EMBREY,† ROD A. HERMAN,† JEFFREY D. WOLT,‡ DONALD WESTON,§ AND LARRY M. MAYER||

Dow AgroSciences LLC, 9330 Zionsville Rd, Indianapolis, Indiana 46268, Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames, Iowa 50011, University of California, 3060 Valley Life Sciences Building, Berkeley, California 94720-3140, and Darling Marine Center, University of Maine, Walpole Maine 04573

Quantitative monitoring of *Bacillus thuringiensis* (Bt) insecticidal crystal proteins in soil has been hampered by the lack of efficient extraction/detection methods. A novel approach for simple and effective Bt protein extraction was explored by evaluating extraction solutions from invertebrate gut fluids. Marine worm gut fluids were identified as promising for extracting Bt protein from soil. An artificial gut fluid based on these marine worm gut fluids was developed using commercially available chemicals and was evaluated for its ability to extract Bt proteins from soil. On the basis of experiments with Cry1 proteins, the artificial gut fluid in combination with ELISA was highly effective for protein extraction and analysis in a variety of soil types and was well-correlated with bioassay results. Coupling of immunoassay with this extraction method provides, for the first time, an efficient, accurate, and quantitative assay for routine measurement of Bt protein residues in soil.

KEYWORDS: ***Bacillus thuringiensis*; Bt proteins; soil extraction; gut fluid; ELISA**

INTRODUCTION

Since the introduction of genetically engineered (GE) crops a decade ago, the planting area of GE crops has increased significantly. In 2003, more than 167 million acres of GE crops were planted by 7 million farmers globally (*1*). In particular, crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) have demonstrated clear advantages in crop production, such as reduced use of pesticides, species selectivity, and suitability for insect resistance management (IRM). As Bt crops increasingly dominate crop productions systems, the potential ecological impacts of these crops remain of interest, particularly with respect to fate in soil. The ability to assess potential for GE protein accumulation in soil has been hampered by the lack of efficient quantitative detection methods because of low extractability of Bt proteins from soil (*2*, *3*). Insect bioassay has been the only available method capable of measuring a reasonable fraction of active Bt protein in soil (*2*, *4*−*9*), but this approach is subject to variability and requires considerable effort to obtain quantitative results.

A rapid, selective, and sensitive quantitative method for monitoring protein levels in soils is of significant importance for exposure characterization and environmental risk assessment. Immunoassay is a useful tool for protein quantification (*10*, *11*). Many studies have been reported to detect Bt proteins in soil using commercial immunoassay kits following multiple extractions with a buffer system, however, the extraction efficiency of protein from soils was poor (*2*, *3*, *12*). Thus, a sufficient and simple extraction method is needed in tandem with ELISA for Bt protein monitoring in soils.

Given that invertebrates must have the biochemical tools necessary to extract nutritionally important soil or sediment-bound proteins, and since susceptible insect pests can liberate Bt proteins from soil (*2*, *4*−*9*), we have been exploring the digestive fluid of deposit feeders as a potential in vitro extraction solution for Bt proteins. In this study, we evaluated gut fluids from different invertebrate sources for their ability to extract Bt proteins from soil and developed an artificial gut fluid for efficient soil extraction. Bt proteins present in multiple commercially available GE crops (Cry1Ab, Cry1Ac, and Cry1F) were added to a variety of soils and the extraction efficiency of the fluids was investigated. Results were then compared to those obtained from insect bioassays.

MATERIALS AND METHODS

Materials. The Cry1F, Cry1Ac, and Cry1Ab lyophilized proteins (core toxins) used in this study were expressed in transgenic *Pseudomonas fluorescens* strains and were purified at Dow AgroSciences LLC (Indianapolis, IN).

* Author to whom correspondence should be addressed. Phone: (317) 337-4118; fax: (317) 337-3235; e-mail: gshan@dow.com.
† Dow AgroSciences LLC.
‡ Iowa State University.
§ University of California.
|| University of Maine.

10.1021/jf0511493 CCC: $30.25
Published on Web 00/00/0000 **PAGE EST: 4.9**

Table 1. Characteristics of Soils Employed in the Studies

soil ID#	textural class[a]	moisture content (%)[b]	pH	total % carbon[c]	CEC[d]	particle distribution (%) sand	silt	clay	origin
621	clay loam	25.7	6.3	2.3	23.3	42	28	30	CA
623	loam	40.8	7.7	3.4	45	17	32	51	TX
640	loamy sand	17.9	7.1	0.7	13.8	37	44	19	MS
656	sand	4.8	6.7	0.4	2.8	85	12	3	NC
659	silty clay loam	22.0	5.2	1.2	12	18	50	32	Piedmont Italy
663	silt loam	24.3	7.3	1.2	11.7	20	58	22	IA

[a] On the basis of USDA textural classification (Soil Survey Manual, 1993). [b] Moisture content (%) at 33 kPa. [c] The total carbon was measured using high-temperature induction furnace combustion method. [d] CEC: cation exchange capacity was measured as CEC–NH_4^+ exchange method.

Six physicochemically diverse soils from different geographic locations were used in this study (**Table 1**). Since rapid biodegradation could confound determinations of extraction efficiency, comparisons were made between viable and sterilized soils. Subsamples of field-moist soil were sterilized by gamma irradiation or autoclaving (soil 621only).

Aliquots (150 mg) of field-moist soils were fortified with purified insecticidal crystal protein at an approximate rate of 0.067−0.167 μg per gram moist soil by adding solutions with a small amount of buffer (up to 50 μL). Fortified soil samples were thoroughly mixed with a vortex or Geno/Grinder (Certiprep Inc., Metuchen, NJ). At least two replicates were used for each treatment.

Three biological (insect homogenate and two marine invertebrate gut fluids) and two chemical (phosphate-buffered saline and the artificial gut fluid) extraction systems were evaluated as follows.

Insect Homogenate. Neonate tobacco budworms, *Heliothis virescens*, for bioassay were obtained from the Dow AgroSciences insectary. Fifth-instar tobacco budworms (150) were frozen for 15 min at −20 °C and were homogenized in 5 mL of 0.01 M phosphate-buffered saline (PBS) for 5 min. The homogenate was centrifuged at 10 000*g* for 10 min and the supernatant was transferred into an empty polypropylene tube. The supernatant was immediately used for extraction or was stored at −80 °C for subsequent use.

Marine Invertebrate Gut Fluids. The gut fluids of deposit-feeding marine invertebrates are very effective extraction solutions for hydrophobic organic compounds (*13*). Two species were used for these experiments: an annelid, *Arenicola brasiliensis*, and the echiuran, *Urechis caupo*, both collected from near San Francisco, California. Animals were held in seawater for up to 24 h to allow evacuation of sediments from the gut, and midgut fluids were then removed by dissection. Fluids from the midgut have greater enzyme activities and surfactant concentration relative to more anterior or posterior gut segments (*14*). Fluids from multiple individuals were pooled and stored at −80 °C until use. The biochemistry of the gut fluids has been characterized elsewhere with respect to total organic carbon, total amino acids, lipids, enzyme activities, surfactancy, protein, enzyme activity, and pH (*13*).

Chemical Extraction Systems. Phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 (PBST) was used as obtained from Sigma Chemical Co. (St. Louis, MO). Artificial gut fluid, modeled after a deposit-feeding marine annelid (*15*), consisted of NaCl (5.192 g), Na_2SO_4 (0.869 g), $NaHCO_3$ (0.043 g), KCl (0.147 g), $CaCl_2$ (0.329 g), $MgCl_2$ (2.341 g), sodium taurocholate (1.748 g), and bovine serum albumin (1.25 g) in 250 mL of distilled water. This solution was stored at 2−8 °C for a maximum of 1 week or was kept at −20 °C for long-term storage.

Soil Extraction. Extraction solutions (750 μL) were added to a 2-mL polypropylene tube containing 0.15−0.3 g of field-moist soil as well as two stainless steel beads. The mixture was homogenized with GenoGrinder (Spex Certiprep) set at 1500 strokes per minute for 1 min. The suspension was then centrifuged at 10 000*g* for 2 min, and the supernatant was removed. The pellet was then extracted two more times as described above. All three supernatants were assayed separately.

Immunoassays for Cry1F, Cry1Ac, and Cry1Ab. Three specific sandwich ELISA kits (Cry1F: Catalog Number 7020000; Cry1Ac: Catalog number 7140220; and Cry1Ab: catalog number 7110000) purchased from Strategic Diagnostics, Inc. (SDI) (Newark, DE) were used to quantify the levels of Bt proteins in the extracts. The protein concentration in the pooled extract was diluted two times with PBST, an aliquot of the diluted sample (100 μL/well) was incubated in the wells of a 96-well plate coated with specific antibodies for 1 h, and then the plate was washed four to five times with PBST. Horseradish peroxidase-conjugated antitoxin antibodies (100 μL/well) were added and incubated for 1 h at room temperature. Following another washing step, tetramethylbenzidine substrate solution (from the SDI kit) was added (100 μL/well). The color development was stopped after 15 min with 1 M HCl (100 μL/well), and absorbance readings were made at 450 nm minus 650 nm. All determinations were conducted in triplicate. The resulting color intensity, measured as optical density (OD), is related to the concentration of protein in the sample (i.e., lower protein concentrations result in lower color development). Standard curves were obtained by plotting absorbance analyte concentration, which were fitted to a quadratic equation $y = Ax^2 + Bx + C$. Absorbance measurements were made with a MAXline Vmax microplate reader with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Insect Bioassay. Fifty microliters of purified deionized water (pH 7.5) containing 0.324 mg/mL of Cry1F was applied to 1.0 g of soil. Soil samples were diluted to a final volume of 30 mL with 0.2% agar, and the soil was suspended by shaking. The soil suspensions were serially diluted 3-fold with 0.2% agar to produce a total of three concentrations. Fifty microliters of the suspensions was applied to each well of a CD-International (Pitman, NJ) bioassay tray containing approximately 0.5 mL of Southland Products (Lake Village, AR) multispecies insect diet per well (surface area per well approximately 1.5 cm^2) to produce final concentrations of 18, 6, and 2 ng of Cry1F per cm^2. A single neonate tobacco budworm, *Heliothis virescens*, was placed in each well. Mortality and insect weights were collected for groups of 16 insects after 6 days of exposure to the treated diet. The assay was conducted with one assay containing 16 insects per treatment and a second assay containing 48 insects per treatment.

RESULTS

Screening of Extraction Buffers. The various extraction solutions were compared with regard to recovery of spiked Cry1F protein from autoclaved soil 621. Extraction with PBST recovered 37 ± 6% of the Cry1F over three consecutive extractions (**Table 2**). Insect body fluid which was from whole body homogenates showed improved extraction compared with PBST, however, recovery was still poor (52 ± 8%). The average recovery achieved with *A. brasiliensis* gut fluid after three extractions was 88 ± 10%. Gut fluid from *U. caupo* produced a moderately lower recovery of 72 ± 11%. An artificial fluid mimicking *A. brasiliensis* gut fluid was prepared and tested. This artificial gut fluid appeared to extract all of the Cry1F from the spiked soil sample after three extractions (102 ± 10%). This artificial gut fluid was chosen for further evaluating the extraction of Cry1 proteins from different soils.

Efficiency of Extraction of Bt Proteins from Soil. Three Bt proteins (Cry1F, Cry1Ac, and Cry1Ab) and six different soils were tested using the artificial gut fluid. No significant difference

Table 2. Comparison of Cry1F Protein Extraction Efficiency with Different Buffers[a]

ext buffer	Cry1F spiked (ng/200 mg soil)	total Cry1F recovered (ng)	ext 1 (%)	ext 2 (%)	ext 3 (%)	total recovery (%)
PBST	20	7.5 ± 1.1	12.7	13.5	11.1	37.3 ± 5.5
insect fluid	*20*	*10.3 ± 1.5*	*42.6*	*7.8*	*1.4*	*51.8 ± 7.5*
U. caupo gut fluid	25	17.9 ± 2.1	57.7	9.2	5.1	72.0 ± 10.5
A. brasiliensis gut fluid	25	21.9 ± 2.0	62.4	15.1	10.0	87.5 ± 10.0
artificial fluid	25	25.6 ± 1.9	80	15	7.3	102.3 ± 9.5

[a] The autoclaved soil 621 was spiked with Cry1F in 50 μL solution and was mixed well. Spiked soils were incubated for approximate 30 min at room temperature prior to extraction. Each sample was extracted three times, and the supernatant was assayed separately by ELISA. Recoveries represent the mean and standard deviation of three replicate determinations.

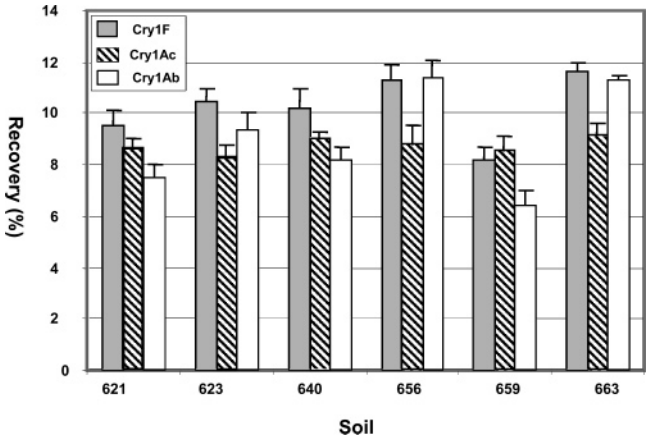

Figure 1. Comparison of protein extraction recoveries in different soils. The soils (150 mg) were spiked with 10 ng Cry1Ab or Cry1Ac or Cry1F in 50 μL of solution and were mixed well. Spiked soils were then incubated for 30 min at room temperature prior to adding artificial gut fluid for extraction. Spiked soils were extracted three times and the pooled supernatants were assayed by ELISA. Each recovery represents three replicate determinations.

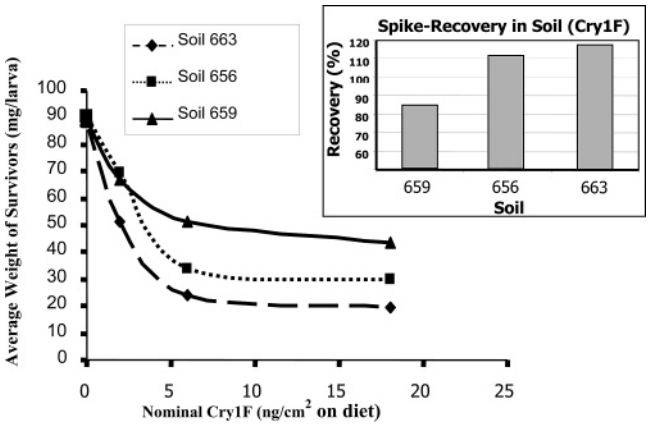

Figure 2. Weight of tobacco budworms exposed to soils containing Cry1F protein. Irradiated soils 659, 656, and 663 were spiked with Cry1F protein and soil suspensions were applied to the surface of insect diet. Diets were fed to neonate tobacco budworms. The mortality and insect weights were recorded after 6 days. The spiked-recovery bars represent the extractability of Cry1F from the soils.

was observed between gamma-irradiated soils and native (nonirradiated) soils when testing Cry1F, Cry1Ab, and Cry1Ac protein recovery (unpublished data). Cry1F showed recoveries ranging from 82% to 116% for gamma-irradiated soils (**Figure 1**). The recoveries for Cry1Ac protein from all soils tested were between 82% and 96% (**Figure 1**). Cry1Ab protein spike recoveries ranged from 64% (soil 659) to 114% (soil 656). Other than soil 659, which has a relatively low extraction recovery (64−84%), no significant extractability difference was observed among the different soil types.

Bioassay of Spiked Soil Samples. Three soil samples (656, 659, and 663) were spiked with Cry1F protein and were tested by insect bioassay for protein recovery. Insect growth inhibition was least for soil 659, followed by 656, and 663, which is consistent with the Cry1F extractabilities using the artificial gut fluid (**Figure 2**).

Persistence of Protein in Soils. Soil 621 (light clay) was chosen to test the protein decay/extractability in aged soils. Solutions containing Cry1Ab, Cry1Ac, and Cry1F were spiked into soils and were incubated at room temperature (ca. 25 °C). The proteins were recovered by extraction with the artificial gut fluid following incubation for 30 min (0.125 day) or 1 day. The average protein recoveries (**Table 3**) were used to approximate the initial degradation rate (k) assuming simple first-order decay:

$$k = \frac{\ln(c_2) - \ln(c_1)}{t_1 - t_2}$$

Table 3. Extractability/Bioavailability of Bt Proteins in a Light Clay Soil (Soil 621)[c]

proteins	protein spiked (ng/150 mg soil)	recovery (%) 30 min (0.125 d)	recovery (%) 24 hours (1 d)	estimated half-life in soil days	literature half-life in soil days
Cry1Ac	10	87 ± 1.7	83 ± 2.8	15	18[a]
Cry1Ab	10	75 ± 4.0	66 ± 3.4	5.5	11[a]
Cry1F	10	92 ± 6.0	46 ± 6.0	1	0.58[b]

[a] From ref *3*. [b] From ref *7*. [c] Samples of irradiated soil 621 (150 mg) were fortified with Cry1 proteins in 50 μL solutions and were mixed well. The samples were kept at room temperature for 30 min and 24 h prior to extraction. The aged soil samples were extracted with artificial gut fluid three times and the supernatants were pooled and assed by ELISAs. Recoveries represent mean and standard deviation for duplicate determinations.

where c_1 and c_2 represent average recoveries at times t_1 (0.0125 d) and t_2 (1 d), respectively. The half-life ($t_{1/2}$) is estimated as 0.693/k. The estimated degradation half-lives (15, 5.5, and 1 days for Cry1Ac, Cry1Ab, and Cry1F proteins, respectively) were relatively consistent with literature half-lives for these proteins as determined by insect bioassay (**Table 3**).

DISCUSSION

ELISA has been widely used as a qualitative and quantitative method for detection of GE protein in plant tissues and in the environment (*10*, *11*), but difficulty in protein extraction from soil has limited ELISA for monitoring proteins in soil. Although protein extractability in soil using buffers is problematic, insect bioassay shows activity of Cry proteins after addition to soils

(*7*−*9*, *16*, *17*). Thus, even though Cry protein residues may be adsorbed and recalcitrant to chemical extracts, those residues may remain in a form which is bioavailable to sensitive insects. For example, Head et al. (*2*) were able to recover approximately 32% of added Cry1Ac spiked to soils using a conventional chemical extraction system and ELISA with a limit of detection of approximately 11.6 ng/g soil, whereas comparable *Heliothis virecens* bioassay detected Cry1Ac at or above 8 ng/g soil.

The ability for insect bioassays to recover adsorbed Cry protein from soils suggests that insect gut fluid effectively extracts Cry proteins from soil. We hypothesized that the investigation of insect gut fluid might assist us in developing an effective artificial fluid for soil extraction. Because of the difficulty in collecting enough insect gut fluid for extraction testing, we used insect whole body fluid for a simple test. Although the extraction recovery was higher than in the commonly used PBST (52% versus 37%), it still was less than desired for routine recovery of bioactive protein residues from soil. One possible reason limiting the extraction recovery of insect homogenates is the effect of proteases in insect fluid, which may degrade the extracted proteins during process. In addition, it is unlikely that the whole body homogenate adequately reflected digestive biochemistry of the tobacco budworm.

We chose to investigate the gut fluid of marine invertebrates because their size facilitated the collection of larger volumes of fluid, their gut fluid is an effective extraction solution from sediments for hydrophobic compounds, and their gut fluid chemistry has been well characterized (*13*). *A. brasiliensis* and *U. caupo* were selected for testing because they are both large (about 15 cm) and yield large volumes of midgut fluid (about 1 mL per individual). Among marine invertebrates, the efficiency of these two gut fluids in extracting the polycyclic aromatic hydrocarbon, benzo(*a*)pyrene, from sediments ranked in the middle of the range (*13*). Investigations with Cry1F in soil demonstrated that *A. brasiliensis* and *U. caupo* gut fluids provided excellent extraction recovery (88% and 72%, respectively) and about 2- or 3-fold better than the more traditional PBST solution. This suggested that such fluid might be a suitable model for an artificial extraction fluid. Because of the limited availability of the marine worms and potential variability between individual worms, simply relying on the collection of natural gut fluid is not feasible as a general laboratory technique. The preferred approach was to develop an artificial fluid using commercially available chemicals on the basis of the composition of the actual gut fluid.

With a thorough study of gut fluid physical and biochemical properties, Mayer et al. (*13*) showed that *A. brasiliensis* gut fluid contained lipids, hydrocarbons, amino acids, surfactants, salts, and digestive enzymes, including esterase, protease, lipase, chitinase, and glucosidase. In general, the interaction of proteins or lipids with the gut lining is a passive process and is a function of the concentration in the digestive fluid. Thus, solubilization of protein in the gut digestive fluid is a critical step in facilitating exposure (*18*). Through studies on hydrophobic organic compounds, such as polycyclic aromatic hydrocarbons, it has been demonstrated that gut fluid solubilization is related to compound bioavailability (*19*, *20*). In an attempt to develop cocktail formulations for hydrophobic contaminants from sediments (*15*), protein and surfactant were discovered as key components for solubilizing target compounds. These two elements are key components in forming hydrophobic microenvironments, which enhances protein solubilization. On the basis of the previous study (*15*), 10 mM sodium taurocholate and 5 g/L bovine serum albumin (BSA) were chosen as components of the artificial gut fluid.

In addition to protein and surfactant content, the ion strength and pH of base buffers play an important role in the system. During investigations of Cry1Ac soil extraction, Palm et al. (*3*) found that high pH favors protein extraction in soil. This is consistent with the improved solubility of Cry1Ac and Cry1F proteins that is seen under basic conditions (Gao, Y.; Herman, R. Dow AgroSciences, personal communication, 2002). It was also concluded that ionic components are important for Bt protein extraction in soil such as KCl (*3*). Marine invertebrate gut fluid contains high concentrations (3−5%) of salts including NaCl, KCl, $CaCl_2$, $MgCl_2$, and Na_2SO_4, which is similar to seawater with a neutral pH. In this study, we chose to use a high-salt artificial seawater with a near neutral pH of 7.2 (*21*) as the base buffer to mimic gut fluid. A high pH solution may enhance protein solubilization, however, it usually causes an adverse effect on the ELISA system, and high dilutions (such as 10×) are needed prior to conducting the ELISA to minimize the high pH interference. Such dilution will decrease assay sensitivity.

The artificial gut fluid was tested in different soil types with three Cry1 proteins. The six soils included in the study were from various geographic locations and had diverse properties that could have influenced recovery of protein residues, including pH (5.2−7.7), % total carbon (0.4−3.4%), and varying particle distributions (**Table 1**). After fortifying soils with the Cry1 proteins at 10 ng per 150 mg soil, very good recoveries (>75%) were generally achieved with artificial gut fluid for all three Cry1 proteins in all six soil samples tested. Exceptions were observed for Cry1Ab with soils 659 where recoveries were lower (64%) but were still much improved over conventional techniques. These acid, clay-textured soils may have had sufficient buffering capacity to reduce the efficiency of protein extraction.

One advantage of using artificial gut fluid as extraction buffer is that it allows direct analysis on ELISA plates. No matrix effects were observed with nondiluted extracts from various soils (data not shown). The limit of detection for this method (constrained by ELISA sensitivity) for all three proteins was <4.5 ng extractable protein per gram soil which is more sensitive than many insect bioassays. This extraction method was also tested with corn and cotton leaf tissues, and extraction of Cry1F from leaf samples was equally efficient (Dow AgroSciences, unpublished data).

Not only did the artificial gut fluid provide a tool to efficiently extract protein from soil, but it also appeared to correlate with the bioavailability of Bt protein in soil (on the basis of insect bioassay). Though the number of comparisons were limited, among the three soils tested (656, 659, and 663), the rank order of extractability by artificial gut fluid matched with the order of protein bioavailability derived by insect assays (**Figure 2**).

The bioavailability or the extractability of protein in aged soils is important for field studies and exposure assessment. Protein degradation by soil microorganisms and irreversible binding with soil particles are considered as major factors that may affect protein availability in soil (*16*). Aging studies with irradiated/sterilized soil indicated that the decay of proteins (or decreased extractable proteins) in this study was mainly caused by irreversible binding or nonmicrobially mediated degradation. One may suspect that protein breakdown or denaturation at room temperature might contribute to the decay. However, a separate study demonstrated that all three Cry1 proteins are stable in

neutral pH buffer at RT up to 2−3 days (unpublished data) suggesting that degradation due to exposure to room temperature was not significant. The estimated half-lives of Cry1Ac, Cry1Ab, and Cry1F protein in soil 621 (clay loam) were 15 days, 5.5 days, and 1 day, respectively (**Table 3**), which is similar to the reported half-lives of pure toxins in soil using bioassays or immunoassays. Herman et al. (*7*, *8*) reported that the half-life of microbial derived Cry1F in light clay soil is 0.6 days. Half-lives of purified core Cry1Ac protein in soil ranged from 18 to 40 days dependent upon soils (*3*, *22*), while Cry1Ab had an approximate half-life of 11 days in a fine sandy loam soil (*3*). This further suggests that the artificial gut fluid may be a useful tool for predicting bioavailability of Bt proteins in soil.

In conclusion, a biomimetic approach was explored to develop an effective means of extracting Bt protein from soil on the basis of invertebrate gut fluids. Coupled with a rapid and sensitive immunoassay, for the first time it is possible to conduct efficient, accurate, and quantitative assays for persistence of Cry1 proteins in soils. The sensitivity of the method was <4.5 ng protein per gram soil. Finally, the good correlation between insect bioassay and the protein extractability from soil suggests that this biomimetic gut fluid may be a useful tool to predict protein bioavailability in the environment and thus aid in environmental risk assessments.

ACKNOWLEDGMENT

We thank Tim Mitel at Regulatory Laboratories of Dow AgroSciences (DAS) for providing soil samples and relevant information and Nick Poletika of DAS for providing technical insight. We thank Laura Tagliani, Penny Hunst, Larry Sernyk, Katherine Armstrong, and John Cuffe of DAS for reviewing the manuscript.

ABBREVIATIONS USED

BSA: bovine serum albumin; Bt: *Bacillus thuringiensis*; ELISA: enzyme-linked immunosorbent assay; GE: genetically engineered; ICP: insecticidal crystal protein; IRM: insect resistance management; PBS: phosphate-buffered saline; PBST: phosphate-buffered saline with 0.05% of Tween 20.

LITERATURE CITED

(1) James, C. Preview: Global status of commercialized transgenic crops: 2003. *ISAAA Briefs*; No. 30; ISAAA: Ithaca, NY, 2004.
(2) Head, G.; Surber, J. B., Jr.; Watson, J. A.; Martin, J. W.; Duan, J. J. No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. *Environ. Entomol.* **2002**, *31*, 30−36.
(3) Palm, C. J.; Donegan, K.; Harris, D.; Seidler, R. J. Quantification in soil of *Bacillus* thuringiensis var *kurstaki δ*-endotoxin from transgenic plants. *Mol. Ecol.* **1994**, *3*, 145−151.
(4) Sims, S. R.; Holden, L. R. Insect bioassay for determining soil degradation of *Bacillus* thuringiensis subsp. *kurstaki* Cry1Ab protein in corn tissue. *Environ. Entomol.* **1996**, *25*, 659−664.
(5) Sims, S. R.; Ream, J. E. Soil inactivation of the *Bacillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: laboratory microcosm and field studies. *J. Agric. Food Chem.* **1997**, *45*, 1502−1505.
(6) Saxena, D.; Folres, S.; Stotzky, G. Vertical movement in soil of insecticidal Cry1Ab protein from *Bacillus thuringiensis. Soil Biol. Biochem.* **2002**, *34*, 111−120.
(7) Herman, R. A.; Evans, S. L.; Shanahan, D. M.; Mihaliak, C. A.; Bormett, G. A.; Young, D. L.; Buehrer, J. Rapid degradation of Cry1F delta-endotoxin in soil. *Environ. Entomol.* **2001**, *30*, 642−644.
(8) Herman, R. A.; Wolt, J. D.; Halliday, W. R. Rapid degradation of the Cry1F insecticidal crystal protein in soil. *J. Agric. Food Chem.* **2002**, *50*, 7076−7078.
(9) Herman, R. A.; Scherer, P. N.; Wolt, J. D. Rapid degradation of a binary PS149B1 *δ*-endotoxin of *Bacillus thuringiensis* in soil, and a novel mathematical model for fitting curve-linear decay. *Environ. Entomol.* **2002**, *31*, 208−214.
(10) Lipton, C. R.; Dautlick, J. X.; Grothaus, G. D.; Hunst, P. L.; Magin, K. M.; Mihaliak, C. A.; Rubio, F. M.; Stave, J. W. Guidelines for the Validation and Use of Immunoassays for Determination of Introduced Proteins in Biotechnology Enhanced Crops and Derived Food Ingredients. *Food Agric. Immunol.* **2000**, *12*, 156−164.
(11) Shan, G.; Lipton, C.; Gee, S. J.; Hammock, B. D. Immunoassay, biosensors and other nonchromatographic methods. In *Handbook of Residue Analytical Methods for Agrochemicals*; Lee, P. W., Ed.; Wiley: West Sussex, U.K., 2003; pp 623−679.
(12) Hopkins, D. W.; Gregorich, E. G. Detection and decay of the Bt endotoxin in soil from a field trial with genetically modified maize. *Eur. J. Soil Sci.* **2003**, *54*, 793−800.
(13) Mayer, L. M.; Weston, D. P.; Bock, M. J. Benzo[*a*]pyrene and zinc solubilization by digestive fluids of benthic invertebrates—a cross-phyletic study. *Environ. Toxicol. Chem.* **2001**, *20*, 1890−1900.
(14) Mayer, L. M.; Schick L. L.; Self, R. F. L.; Jumars, P. A.; Findlay, R. H.; Chen, Z.; Sampson, S. Digestive environments of benthic macroinvertebrate guts: Enzymes, surfactants, and dissolved organic matter. *J. Mar. Res.* **1997**, *55*, 785−812.
(15) Voparil, I. M.; Mayer, L. M. Commercially available chemicals that mimic a deposit feeder's (*Arenicola marina*) digestive solubilization of lipids. *Environ. Sci. Technol.* **2004**, *38*, 4334−4339.
(16) Stotzky, G. Persistence and biological activity in soil of insecticidal proteins from *Bacillus* thuringiensis and of bacterial DNA bound on clays and humic acids. *J. Environ. Qual.* **2000**, *29*, 691−705.
(17) Tapp, H.; Stotzky, G. Persistence of the insecticidal toxin from *Bacillus thuringiensis subsp. kurstaki* in soil. *Soil Biol. Biochem.* **1998**, *30*, 471−476.
(18) Shiau, Y. F. In *Physiology of the gastrointestinal tract*, 2nd ed.; Johnson, L. R., Ed.; Raven Press: New York, 1987; pp 1527−1556.
(19) Weston, D. P.; Maruya, K. A. Predicting bioavailability and bioaccumulation with in vitro digestive fluid extraction. *Environ. Toxicol. Chem.* **2002**, *21*, 962−971.
(20) Weston, D. P.; Mayer, L. M. Comparison of in vitro digestive fluid extraction and traditional in vivo approaches as measures of polycyclic aromatic hydrocarbon bioavailability from sediments. *Environ. Toxicol. Chem.* **1998**, *17*, 830−840.
(21) Parsons, T. R.; Maita, Y.; Lalli, C. M. *A Manual of Chemical and Biological Methods for Seawater Analysis*; Pergamon press: Oxford, U.K., 1984.
(22) Palm, C. J.; Schaller, K. K.; Donegan, K. K.; Seidler, R. J. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki δ*-endotoxin. *Can. J. Microbiol.* **1996**, *42*, 1258−1262.

Received for review May 18, 2005. Revised manuscript received June 15, 2005. Accepted June 16, 2005.

JF0511493