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In situ studies on the time-dependent degradation of recombinant corn DNA and protein in the bovine rumen

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ABSTRACT: An in situ technique was adopted to investigate the time-dependent ruminal degradation of chloroplast compared with recombinant DNA of Bt176 corn using conventional and quantitative PCR assays. In parallel, the Cry1Ab protein content and fragment sizes were determined by ELISA and immunoblotting techniques. Triplicate nylon bags filled with 5 g of each substrate (whole-plant isogenic, whole-plant transgenic, ensiled isogenic, and ensiled transgenic corn) were positioned within the rumen of 5 rumen-cannulated, nonlactating cows and incubated for 2, 4, 8, 16, 24, and 48 h. To investigate the DNA degradation process, PCR assays were developed to detect fragments of the endogenous highly abundant rubisco gene (173, 896, 1,197, and 1,753 bp) and of the recombinant cry1Ab gene (211, 420, 727, and 1,423 bp). Short fragments of rubisco (<431 bp) and cry1Ab DNA (211 bp) were amplifiable in whole-plant and ensiled corn samples incubated in the rumen for 48 h, whereas the traceability of larger fragments depended on previous processing of the sample (whole-plant or ensiled corn), the length of the target sequence, and concomitantly on the length of time incubated in the rumen. Quantification of rubisco and cry1Ab gene fragments applying real-time PCR assays revealed degradation to <20% of initial 0-h values within 2 h and <0.5% after 48 h of ruminal incubation. Analysis of Cry1Ab protein in whole-plant corn using the ELISA technique revealed a decrease to 28.0% of the initial value within 2 h and to 2.6% within 48 h. The concentration of Cry1Ab protein of ensiled corn was only 10% that of whole-plant corn. Ensiled corn Cry1Ab protein decreased to 10% of initial values after 48 h of ruminal incubation. Using an immunoblotting technique, the full-size Cry1Ab protein was only detectable up to 8 h; thereafter, only fragments of approximately 17 and 34 kDa size were found. In conclusion, ruminal digestion decreased the presence of functional cry1Ab gene fragments. It is unlikely that full-size, functional Cry1Ab protein will be present after 8 h of incubation in the rumen. Therefore, results based on ELISA measurements should be interpreted carefully and verified by another detection method that discriminates between the full-size and fragmented Cry1Ab protein.

Key words: cattle, enzyme-linked immunosorbent assay, in situ disappearance kinetics, polymerase chain reaction, recombinant DNA, recombinant protein

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INTRODUCTION

Genetic engineering has enabled the recombination of DNA to modify an organism or populations of organisms. The addition of foreign genes is used to create plants, such as transgenic corn and soybeans that are capable of synthesizing novel proteins, resulting in insect resistance and herbicide tolerance. Genetically modified Bt176 corn has been engineered to express the cry1Ab gene, isolated from the naturally occurring soil bacterium Bacillus thuringiensis (Bt), to protect itself against the European corn borer. Since the release of those plants into the food and feed market, the public has expressed concerns about the effect and the digestive fate of recombinant DNA and proteins of genetically modified crops. Studies conducted with cows have illustrated that plant-derived and transgenic DNA and protein are degraded throughout the digestive tract (Chowdhury et al., 2004; Einspanier et al., 2004; Lutz...
et al., 2005b). However, because those studies did not consider the long mean retention time of feed in the rumen of cows, additional data on the time-dependent degradation of plant DNA and the recombinant cry1Ab gene, as well as the Cry1Ab protein are crucial. In addition, knowledge about the degradation of transgenic DNA over time is necessary to estimate the likelihood of functional genes to transfer from feed to ruminal microorganisms. In the current study, we adopted a nylon bag technique (Ørskov and McDonald, 1979; Flachowsky et al., 1988; Madsen and Hvælplund, 1994) to analyze in situ degradation of the chloroplast-specific rubisco gene, the recombinant cry1Ab gene, and the Cry1Ab protein in the rumen of cows.

MATERIALS AND METHODS

Feeding Experiment

Five mature, rumen-cannulated, nonlactating Holstein-Friesian cows (average BW = 700 kg) were housed at the Department of Animal Sciences of the Technical University Munich, Germany. A diet (as-fed basis) of 13 kg of isogenic corn silage (39% DM/d), 0.8 kg of soybean meal/d, and 50 g of mineral supplement (R-Lactol, Raiffeisen Kraftfutterwerke Sued GmbH, Wuerzburg, Germany)/d was fed 12 d before and during the experimental period. The diet was fed in 2 equal meals at 0700 and 1600. All animals had access to water ad libitum.

In Situ Experiments and Animal Performance

The study was performed according to strict federal and international guidelines on animal experimentation. The experiment was set up according to the requirements of the Bavarian State animal welfare committee.

Genetically modified Bt176 corn (Navares) and the non-Bt isoline (Antares; Syngenta International AG, Basel, Switzerland) were grown on adjacent experimental fields at the Bavarian State Research Center for Agriculture (Poing-Grub, Germany) to minimize variation based on different environmental conditions. After harvest, the whole corn plants were chopped (particle size of approximately 1 cm) and either frozen to −20°C or ensiled. Subsequently, the following 4 substrates were used for the in situ experiment: whole-plant isogenic (53% DM; 90 g of CP, 27 g of crude fat, and 178 g of NDF/kg of DM); whole-plant transgenic (37.9% DM; 80 g of CP, 28 g of crude fat, and 186 g of NDF/kg of DM); ensiled isogenic (51.7% DM; 90 g of CP, 24 g of crude fat, and 183 g of NDF/kg of DM); and ensiled transgenic corn (36.3% DM; 84 g of CP, 29 g of crude fat, and 182 g of NDF/kg of DM). Samples were lyophilized and milled to pass a 3-mm screen. Five grams of an individual lyophilized sample on an as-fed basis were weighed into bags of precision woven nylon cloth (10 cm × 20 cm) with an aperture of 53 ± 10 μm (BG1020; Bar Diadem Inc., Parma, ID). All nylon bags were sealed and connected to a 55-cm, coated flexible steel cable with lacing cords. Before the 0700 feeding, this plastic carrier was placed in the rumen and attached to the cap of the fistula on a 40-cm nylon cord. To exclude the possibility of distinct degradation patterns of DNA and protein dependent on the sample’s position on the carrier, triplicates of each variety were attached randomly to the carrier. After 2, 4, 8, 16, 24, and 48 h of incubation in the rumen, the bags were quenched in iced water to stop the microbial activity and subsequently washed in a washing machine using a standard cold rinse cycle. The bags were then lightly squeezed to eliminate excess water and lyophilized. To estimate the initial DNA and protein concentration, bags containing either whole-plant or ensiled isogenic or transgenic corn were washed and lyophilized without ruminal incubation in duplicate.

To ascertain equal ruminal fermentation conditions in all cows, we determined the pH values (Schott CG 842 pH meter, Mainz, Germany) and the ammonia content of ruminal fluid collected from the ventral sac according to Voigt and Steeger (1967) before and 30, 60, 90, 150, 210, and 270 min after feeding. Furthermore, ruminal VFA were measured according to Geissler et al. (1976) in samples taken before and 210 min after feeding.

Deoxyribonucleic Acid Extraction

Lyophilized and frozen samples (50 mg) of at least two different positions on the rumen-placed carrier per time point and substrate (5 cows × 2 bags; n = 10) were processed. Using the bead-beating FastPrep technique (BIO101, Carlsbad, CA), 50 mg were repeatedly ground with 0.8 g of Matrix Green ceramic beads (BIO101) at 5.0 m/s for 40 s. To improve DNA yield, samples were refrozen in liquid N2 for 10 min before being reground. The resulting fine powder was dissolved in 600 μL of lysis buffer (C1) and 10 μL of RNase-A (Nucleo Spin Plant Kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany), mixed thoroughly, and incubated for at least 30 min at 60°C. All succeeding DNA purification steps were performed using a silica spin column following the manufacturer’s protocol. The DNA was finally eluted in 50 to 100 μL of CE-buffer (Nucleo Spin Plant Kit) depending on the concentration.

Concentrations of DNA were determined by UV absorption at 260 nm, and the DNA integrity was estimated by 260/280 UV absorption ratio.

PCR Analysis

Oligonucleotides. Primer sets used for PCR and the respective DNA fragments amplified are described in Table 1. Five primer pairs were designed to detect different fragment lengths (173, 430, 896, 1,197, and 1,753 bp) of the highly abundant chloroplast-specific rubisco gene (Zea mays complete chloroplast genome; GenBank...
PCR Conditions. All PCR reactions were performed in a PCR thermocycler (Biometra, Göttingen, Germany) and contained 150 ng of DNA, 1× PCR reaction buffer (ABgene, Epsom, UK), 2.5 mM of MgCl₂ (ABgene), 0.8 μM of both forward and reverse primer (Metabion, Martinsried, Germany), 4.0 mM of dNTP (ABgene), and 0.5 IU of Thermoprime Plus DNA Polymerase (ABgene). The master mixes for amplification of the 1,753-bp fragments included 3.5 mM of MgCl₂. Cycling conditions are described in Table 2.

In case amplicons were obtained for one primer pair, the respective next higher fragment length was attempted to be amplified. If a sample at a given time tested positive for a specific fragment, the subsequent time point was tested with the same primer pair.

All PCR assays included both positive and negative controls. As a positive control, DNA from Bt176 corn leaf tissue (Navaires) was used. Negative controls for the *rubisco* and *cry1Ab* primer sets did not contain template DNA. Additional negative controls for the amplification of *cry1Ab* fragments consisted of non-recombinant corn DNA (Antaeres).

Agarose Gel Electrophoresis. The PCR amplicons (15.0 μL) were electrophoresed at 100 V on a 1.5 to 2% (wt/vol) agarose gel and visualized using an UV transilluminator. The gels were digitized using a video documentation system (Vilber Lourmat, Marne-la-Vallée Cedex 1, France).

Sequencing. The PCR products were commercially sequenced to confirm nucleotide sequence identity (Medigenomix, Martinsried, Germany).

Relative Quantification of Rubisco and Cry1Ab DNA (Real-Time PCR)

Quantification of the *rubisco* and *cry1Ab* gene was performed on at least duplicate samples of two different positions on the rumen-placed carrier using the LightCycler instrument (Roche, Mannheim, Germany). *rubisco* and the *cry1Ab* gene concentrations of samples

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**Table 1. Primers, targets, and amplicon sizes for PCR analysis of rubisco and cry1Ab DNA**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′ – 3′)</th>
<th>Target</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry03</td>
<td>CTC TCG CCG TTC ATG TCC GT</td>
<td><em>cry1Ab</em></td>
<td>211 bp</td>
<td>Hupfer et al. (1998)</td>
</tr>
<tr>
<td>Cry04</td>
<td>GGT CAG CTC TGG GCT GAT GT</td>
<td><em>cry1Ab</em></td>
<td>420 bp</td>
<td>Studer et al. (1997)</td>
</tr>
<tr>
<td>Cry1A1</td>
<td>CCG CCC CGA GTT CAC CTT</td>
<td><em>cry1Ab</em></td>
<td>727 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Cry1A2</td>
<td>CTG CTG GGG ATG TGG TAG</td>
<td><em>cry1Ab</em></td>
<td>1,423 bp</td>
<td>This study</td>
</tr>
<tr>
<td>cry 727 rev</td>
<td>TAG GTG GGG AAC AGG CTC AC</td>
<td><em>cry1Ab</em></td>
<td>1,423 bp</td>
<td>This study</td>
</tr>
<tr>
<td>cry 1423 rev</td>
<td>AGG TTG GGC TCT TTC GTA ACA</td>
<td><em>cry1Ab</em></td>
<td>1,423 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 173F</td>
<td>AGC TAT TCG TGG GCC TTG AGG CAC</td>
<td><em>rubisco</em></td>
<td>173 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 173R</td>
<td>TGG TAT CCA TCG GCT TTA GAC AC</td>
<td><em>rubisco</em></td>
<td>430 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 896F</td>
<td>AGG TAA TCC AGA AGG GGA AGC C</td>
<td><em>rubisco</em></td>
<td>896 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 1197F/1753F</td>
<td>GGC CTA CTT CTT CAC ATT CAC C</td>
<td><em>rubisco</em></td>
<td>1,197 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 1197R</td>
<td>CAT TCT CAT GGC CTT TCT ATC C</td>
<td><em>rubisco</em></td>
<td>1,753 bp</td>
<td>This study</td>
</tr>
<tr>
<td>Rub 1753R</td>
<td>AAA CTC GCT ATA GTC GGC TAC G</td>
<td><em>rubisco</em></td>
<td>1,753 bp</td>
<td>This study</td>
</tr>
</tbody>
</table>

1With forward primer cry for.
2With reverse primer Rub173R.
3With forward primer Rub896F.
4With forward primer Rub1197/1753R.

---

**Table 2. Cycling conditions for PCR analysis of rubisco and cry1Ab DNA**

<table>
<thead>
<tr>
<th>Target</th>
<th>Fragment length</th>
<th>Denaturation</th>
<th>Amplification steps</th>
<th>Cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cry1Ab</em></td>
<td>211 bp</td>
<td>94°C, 3 min</td>
<td>94°C, 30 s; 63°C, 30 s; 72°C, 30 s</td>
<td>35</td>
<td>72°C, 2 min</td>
</tr>
<tr>
<td>420 bp</td>
<td>94°C, 3 min</td>
<td>94°C, 30 s; 56°C, 30 s; 72°C, 30 s</td>
<td>35</td>
<td>72°C, 2 min</td>
<td></td>
</tr>
<tr>
<td>727 bp</td>
<td>94°C, 5 min</td>
<td>94°C, 30 s; 56°C, 30 s; 72°C, 30 s</td>
<td>35</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
<tr>
<td>1,423 bp</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s; 56°C, 45 s; 72°C, 45 s</td>
<td>40</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
<tr>
<td><em>rubisco</em></td>
<td>173 bp</td>
<td>94°C, 3 min</td>
<td>94°C, 30 s; 60°C, 30 s; 72°C, 30 s</td>
<td>35</td>
<td>72°C, 5 min</td>
</tr>
<tr>
<td>430 bp</td>
<td>94°C, 3 min</td>
<td>94°C, 30 s; 60°C, 30 s; 72°C, 30 s</td>
<td>35</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
<tr>
<td>896 bp</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s; 58°C, 45 s; 72°C, 45 s</td>
<td>40</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
<tr>
<td>1,197 bp</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s; 58°C, 45 s; 72°C, 45 s</td>
<td>40</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
<tr>
<td>1,753 bp</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s; 58°C, 45 s; 72°C, 45 s</td>
<td>40</td>
<td>72°C, 5 min</td>
<td></td>
</tr>
</tbody>
</table>
incubated in the rumen were expressed as a percentage of initial values.

For *rubisco* gene quantification, the reaction mixture (final volume = 10 μL) contained 50 ng of extracted DNA, 1 μL of LightCycler DNA Master SYBR Green I (10×), 3 mM of MgCl₂ (Roche), and 0.4 μM of forward and reverse primer (Rub173F and Rub173R). Amplification involved one cycle at 95°C for 10 min for initial denaturation and 45 cycles of 95°C for 15 s, 61°C for 10 s, and 72°C for 20 s. Amplified products underwent melting curve analysis by slow heating with a 0.1°C/s increment from 65 to 95°C with fluorescence collection at 0.1°C intervals after the last cycle to specify the integrity of amplification. Additionally, the product size of all samples was verified by electrophoresis after the PCR run. Dilutions of purified cloned DNA were used to construct gene-specific calibration curves.

For relative quantification of genetically modified Bt176 corn, a commercially available Bt176 quantification kit (Roche) was used according to the manufacturer’s instructions.

In addition to the *cry1Ab* gene, a reference gene (corn *invertase*, provided in the kit) was measured and the ratio between the *cry1Ab* and *invertase* gene was calculated to ensure unvarying degradation patterns of both genes. To exclude possible unspecific amplification for the *cry1Ab* gene quantification, isogenic corn samples of all time points and substrates also were analyzed.

**Cry1Ab Protein Analysis**

*Enzyme-Linked Immunosorbent Assay.* Estimation of the Cry1Ab protein was performed using a commercially available ELISA kit according to the manufacturer’s instructions (Agdia Inc., Elkhart, IN). Frozen whole-plant or ensiled transgenic corn samples (20 mg) obtained from all animals at all time points and originating from at least 2 positions on the rumen-placed plastic carrier (n ≥ 10) were ground using the FastPrep homogenizing instrument with 0.8 g of Matrix Green ceramic beads at 6 m/s for 40 s until the material was pulverized. The resulting powder was dissolved in 1 mL of MultiEvent buffer (provided in the kit). Dilutions of 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, and 1.6 ng/mL of a control Cry protein (provided in the kit) were used to create a standard curve. Results are expressed as Cry1Ab protein (ng/g of fresh weight). Samples of non-recombinant corn served as negative controls. All samples were measured at least in duplicates.

**Immunoblotting.** An immunoblotting technique (Lutz et al., 2005b) was used to determine the fragment size of the Cry1Ab protein detected by ELISA. Briefly, whole-plant and ensiled transgenic corn samples were prepared as described for the ELISA assay, except that PBS (pH 7.4) with protease inhibitors (Merck KGaA, Darmstadt, Germany) was used as extraction buffer. The SDS-PAGE conditions included a 4 to 12% gradient Bis-Tris gel (NuPage, Invitrogen GmbH, Karlsruhe, Germany), 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×). After separation and transfer onto a nitro-cellulose membrane, Cry1Ab protein was detected using a polyclonal rabbit antiCry1Ab/1Ac antibody (Agdia Inc.; final concentration 5 μg/mL; 60 min) followed by a secondary antibody solution (biotinylated antirabbit IgG in casein solution; final concentration 1.5 μg/mL; 30 min). For signal amplification, membranes were incubated in Vectastain ABC-AMP Reagent (Vector Laboratories, Inc., Burlingame, CA; 10 min). Signals were visualized by chemiluminescence (DuoLuX, Vector Laboratories, Inc.). Isogenic corn served as a negative control; the sample without incubation time in the rumen (0 h) and Cry1Ab/1Ac protein included in the ELISA kit served as positive controls.

**Statistical Analyses**

Results of PCR for a specific time point were regarded as positive when >50% of all extracted DNA samples yielded amplicons of the correct size (bp) and identity (sequence analysis).

Data of ELISA were analyzed by using the MIXED procedure of SAS (Version 8.2; SAS Inst., Inc., Cary, NC). At 2 h, the influence of ruminal position was tested for significance for the transgenic whole-plant corn samples. The model included animal (n = 5) and position (n = 3) as class variables. Because degradation was not significantly influenced by position, further evaluations were restricted to two ruminal positions. The model of the MIXED procedure included animal as a random effect, incubation time as repeated measure, and corn type and ruminal position as class variables. If the *F*-test of the MIXED procedure was significant, differences were separated using the Duncan’s multiple range test.

**RESULTS**

**PCR Analysis of Rubisco and Cry1Ab**

Amplicons of the *rubisco* gene included fragment lengths of 173 and 430 bp in all samples of whole-plant and ensiled corn (Figure 1). Fragments of 896 and 197 bp were found up to 24 h in whole-plant samples but could not be detected in any of the ensiled samples tested. *Rubisco* gene fragments of 1,753 bp could be amplified up to 8 h after rumen incubation in whole-plant corn. Analyzing all amplifiable fragments of the *rubisco* gene did not show a qualitative difference between isogenic and transgenic corn.

Fragments of the *cry1Ab* gene with the size of 211 bp were found in all samples of whole-plant and ensiled transgenic corn (Figure 2). The 420-bp fragment could be amplified in all samples of whole plant but only up to 8 h of ensiled corn. Fragment lengths of 727 and 1,423 bp were detected in whole-plant corn up to 16 and 4 h, respectively, but were not found in any sample of ensiled corn. A summary of all tested amplicons in
Ruminal degradation of DNA and protein

**Rubisco gene fragments**

![Image of gel electrophoresis](image)

**Figure 1.** Polymerase chain reaction amplification and agarose gel electrophoresis of *rubisco* DNA. Primer pairs for detection of 173, 430, 896, and 1,753 bp were used. Lanes 1 to 10: 1 = marker, 2 = 0 h, 3 = 2 h, 4 = 4 h, 5 = 8 h, 6 = 16 h, 7 = 24 h, 8 = 48 h, 9 = positive control (transgenic corn leaves), and 10 = negative control (H₂O).

whole-plant and ensiled corn samples as well as a comparison between the *rubisco* and the *cry1Ab* gene is shown in Figure 3.

**Relative Quantification of Rubisco and Cry1Ab DNA (Real-Time PCR)**

Ruminal fermentation conditions were comparable in all animals (Table 3). In addition, conventional PCR (and ELISA measurements, see subsequent) yielded similar results for all cows. For technical reasons, quantitative PCR was performed in 3 animals only, and results are shown for one representative animal.

As demonstrated in Figure 4A and B for transgenic whole-plant and ensiled corn samples, no consistent difference in the degradation pattern between the positions was apparent. Therefore, DNA originating from the 2 different bags was extracted and pooled for quantification of *rubisco* and *cry1Ab* gene fragments.

In addition, comparing the degradation of *rubisco* gene fragments between isogenic and transgenic corn samples during ruminal incubation revealed no marked differences in whole-plant (Figure 4C) or ensiled corn (Figure 4D). Less than 20% of the initial *rubisco* gene fragment was measured after 2 h of ruminal incubation both in isogenic and transgenic whole-plant corn. Values were <5% of initial by 4 h; final values were <0.1% for Bt and non-Bt corn by 48 h of ruminal incubation.

Initial values for the *rubisco* gene measured in ensiled plant samples were approximately 0.9% of initial values found in whole-plant corn samples. A marked decrease to <6% of the initial values for both isogenic and transgenic corn after 4 h of incubation in the rumen was measured. Values dropped to <0.5% by 48 h of ruminal incubation.

Quantifying the *cry1Ab* gene using real-time PCR (Figure 5) showed a sharp decrease during the first 4 h of incubation to <1% of the initial value for whole-plant corn samples, which ended up in final values of <0.5% after 48 h. Quantification of the *cry1Ab* gene fragment in ensiled corn samples reached the detection limit of the commercial kit after 8 h of ruminal incubation; therefore, data are not shown. Results after that time point were not reliably distinguishable from values of isogenic corn samples; however, the 0-h value of ensiled corn was only 0.62% of the initial value of whole-plant transgenic corn. Comparing the degradation pattern of *rubisco* DNA (Figure 4) and *cry1Ab* DNA (Figure 5) did not reveal obvious differences between both genes.

**Cry1Ab Protein Analysis**

*Enzyme-Linked Immunosorbent Assay.* Because the results for the 2-h transgenic whole-plant samples obtained from all 3 positions on the rumen carrier did
Figure 2. Polymerase chain reaction amplification and agarose gel electrophoresis of cry1Ab DNA. Primer pairs for detection of 211, 420, 727, and 1,423 bp were used. Lanes 1 to 11: 1 = marker, 2 = 0 h, 3 = 2 h, 4 = 4 h, 5 = 8 h, 6 = 16 h, 7 = 24 h, 8 = 48 h, 9 = negative control (isogenic corn leaves), 10 = positive control (transgenic corn leaves), and 11 = negative control (H2O).

not vary (n = 15; P = 0.20), only samples of 2 different positions on the carrier were examined for the remaining incubation time points (final n = 120; P = 0.50).

After 2 h of ruminal incubation, a sharp decrease of the cry1Ab signal to 27 ± 8.0% (43.3 ± 12.46 ng of fresh weight/g; average of all animals ± SD) of the initial

Figure 3. Comparison of the maximally detectable fragment sizes of rubisco and cry1Ab DNA by conventional PCR.
Table 3. Ruminal pH, ammonia, and volatile fatty acid concentrations of cows fed an isogenic corn silage-based diet

<table>
<thead>
<tr>
<th>Item</th>
<th>Cow 1</th>
<th>Cow 2</th>
<th>Cow 3</th>
<th>Cow 4</th>
<th>Cow 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH(^1)</td>
<td>6.9 ± 0.2</td>
<td>6.8 ± 0.2</td>
<td>6.7 ± 0.3</td>
<td>6.7 ± 0.3</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td>NH(_3), mg/100 mL(^2)</td>
<td>7.8 ± 4.5</td>
<td>8.5 ± 5.4</td>
<td>8.3 ± 3.6</td>
<td>6.6 ± 4.4</td>
<td>8.0 ± 3.0</td>
</tr>
<tr>
<td>VFA, mmol/L(^3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid (C2)</td>
<td>49.1 ± 1.2</td>
<td>52.5 ± 3.5</td>
<td>60.0 ± 0.0</td>
<td>50.0 ± 2.3</td>
<td>50.9 ± 1.2</td>
</tr>
<tr>
<td>Propionic acid (C3)</td>
<td>14.8 ± 0.0</td>
<td>11.5 ± 1.0</td>
<td>13.5 ± 0.0</td>
<td>12.5 ± 0.0</td>
<td>12.5 ± 0.0</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>10.2 ± 0.0</td>
<td>9.6 ± 0.8</td>
<td>14.8 ± 0.0</td>
<td>10.2 ± 0.0</td>
<td>10.2 ± 0.0</td>
</tr>
<tr>
<td>C2:C3</td>
<td>3.3:1</td>
<td>4.6:1</td>
<td>4.4:1</td>
<td>4.1:1</td>
<td>4.2:1</td>
</tr>
</tbody>
</table>

\(^1\)Means ± SD of measurement before and 30, 60, 90, 150, 210, and 270 min after feeding.
\(^2\)Means ± SD of measurement before and 30, 60, 90, 150, 210, and 270 min after feeding.
\(^3\)Means ± SD of measurement before and 210 min after feeding.

value (155.3 ng/g) was measured (Figure 6A). Quantities of protein diminished gradually to 18 ± 5.2% (28.2 ± 9.31 ng/g) after 4 h and ended with final values of 2.6 ± 1.0% (4.1 ± 1.52 ng/g) after 48 h. The 0-h value of immunoactive Cry1Ab protein in ensiled corn was only approximately 10% (15.8 ng of fresh weight/g) of initial whole-plant corn values (153.3 ng/g). The signal for the Cry1Ab protein gradually diminished over the entire incubation period (48 h = 1.6 ± 0.4 ng/g).

**Immunoblotting.** Evaluation and validation of the immunoblotting assay was performed on fresh transgenic corn leaves and showed a detection limit of 50 mg (fresh weight) of transgenic corn, equivalent to approximately 2.0 ng of Cry1Ab protein/g of fresh weight according to ELISA concentration measurement (Lutz et al., 2005a). The full-sized protein of 60 kDa could be detected up to 8 h in whole-plant corn (Figure 6B). Bands of immunoactive fragments with the length of 17 kDa were observed after 2 h and increased thereafter. After 16 h of incubation, bands of approximately 34 kDa were evident and intensified at later time points.

Bands for Cry1Ab protein were not detected in any of the ensiled corn samples (data not shown).

**DISCUSSION**

The present in situ study was performed to analyze the time-dependent fragmentation and quantitative degradation of feed DNA and recombinant Cry1Ab protein. To study precise degradation profiles, an in situ technique using nylon bags was adopted. This method has been applied previously to investigate the ruminal degradability of feed from Bt and non-Bt corn and revealed no effect of a genetic modification on the determined values (Aulrich et al., 2001; Folmer et al., 2002; Donkin et al., 2003).

In our studies, conventional PCR techniques using the highly abundant rubisco DNA as a marker gene provided comparable results to those using cry1Ab DNA. Only small fragments of the rubisco gene and the cry1Ab gene were amplifiable in samples of whole-plant and ensiled corn. In ensiled corn samples, fragments spanning the size of 896 bp for rubisco and 727 bp for cry1Ab could not even be detected in the 0-h samples. This parallels observations of Hupfer et al. (1999) and our own laboratories (Lutz et al., 2005a), in which only small DNA fragments persisted after 60 or 106 d of ensiling. Compared with ensiled corn, rubisco and cry1Ab gene segments of whole-plant corn samples were detectable for longer periods of ruminal incubation. Fragments of DNA with comparable sizes to a potential functional bioactive gene (e.g., beta-lactamase [bla] introduced in Bt176 corn with a size of 861 bp; Accession No. U03991) were detectable up to 16 h for rubisco (896 bp) and 24 h for cry1Ab (727 bp). However, Badosa et al. (2004) demonstrated no transfer of the bla gene from Bt176 to corn-associated bacteria under field conditions. Even under optimized laboratory conditions, the transformation of highly competent bacteria with transgenic plant DNA extracts was only observed when highly homologous regions were present (Schluter et al., 1995; Nielsen et al., 1998; de Vries et al., 2001). Thus, we showed that feed DNA can survive in ruminal fluid for a significant time, but functional activity of that DNA is unlikely to remain after exposure to the ruminal environment.

Our results regarding conventional PCR analysis differ from those of Phipps et al. (2003) and Chowdhury et al. (2004). Phipps et al. (2003) detected high-copy rubisco DNA only up to 1,197 bp and single-copy transgenes of approximately 200 bp in ruminal samples after feeding genetically modified soybean meal and ground, genetically modified corn grains. However, in that study, samples from various time points were bulked into single samples, leading to a decrease in the detection of larger fragments. Chowdhury et al. (2004) showed no time-dependent fragmentation but obtained similar amplicons in ruminal samples after 5 and 18 h of feeding.

Real-time PCR results for rubisco and cry1Ab DNA confirmed those of conventional PCR, showing decreasing amounts with increasing ruminal incubation time. After 4 h, <10% of the initial values for rubisco and cry1Ab genes were found in all substrates, reflecting the rapid microbial and enzymatic digestion of DNA in ruminal fluid (Duggan et al., 2000). This agrees with
findings of Einspanier et al. (2004), who reported a significant decrease in the amount of chloroplast DNA after gastric digestion.

It should be noted, however, that generally with real-time PCR, only small DNA fragments are analyzed (in our case <174 bp of plant and transgenic DNA). As shown by conventional PCR, those lengths were still detectable after 48 h, even in ensiled corn samples, whereas longer fragment lengths could not be amplified by 48 h of ruminal incubation. Therefore, the results obtained by quantitative PCR do not provide evidence for the persistence of potential full-sized functional genes during the ruminal fermentation process.

Analyzing the Cry1Ab protein in ensiled and whole-plant transgenic corn by ELISA showed a continual decrease of the immunoactive signal with advancing
Ruminal degradation of DNA and protein

Figure 5. Real-time quantification of cry1Ab DNA in whole-plant corn samples (one representative animal); mean of 2 different sample positions on the rumen-placed carrier.

Figure 6. Immunoactivity of the Cry1Ab protein. Panel A = ELISA measurements of Cry1Ab protein in whole-plant and ensiled corn after incubation in the bovine rumen; mean ± SD of 5 animals (samples of 2 different positions); panel B = immunoblotting of the time-dependent degradation of the Cry1Ab protein. The arrows indicate the Cry1Ab protein specific size of approximately 60 kDa. Lane 1 = Antares, Lanes 2 to 9 = Navares, 2 = 0 h, 3 = 2 h, 4 = 4 h, 5 = 8 h, 6 = 16 h, 7 = 24 h, 8 = 48 h, and 9 = positive control (Cry1Ab/1Ac protein included in the ELISA kit).

Ruminal incubation time. The initial value of ensiled corn samples constituted only 10% of those of whole-plant corn, demonstrating a degradation of recombinant protein during the ensiling process. Our own investigations revealed a clear decrease of Cry1Ab protein during the ensiling process, probably because of low pH conditions and microbial activity (Lutz et al., 2005a).

To the best of our knowledge, there are no data available on the exact time-dependent degradation kinetics of ruminally incubated recombinant protein. However, Chowdhury et al. (2003) and Lutz et al. (2005b) noted a marked decrease in the content of Cry1Ab protein after ruminal digestion.

Immunoblotting showed the full-size protein (60 kDa) up to 8 h, which is in contrast to our recently acquired in vivo data, where ruminal samples were taken after slaughtering and no full-size protein was found (Lutz et al., 2005b). Moreover, specific fragments of 17 and 34 kDa were detected, which were previously described by Lutz et al. (2005b) in gastrointestinal samples. Chowdhury et al. (2003) did not report detection of these smaller fragments, which could be due to the use of different antibodies or a different fragmentation pattern of Bt176 corn compared with the Bt11 corn evaluated by those authors. In summary, the results obtained by immunoblotting confirm that ELISA measurements require careful interpretation because currently used, commercially available antibodies obviously also bind to immunoactive fragments whose potential activity has yet to be investigated.

Using PCR, ELISA, and immunoblotting techniques, we demonstrated that digestion of corn in the rumen of cows results in extensive time-dependent degradation and fragmentation of recombinant DNA and protein from Bt corn. It is unlikely that immunoactive protein fragments exhibit activity, but the potential bioactivity of Cry1Ab protein fragments warrants further investigation.

LITERATURE CITED


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