JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Degradation of Cry1Ab Protein from Genetically Modified Maize in the Bovine Gastrointestinal Tract

BODO LUTZ,[†] STEFFI WIEDEMANN,[†] RALF EINSPANIER,[§] JOHANN MAYER,[#] AND CHRISTIANE ALBRECHT*,[†]

Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany; Institute of Veterinary Biochemistry, Free University of Berlin, Oertzenweg 19b, D-14163 Berlin, Germany; and Bavarian State Research Center for Agriculture, Prof.-Dürrwaechter-Platz 1, D-85586 Poing, Germany

Immunoblotting assays using commercial antibodies were established to investigate the unexpected persistence of the immunoactive Cry1Ab protein in the bovine gastrointestinal tract (GIT) previously suggested by enzyme-linked immunosorbent assay (ELISA). Samples of two different feeding experiments in cattle were analyzed with both ELISA and immunoblotting methods. Whereas results obtained by ELISA suggested that the concentration of the Cry1Ab protein increased during the GIT passage, the immunoblotting assays revealed a significant degradation of the protein in the bovine GIT. Samples showing a positive signal in the ELISA consisted of fragmented Cry1Ab protein of approximately 17 and 34 kDa size. Two independent sets of gastrointestinal samples revealed the apparent discrepancy between the results obtained by ELISA and immunoblotting, suggesting that the antibody used in the ELISA reacts with fragmented yet immunoactive epitopes of the Cry1Ab protein. It was concluded that Cry1Ab protein is degraded during digestion in cattle. To avoid misinterpretation, samples tested positive for Cry1Ab protein by ELISA should be reassessed by another technique.

KEYWORDS: ELISA; Cry1Ab protein; genetically modified plants; feed analysis; feeding experiments; immunoblotting; digestion

INTRODUCTION

The recent regulation 1830/2003 of the European Parliament and of the Council of the European Union on genetically modified food and feed comprised the traceability and labeling of genetically modified organisms (GMO) as well as the survey of food and feed products produced from GMO (*1*). The implementation of these aspects in European law and the further planned licensing of GMO food and feed by the European Community have provoked a strong public discussion about the pros and cons of genetically modified plants, especially with regard to their safety for the consumer. Genetically modified plants such as maize will increasingly be used not only as nutritional components or feed source for farm animals but also for human consumption (*2*). Bt-maize has been genetically modified to express an insecticidal crystal protein called a Cry protein (for example, Cry1Ab protein) from *Bacillus thuringiensis* (*3*). The modified plant produces this larvicidal toxin against lepidopteran pests, especially the European corn borer (*Ostrinia nubilalis*). Recently, case studies investigating the fate of plant DNA (*4*−*8*) and Cry1Ab protein (*9*, *10*) in farm animals and rodents were reported. These studies revealed an apparent persistence of the Cry1Ab protein immunoactivity in the bovine gastrointestinal tract (GIT). Analyses performed with a commercially available enzyme-linked-immunosorbent assay (ELISA) suggested that Cry1Ab protein accumulated in the course of the GIT passage. As no traces of Cry1Ab protein in animals fed isogenic maize were detected, potential cross-reaction of the monoclonal antibody used in the ELISA was ruled out. However, it cannot be excluded that the signals detected with the ELISA technique represented an immunoreaction between the monoclonal antibody and fragmented, yet still immunoactive parts of the Cry1Ab protein. Thus, data based only on ELISA measurement could result in an overestimation of the full-length Cry1Ab protein.

To examine this hypothesis, immunoblotting assays with polyclonal and monoclonal antibodies against epitopes of the Cry1Ab protein were developed. Two independent sets of GIT samples (gastrointestinal contents of rumen, jejunum, and colon) as well as feces, collected from two different feeding experiments, were analyzed both with ELISA and with the immunoblotting technique.

* Corresponding author (e-mail christiane.albrecht@wzw.tum.de; telephone +49 8161 71 3994; fax +49 8161 71 4421).
[†] Technical University Munich.
[§] Free University of Berlin.
[#] Bavarian State Research Center for Agriculture.

10.1021/jf049222x CCC: $30.25
Published on Web 02/08/2005

MATERIALS AND METHODS

Feeding Studies. Isogenic maize (Antares) and transgenic maize (Navares) (Syngenta International AG, Basel, Switzerland) were planted and grown on experimental fields of the Bavarian State Research Center for Agriculture (Grub, Germany). The harvested plants were chopped (average size of ~1 cm) and processed to maize silage. Feeding experiment A consisted of six lactating cows (3.5 ± 1.2 years old), kept under standard conditions and fed with either isogenic or transgenic Bt176 maize silage. Feed consumed per day consisted of 23.0 ± 2.0 kg of isogenic maize or 27.5 ± 1.5 kg of transgenic maize and 3.0 kg of hay, 2.5 kg of concentrated feed, and 0.17 kg of additional cattle feed.

Feeding experiment B has been described in detail elsewhere (*10*). Briefly, 11 cows (4.4 ± 2.9 years old) in each group were fed with silage either from genetically modified maize (Navares) or from the isogenic line (Antares) for 4 weeks. The daily diet contained 88.5% maize silage, 2% barley straw, 4.5% wheat, 4.5% rape-bruised grain extract, and 0.5% mineral feed. Each cow was fed with 19 kg of maize silage in a total fed ration of 21.5 kg per day.

All animals were in good health. The samples of rumen, jejunum, and colon content were taken immediately after slaughter, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Cry1Ab Protein Measurement (ELISA). Determination of the Cry1Ab protein was carried out by using a commercially available ELISA kit according to the manufacturer's instructions (Agdia Inc., Elkhart, IN). To pulverize the samples, 0.2 g of material was ground using the FastPrep system (BIO101, Carlsbad, CA) with 0.8 g of Green Matrix at 6 m/s for 40 s. The procedure was repeated until the sample was pulverized; the finally obtained powder was dissolved in 1000 μL of multievent buffer (MEB, provided in the kit). The control Cry protein, provided by the supplier, was diluted and used in the following concentrations to create a standard curve: 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, and 1.6 ng/mL. The results were expressed as Cry1Ab protein (nanograms) per gram of wet sample.

Immunoblotting (Western Blotting). The samples were prepared as described above (Cry1Ab Protein Measurement), except that PBS (pH 7.4) with protease inhibitors (Merck KGaA, Darmstadt, Germany) was used as extraction buffer; 17.75 μL of extracted protein, 1.0 μL of 1,4-dithiothreitol (1 mM; Merck KGaA), and 6.25 μL of SDS sample buffer (4×) were applied to SDS-PAGE in a 4−12% gradient Bis-Tris gel (NuPage, Invitrogen GmbH, Karlsruhe, Germany). 2-Morpholinoethanesulfonic acid was used as running buffer. After separation, the polypeptides were transferred onto a nitrocellulose membrane (pore size = 0.45 μm; Schleicher & Schuell BioScience, Keene, NH). The membranes were blocked with TBS−Tween 20 buffer (0.05 M Tris, 0.15 M NaCl, pH 7.6; 1% Tween 20) + 1% (w/v) nonfat dried milk overnight at 4 °C; subsequently, the membranes were incubated for 60 min in TBS−Tween 20 buffer supplemented either with a monoclonal mouse anti-Cry1Ab/1Ac (final concentration = 5 μg/mL) or with the same polyclonal rabbit anti-Bt-Cry1Ab/1Ac antibody (final concentration = 5 μg/mL; Agdia Inc.) which was applied in the ELISA kit. The membranes were washed four times with casein solution and incubated for 30 min in secondary antibody solution (biotinylated anti-rabbit IgG and biotinylated anti-mouse IgG in casein solution, respectively; final concentrations = 1.5 μg/mL). After three additional washing steps with casein solution for 10 min, membranes were incubated in Vectastain ABC-AmP reagent (Vector Laboratories, Inc., Burlingame, CA) for 10 min. Membranes were washed three more times (casein solution, 10 min) and incubated for 5 min with chemiluminescent substrate (DuoLuX, Vector Laboratories, Inc.). Following two final washing steps (0.1 M Tris, pH 9.3, 5 min), the membranes were exposed to a chemiluminescent detection film (Roche, Mannheim, Germany) for 15 min. All washing and incubation steps were performed at room temperature unless indicated otherwise.

RESULTS

ELISA. To ascertain the accuracy of the Cry1Ab ELISA measurements, rumen contents from animals fed isogenic maize were spiked corresponding to the standard curve (mentioned above) with 1.6, 1.0, 0.5, 0.25, 0.12, and 0.06 ng of Cry protein standard (included in the ELISA kit). The levels detected were 79.7 ± 1.6, 68.4 ± 5.3, 65.5 ± 5.5, 81.0 ± 3.7, 87.6 ± 1.2, and 71.7 ± 53.0% (recovery value ± SD) of 1.6, 1.0, 0.5, 0.25, 0.12, and 0.06 ng, respectively. Due to the high SD of 0.06 (71.7 ± 53.0%) results below a corresponding value of 0.12 ng were regarded as unreliable and were excluded from the analysis.

Table 1. ELISA Measurements (Nanograms of Cry1Ab Protein per Gram of Wet Sample) in Rumen, Jejunum, Colon, and Feces of Feeding Experiments A and B

expt	rumen	jejunum	colon	feces
A	5.1 ± 1.0	1.4 ± 0.8	3.8 ± 1.3	4.4 ± 1.4
B	4.3 ± 0.9	2.1 ± 1.3	2.5 ± 1.1	2.8 ± 1.1

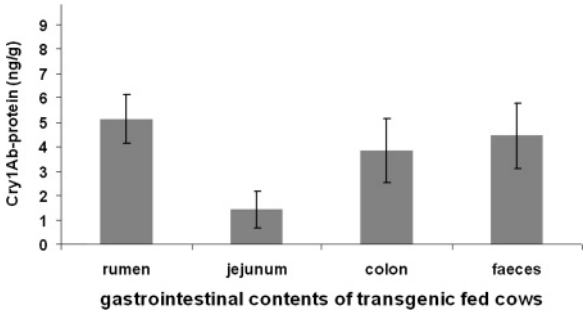

Figure 1. Results obtained by ELISA performed with samples of feeding experiment A. All samples were determined in duplicates. Error bars represent standard deviation (SD).

To rule out cross-reactivity of the antibody used in the ELISA with other proteins of animal, bacterial, and plant origin, samples of animals fed isogenic maize were analyzed in parallel. All isogenic samples were negative. Both sets of GIT samples yielded similar results by ELISA (**Table 1**). In feeding experiment A, 5.1 ± 1.0, 1.4 ± 0.8, 3.8 ± 1.3, and 4.4 ± 1.3 ng of Cry1Ab protein per gram of wet sample were detected in rumen, jejunum, colon, and feces, respectively (**Figure 1**). This compared well with the ELISA results obtained from feeding experiment B (4.3 ± 0.9, 2.1 ± 1.3, 2.5 ± 1.1, and 2.8 ± 1.1 ng) in rumen, jejunum, colon, and feces, respectively, published by Einspanier et al. (*10*). Both data sets suggested that the concentration of Cry1Ab protein was apparently increasing during the GIT passage due to the reduced moisture content in the lower parts of the GIT.

Immunoblotting. To determine the fragment size of the Cry1Ab protein detected by ELISA, that is to assess a potential fragmentation of the Cry1Ab protein, an immunoblotting assay was developed. For evaluation and validation purposes, samples with decreasing amounts of Cry1Ab protein, isolated from fresh plant material of transgenic maize (Navares) as described above, were loaded on a BIS-Tris gel (**Figure 2**). The detection limit corresponded to 50 mg (fresh weight) of transgenic maize (Navares), equivalent to ~2.0 ng of Cry1Ab protein (according to ELISA concentration measurements).

The same GIT samples analyzed by ELISA were tested by immunoblotting. Cry1Ab protein was not detectable in any isogenic (i) sample for feeding experiment A (**Figure 3**) or for feeding experiment B (data not shown). Using the polyclonal antibody the samples of rumen contents showed bands at approximately 34 and 17 kDa (**Figure 3A**,**C**). All other samples (jejunum, colon, feces) displayed only the band at 17 kDa (**Figure 3A**,**B**). The smear at ~60 kDa observed in bovine GIT samples of both isogenic and transgenic maize fed animals (**Figure 3A**,**B**) is presumably due to unspecific binding of the

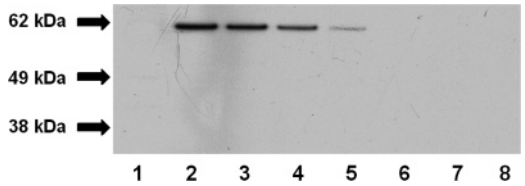

Figure 2. Detection limit of the immunoblotting assay using the polyclonal antibody in a serial dilution of Cry1Ab protein extracted from fresh GM maize plant material (Navares): (lane 1) Antares; (lane 2) Navares (200 mg, ~100%); (lane 3) Navares (75%); (lane 4) Navares (50%); (lane 5) Navares (25%); (lane 6) Navares (10%); (lane 7) Navares (1%); (lane 8) Navares (0.1%); arrows indicate the sizes of the molecular weight standard.

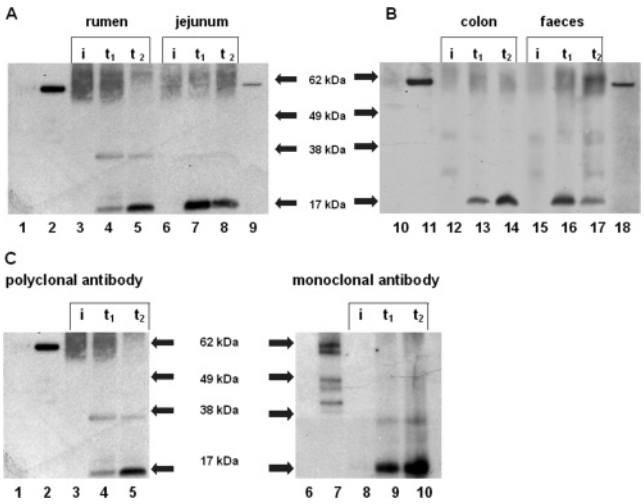

Figure 3. Immunoblotting of samples from bovine gastrointestinal contents (feeding experiment A): (**A**, **B**) polyclonal antibody [(lane 1) Antares; (lane 2) Navares; (lanes 3–5) rumen; (lanes 6–8) jejunum; (lane 9) positive control; (lane 10) Antares; (lane 11) Navares; (lanes 12–14) colon; (lanes 15–17) feces; (lane 18) positive control]; (**C**) comparison of polyclonal and monoclonal antibody in rumen samples [(lanes 1–5) polyclonal antibody; (lanes 6–10) monoclonal antibody; (lanes 1, 6) Antares; (lanes 2, 7) Navares; (lanes 3–5, 8–10) rumen content]; i, samples from a cow fed isogenic maize (feeding experiment A); t, samples of two cows fed transgenic maize (feeding experiment A); positive control, Cry protein provided in the ELISA kit. Arrows indicate the sizes of the molecular weight standard.

polyclonal antibody to other bovine proteins present in the protein extract. Plant extracts of fresh Navares leaves (**Figures 2** and **3**, lanes 2 and 11) did not show any unspecific binding or smear. To test whether the antibody used in the ELISA kit displayed a similar pattern, new membranes with the same samples were prepared and exposed to the monoclonal antibody. The fragmentation pattern in rumen samples was very similar (**Figure 3C**). However, it has to be noted that the sample of Navares plant material (lane 7) showed more bands when using the monoclonal antibody as compared to the polyclonal antibody (lane 2). Immunoblotting analyses with samples of feeding experiment B revealed similar results (data not shown). The positive control represents the Cry protein provided with the ELISA kit.

DISCUSSION

The ELISA technique is widely used for screening of the Cry1Ab/1Ac protein, to determine the expression levels of Cry protein or to track the persistence and the amount of the insecticidal Cry protein in different environments (*12*−*15*). In addition to the Cry1Ab protein from maize, the content or concentration of the Cry1Ac protein in potatoes and cotton is measured in different kinds of samples, for example animal tissues, plants, and soil. Previously we reported the detection of Cry1Ab protein immunoactivity in bovine gastrointestinal contents using a commercial ELISA kit (*10*). To verify the Cry1Ab protein measurements obtained with the ELISA, we evaluated an immunoblotting assay with a polyclonal antibody. This provided information with respect to the size of the reactive protein and the appearance of potential degradation products in the bovine GIT samples. Intriguingly, in all samples analyzed the full-length Cry1Ab protein (60 kDa) was absent or at least below the detection limit of the immunoblotting assay. Only fragments of approximately 34 and 17 kDa size were found, suggesting that the antibody used in the ELISA recognized fragmented yet immunoactive parts of the Cry1Ab protein. To investigate this hypothesis, we tested the monoclonal antibody in immunoblotting. Western blotting with the monoclonal antibody confirmed the previous results obtained with the polyclonal antibody, suggesting significant protein degradation due to physiological processes such as proteolysis by bovine proteases, rumen bacterial decomposition, and the pH in the bovine GIT.

For transgenic maize event Bt176 different transgene products sized 36, 40, and 60 kDa have been reported, potentially indicating that in-plant metabolic processing is occurring or that multiple products are being produced (*16*).

Chowdhury et al. found the full-length Cry1Ab protein within Bt11 maize in different parts of the GIT of calves and pigs (*9*, *17*) using ELISA and immunoblotting technique,whereas in our study only fragmented Cry1Ab protein was detected. The authors (*9*, *17*) suggested that only a trace amount of Cry1Ab protein survived passage through the GIT but was not transferred to liver, spleen, kidney, lymph nodes, or muscles. Discrepancies between the findings of the study of Chowdhury et al. and this investigation could be related to differences in the metabolism of calves and adult lactating cows or the distinct expression levels of the Cry1Ab protein in Bt11 and Bt176 maize. However, previous studies from our laboratories (data not published) or others (*9*, *17*) did not reveal any presence of Cry1Ab protein in tissue samples.

The finding that only small-sized fragments of the Cry1Ab protein were detected throughout the GIT and the feces has also a major impact on environmental concerns. Additional release of full-length Cry1Ab protein (60 kDa) caused by the output of liquid manure of Bt176-fed animals on fields can be almost ruled out. Moreover, a potential effect of fragmented Cry1Ab protein on the epithelial tissue of the bovine GIT and on other organisms cannot be excluded but is less likely.

Haider et al. reported that the insect specificity of *B. thuringiensis* var. *colmeri*, serotype 21, was altered depending on the digestive fluids the crystal endotoxin was processed in (*18*). They showed that processing of the native crystal δ-endotoxin (130 kDa) by gut enzymes of *Pieris brassicae* larvae yielded a 55 kDa protein that was toxic to only lepidopteran cell lines, whereas gut extracts of the dipteran (*Aedes aegypti*) larvae resulted in a 52 kDa fragment that was toxic to all mosquito cell lines but only one lepidopteran cell line (*Spodoptera frugiperda*). Experiments investigating a possible bioactivity of 17 and 34 kDa Cry1Ab protein fragments are lacking. However, this should be investigated as a separate matter.

ELISA is a method widely used for screening purposes with a high throughput capacity that allows rapid, preliminary testing and is easy to handle. However, it is not a suitable method for

drawing definitive conclusions, as it does not provide information on the chemical structure. To comply with legal requirements according to the Commission of the European Communities (93/256/EEC), an identifying analytical procedure consists of a suitable combination of cleanup, chromatographic separation, and spectrometric or immunochemical detection (*19*). The data provided in this paper indicate that results on Cry1Ab protein content based solely on ELISA measurements have to be interpreted carefully. It cannot be ruled out, yet is unlikely, that the signals obtained by ELISA measurements in our studies are restricted to bovine GIT samples due to very specific conditions regarding rumen bacterial composition and pH changes. However, to ensure adequate measurements and to confirm the presence of the full-length Cry1Ab protein, the use of an alternative method, for example immunoblotting or a bioassay with susceptible insect species, is advisable.

ACKNOWLEDGMENT

We thank Tamara Stelzl, Inge Celler, Christine Fochtmann, and Robert Gossmann for technical assistance. We are indebted to the staff of the Bavarian State Research Center for Agriculture for organization, logistics, and technical assistance during the feeding experiments.

LITERATURE CITED

(1) Regulation (EC) 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending. Directive 2001/18/EC. *Off. J. Eur Communities* **2003**, *L 268*, 24−28.

(2) International Service for the acquisition of agri-biotech applications SEAsia Center and CAB International: Crop Biotech Update, Special Edition 14, January 2004; Global Status of Commercialized Transgenic Crops 2003; http://www.biotechknowledge.com.

(3) Novartis Seeds AG. Genetically modified plant: maize protecting itself against cornborers−BT-176 MAIZE. **1994**, 1−11.

(4) Klotz, A.; Mayer, J.; Einspanier, R. Degradation and possible carry over of feed DNA monitored in pigs and poultry. *Eur. Food Res. Technol.* **2002**, *214*, 271−275.

(5) Schubbert, R.; Lettmann, C. M.; Doerfler, W. Ingested foreign DNA persists in the gastrointestinal tract and enters the bloodstream of mice. *Mol. Gen. Genet.* **1994**, *242*, 495−504.

(6) Schubbert, R.; Renz, D.; Schmitz, B.; Doerfler, W. Foreign (M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 961−966.

(7) Doerfler, W. Uptake of foreign DNA from the environment: the gastrointestinal tract and the placenta as portals of entry. In *Foreign DNA in Mammalian Systems*; Wiley-VCH Verlag: Weinheim, Germany, 2000; Chapter 11, pp 147−157.

(8) Einspanier, R.; Klotz, A.; Kraft, J.; Aulrich, K.; Poser, R.; Schwägele, F.; Jahreis, G.; Flachowsky, G. The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material. *Eur. Food Res. Technol.* **2001**, *212*, 129−134.

(9) Chowdhury, E. H.; Shimada, N.; Murata, H.; Mikami, O.; Sultana, P.; Miyazaki, S.; Yoshioka, M.; Yamanaka, N.; Hirai, N.; Nakajima, Y. Detection of Cry1Ab protein in gastrointestinal contents but not visceral organs of genetically modified Bt11-fed calves. *Vet. Hum. Toxicol.* **2003**, *45*, 71−75.

(10) Einspanier, R.; Lutz, B.; Rief, S.; Berezina, O.; Zverlov, V.; Schwarz, W.; Mayer, J. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgenic maize. *Eur. Food Res. Technol.* **2004**, *218*, 269−273.

(11) Clements, M. J.; Campbell, K. W.; Maragos, C. M.; Pilcher, C.; Headrick, J. M.; Pataky, J. K.; White, D. G. Influence of Cry1Ab protein and hybrid genotype on fumonisin contamination and *Fusarium* ear rot of corn crop. *Science* **2003**, *43*, 1283−1293.

(12) Palm, C. J.; Donegan, K. 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.

(13) Theunis, W.; Aguda, R. M.; Cruz, W. T.; Decock, C.; Peferoen, M.; Lambert, B.; Bottrell, D. G.; Gould, F. L.; Litsinger, J. A.; Cohen, M. B. *Bacillus thuringiensis* isolates from the Philippines: habitat distribution, δ-endotoxin diversity, and toxicity to rice stem borers (Lepidoptera: Pyralidae). *Bull. Entomol. Res.* **1998**, *88*, 335−342.

(14) Olsen, K. M.; Daly, J. C. Plant−toxin interactions in transgenic Bt cotton and their effect on mortality of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Entomol. Soc. Am.* **2000**, *93*, 1293−1299.

(15) Zwahlen, C.; Hilbeck, A.; Gugerli P.; Nentwig, W. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the fields. *Mol. Ecol.* **2003**, *12*, 765−775.

(16) Andow, D. A.; Hilbeck A. Science-based risk assessment for nontarget effects of transgenic crops. *BioScience* **2004**, *54*, 637−649.

(17) Chowdhury, E. H.; Kuribara, H.; Hino, A.; Sultana, P.; Mikami, O.; Shimada, N.; Guruge, K. S.; Saito, M.; Nakajima, Y. Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *J. Anim. Sci* **2003**, *81*, 2546−2551.

(18) Haider, M. Z.; Knowles, B. H.; Ellar, D. J. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem.* **1986**, *156*, 531−540.

(19) Commission Decision of 14 April 1993 (93/256/EEC). *Off. J. Eur. Communities* **1993**, *L 118*, 64−74.

Received for review May 17, 2004. Revised manuscript received November 12, 2004. Accepted November 15, 2004. This study was supported by the German Federal Ministry of Research and Technology (BMBF Grant 0312631D) and the Federal Agency for Nature Conservation (BfN Grant 20767432).

JF049222X