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Safety Assessment of Cre Recombinase

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Cre recombinase, when used as a tool in agricultural biotechnology, can precisely excise DNA sequences that may be useful in the introduction of a new trait but are not needed in the commercial product. Although the *cre* genetic material would not be present in the final product, the present studies were performed to assess the safety of Cre recombinase to provide confirmatory evidence of the safe use of Cre-*lox* technology in agricultural biotechnology. Cre recombinase shares no relevant sequence similarity to known allergens or toxins. When Cre recombinase was exposed to a pH 1.2 solution of simulated gastric fluid lacking pepsin, CD spectroscopy showed that there was a loss of secondary structure and that the protein was no longer active in a functional assay. Cre recombinase was degraded rapidly when exposed to pepsin in a standardized gastric digestion model; therefore, Cre recombinase would not survive the harsh gastric environment. When orally administered to mice as an acute dosage of 53 mg/kg of body weight, no treatment-related adverse findings were observed. These data support the conclusion that human and animal dietary exposure to Cre recombinase pose no known safety concerns; consistent with the fact that bacteriophage P1, the source of the *cre* gene and expressed protein, is commonly encountered in the environment and in normal enteric bacteria without reports of adverse consequences.

KEYWORDS: Agricultural biotechnology; Cre recombinase; digestibility

INTRODUCTION

Agricultural biotechnology has been used to improve a variety of agronomically important crops such as corn, soybean, potato, and cotton. Selectable marker genes, such as ones that confer resistance to antibiotics, have been used in conjunction with the gene of interest to readily select plants that express the desired trait(s). Selectable marker genes, however, are not typically needed in the final commercial product once the desired plants have been selected. While the presence of the commonly used marker genes/proteins in food and feed derived from genetically modified plants do not pose a health concern (*1*−*3*), it may be preferred to remove these markers from the final product to enhance acceptance because these genes are not needed in the final product (*4*). The methods used to remove marker genes include the use of the Cre-*lox* recombination system (*5*). The Cre-*lox* recombination system has been used in a variety of organisms (*5*−*8*) to precisely excise selected DNA.

Cre recombinase, from bacteriophage P1, catalyzes the excision of DNA sequence present between two tandemly repeated 13 bp recognition sequences called *lox* sites. Specifically, Cre recombinase binds to 13 bp inverted repeats within a *lox* site, and the dimerized Cre recombinase then catalyzes a crossover between the two *lox* sites, which results in the excision of the DNA fragment between them (*9*−*11*). When used in agricultural biotechnology, the selectable marker DNA may be specifically excised by designing the inserted DNA construct to contain a *lox* site on each end of the DNA segment to be removed and then crossing plants containing this DNA insert with plants expressing Cre recombinase. Conventional breeding is then used to identify progeny plants that retain the desired genetic trait(s) but lack the Cre recombinase genetic construct and the DNA segment flanked by *lox* sites (*8*, *12*). The result is a commercial event that does not contain or produce the protein products of both the selectable marker gene and the Cre recombinase gene. However, even though Cre recombinase protein is absent in the final product, the present safety studies of this protein were conducted to facilitate acceptance and stewardship of products developed using this technology.

MATERIALS AND METHODS

With the exception of the bioinformatic analyses and the data pertaining to the development of the Cre recombinase assay and circular dichroism (CD) spectroscopy, all studies were performed in accordance with the United States Environmental Protection Agency Good Laboratory Practice Standards (40 CFR Part 160). Additionally, the acute oral toxicity study was performed in accordance with guidelines defined by the Organization for Economic Cooperation and Development (420).

Bioinformatic Analyses. The amino acid similarity of Cre recombinase toward allergens, toxins, or other pharmacologically relevant proteins was evaluated using standard bioinformatic methods. The

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allergen sequence database (AD5) was assembled as previously described (*13*) and contained 1191 sequences. The toxin sequence database (TOXIN5) was assembled from public sequence databases using the keyword "toxin" and contained 12 771 sequences. The actual number of unique toxin sequences is less than 12 771 because of the redundancy of the public databases and some entries may contain the word toxin but are not bona fide protein toxins. The ALLPEPTIDES database was comprised of SwissProt (Release 39.0) and The Institute for Genomic Research non-redundant amino acid database (Release 1.0) and contained 2 049 024 sequences.

Because the *E* score is a statistical measure that the alignment is random, an *E* score of 1×10^{-5} (1 e-5) was set as an initial high cutoff value for alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E* score less than 1×10^{-5} was analyzed further to determine if such an alignment represented bona fide sequence homology. Specific FASTA (*14*) parameters used for these analyses have been previously described (*13*).

Protein Production. The enterobacteria phage P1 Cre recombinase protein, Entrez protein accession number YP 006472 (*15*), was produced in *Escherichia coli* as a fusion with intein protein purified on a chitin column and chemically cleaved using dithiothreitol, as previously described (*16*). The coding region of the expression vector was confirmed using DNA sequence analysis. The final material was exchanged into buffer containing 20 mM HEPES, 500 mM NaCl, pH 8, by dialysis. Approximately 700 mg of Cre recombinase was obtained from 0.9 kg of fermentation paste.

Protein Characterization. Total protein concentration was estimated using a Bio-Rad colorimetric protein assay (Hercules, CA) and bovine serum albumin as the standard. Purity and molecular weight were estimated from a stained SDS−polyacrylamide gel, which contained three load levels (1, 2, and 3 μg of total protein) by using a densitometer. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Perseptive Voyager DE-Pro, Perkin-Elmer, Foster City, CA) was used to assess the identity of Cre recombinase after reduction, alkylation, and in-gel trypsin digestion of bands excised from an SDS−polyacrylamide gel (*17*). Monoisotopic peptide masses (MH^+) were observed in reflector mode. Observed mass data were compared to the expected masses for a trypsin digest of Cre recombinase (*18*, *19*). N-terminal sequence analysis (*20*) was performed for 15 cycles using an Applied Biosystems 494 Procise Sequencing System on a sample excised from a polyvinylidene fluoride transfer membrane. Immunoblot analysis was performed using goat anti-Cre recombinase antisera, and the protein was detected with enhanced chemiluminescence reagents.

Cre Recombinase Activity Assay. A functional end point assay was developed based on previously published reports (*16*, *21*). Linear substrate DNA was prepared by annealing an oligonucleotide, containing a unique *Bgl*II restriction site flanked by two *lox* sites followed by either an *Eco*RI or *Hin*dIII site, with its complementary oligonucleotide. The annealed DNA was ligated into pUC18 after restriction with *Eco*RI and *Hin*dIII. A 683 bp *Bgl*II fragment of pTYB11, containing a unique *Kpn*I site, was inserted between the two *lox* sites to increase the distance between them. The resulting 3357 bp plasmid was linearized with *Kpn*I and contained a *lox* site at either 480 or 169 bp from the ends. Two recombination products were expected: a linear 682 bp fragment and a circular 2675 bp product. Functional activity was evaluated by mixing DNA substrate (typically 250 ng) with varying amounts of Cre recombinase and assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM NaCl, pH 7.4) on ice. Assay volumes were 50 μL. Reactions were initiated by incubation at 37 °C for up to 30 min and stopped by heating at 70 °C for 10 min followed by an addition of 5 μL of 10× BlueJuice (Invitrogen, Carlsbad, CA). Samples of each reaction were analyzed on 1% (w/v) agarose gels buffered with 1× Tris−acetate−EDTA and containing ethidium bromide. Gels were calibrated using a DNA marker (1 kb ladder, Invitrogen). The percent of product formation was quantitated optically, using an Alpha Innotech Corporation densitometer by comparison of the relative amount of product bands to negative controls lacking Cre recombinase. One unit of activity was defined as the minimum amount of protein required to achieve maximum activity (*16*, *22*) under specific assay conditions: a reaction time of 30 min at 37 °C using 250 ng of substrate DNA. Specific parameters including assay linearity, pH, temperature, and the effect of freeze−thaw were investigated.

CD Spectroscopy. CD measurements were carried out at Alliance Protein Laboratories (Thousand Oaks, CA) on a Jasco J-715 spectrapolarimeter at room temperature with a 0.05 cm cell for the far-UV analysis and a 1 cm cell for the near-UV analysis. The solvent spectra were subtracted from the sample spectra and then converted to the mean residue ellipticity using the protein concentration (assuming a mean residue weight of 112) and the path length of the cell. CD spectra of Cre recombinase were evaluated at a pH intended to mimic a gastric environment (10 mM HCl, 35 mM NaCl, pH 1.2) and a neutral pH (20 mM HEPES, 500 mM NaCl, pH 8.0).

Digestibility Assay. The digestibility of Cre recombinase was evaluated using a previously described method (*23*). Briefly, Cre recombinase was mixed with a diluted solution of simulated gastric fluid (SGF) at a ratio of 1 part protein to 19 parts SGF such that there were 10 U of pepsin per microgram of protein in a total volume of 900 μL. Digestion was performed at 37 °C. Digestion samples (50 μL) were removed at selected times (0.67, 2.08, 5.08, 10.67, 20.00, 30.03, and 60.00 min) and quenched (*23*). Zero incubation time points were prepared in separate tubes and quenched prior to addition of protein or buffer. Experimental controls were prepared in separate tubes to determine the stability of Cre recombinase in SGF lacking pepsin [0.2% (w/v) NaCl, pH 1.2] and to characterize the SGF lacking Cre recombinase.

Samples were separated on SDS−polyacrylamide gels under reducing conditions (*24*) and analyzed using Coomassie Brilliant Blue G colloidal stain and by Western blot with anti-Cre recombinase goat serum. The 60 min digestion sample was not analyzed. The limit of detection of Cre recombinase for these methods was determined by serial dilution of the time zero (T0) incubation time point.

Acute Oral Toxicity of Cre Recombinase in Mice. The Cre recombinase dosing solution was prepared by dialysis of the protein into a vehicle buffer (10 mM sodium phosphate, pH 7.4) followed by concentration using centrifugal filter devices. The bovine serum albumin (BSA) protein control dose was prepared by mixing lyophilized BSA powder (Calbiochem, La Jolla, CA) with the vehicle buffer. Doses were shipped on ice to Charles River Laboratories, Inc. (Spencerville, OH). The dose solutions were stirred continuously at room temperature for approximately 20 min prior to dosing until completion of dosing. Predose samples were collected following 20 min of stirring. Because the Cre recombinase dose was assessed visually to be a suspension due to limited solubility, homogeneity samples (250 μL) were collected prior to dosing from the top, middle, and bottom of the dosing container. After administration of doses, post-dose samples were taken to assess protein stability. All samples were immediately frozen on dry ice and stored at −80 °C until analyzed. The protein in the pre- and post-dose samples was assessed for purity, molecular weight, concentration, and functional activity.

Three groups (each comprised of 10 males and 10 females) of young, adult mice [Crl: CD-1(ICR)BR (VAF/Plus)] received either a dose of Cre recombinase, the vehicle control (10 mM sodium phosphate buffer, pH 7), or the BSA protein control by oral gavage. Following acute dosing, the mice were observed daily, weighed weekly, and had food consumption measured weekly. Daily clinical observations included changes in the skin and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic and central nervous systems, including tremors and convulsions, changes in level of activity, gait, and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies of bizarre behavior. A general health/mortality check was performed twice daily. A gross necropsy examination was performed on all animals after the scheduled time of euthanasia (day 14). Gross necropsy included examination of the cranial, thoracic, abdominal, and pelvic body cavities. No organ weights were collected.

The mice were housed and cared for based on the standards recommended by the Guide for the Care and Use of Laboratory Animals Mice (*25*). Body weights, body weight changes, and food consumption were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey−Kramer test for group-wise comparisons to the protein

```
SCORES   Init1: 42   Initn: 42   Opt: 69   z-score: 95.0 E(): 2.1
>>AD5:21213898 (141 aa)
 initn:  42 init1:  42 opt:  69 Z-score: 95.0 expect():  2.1
Smith-Waterman score: 69;  27.7% identity in 65 aa overlap
 (184-244:33-97)

           160       170       180       190       200
Cre     RCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTL----VSTAGV
                                        ||:  :|   :|| |:  ||    :::  :
LepD2   KFIALFALVAVASAGKMTFKDCGHGEVTELDITGCSGDTCVIHRGEKMTLEAKFAANQDT
             10        20        30        40        50        60

      210       220       230       240       250       260
Cre     EKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATH
         |:    :||::   |:| |:  |  ::: | |:|:
LepD2   AKVTIKVLTKVAGTTIQVPGLETDGCKFIKCPVKKGEALDFIYSGTIPAITPKVKADVTA
             70        80        90       100       110       120

      270       280       290       300       310       320
Cre     RLIYGAKDDSGQRYLAWSGHSARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLD

LepD2   ELIGDHGVMACGTVHGQVE
            130       140
```

Figure 1. Best allergen sequence similarity. Lep d 2 is a dust mite protein.

```
SCORES   Init1: 40   Initn: 40   Opt: 92   z-score: 111.1 E(): 2.8
>>TXN5:P74828 (464 aa)
 initn:  40 init1:  40 opt:  92 Z-score: 111.1 expect():  2.8
Smith-Waterman score: 92; 26.4% identity in 178 aa overlap
 (165-340:58-212)

          140       150       160       170       180       190
Cre     LAFERTDFDQVRSLMENSDRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRML
                                        |:| :|    | :|  ||: :: |:|   :
P74828  YDTEFLPAALEIIERPVSPTARLTGRVMVAGLAITT----AWLAIGRVEVVAPTQG--RI
         30        40        50        60            70          80

          200       210       220       230       240       250
Cre     IHIGRTKTLVST-AGVEKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSAT
          ||:|| : |  :|: : : :|  : |::   :  :: ||:          ::: | :|
P74828  APIGETKIVQSPESGIVRRILVGEGQKVQKGQVL--ITLDPTM--------SAAEAAQAR
             90        100       110          120              130

          260       270       280       290       300       310
Cre     SQLSTRALEGIFEATHRLIYGAKDDSGQRYLA-WSGHSARVGAARDMARAGVSIPEIMQA
          | :  |::   | :: |  | | :| |: |  :: :|:| : | :||| ::  | | |
P74828  VALLSAQLDA---ARNQAIIDALDGKGFRFAAPAAAGAAEVETHRGLARARLGQIEAMLA
             140          150       160       170       180

          320       330       340
Cre     GGWTNVNIVMNYIRNLDSETGAMVRLLEDGD
        || :: : : :     ::|: |:|| ||:
P74828  GGRSDSGAAASA----SAEAQAQVRKLEQSLPLLEQQIAANEAMAAKGYVSKLRVVEMRR
       190       200           210       220       230       240
```

Figure 2. Best toxin sequence similarity. P74828 is a *Sphingomonas* sp. s88 hypothetical 49.1 kDa protein.

control group, when appropriate. All statistical comparisons were two-tailed with a minimum level of significance of 5% ($p < 0.05$).

RESULTS

Bioinformatic Analyses of Cre Recombinase Protein Sequence. Proteins that share a high degree of amino acid similarity throughout the entire length are often homologous. Homologous proteins share secondary structure and common three-dimensional folds. Proteins homologous to toxins are more likely to be toxic, and proteins homologous to allergens are more likely to share linear and/or conformational cross-reactive allergenic epitopes than are unrelated proteins. Therefore, the FASTA program was used to infer higher order structural similarities (i.e., secondary and tertiary protein structures). A separate search tool, ALLERGENSEARCH, was used to locate short linear sequence identities (*13*).

The highest scoring similarity to a protein in the allergen database was a dust mite protein, Lep d 2. The alignment contained 27.7% amino acid identities in a 65 amino acid overlap (**Figure 1**). This result does not indicate structural homology (*26*), supported by the large observed *E* score value of 2.1. No hits of eight linearly contiguous amino acid identities were observed between Cre recombinase and the allergen database. Eight linearly contiguous amino acid identities represent the smallest likely IgE binding epitope (*27*).

The best similarity to a protein in the toxin database was a *Sphingomonas* sp. s88 hypothetical 49.1 kDa protein (**Figure 2**). The alignment contained 26.4% amino acid identities in 178 aa overlap, with a total of eight gaps. The *E* score of 2.8 did not indicate a relevant similarity (*26*). This protein is not itself a toxin but is similar to proteins that comprise the hemolysin secretion apparatus. The toxin database contains many sequences that are not actual toxins (e.g., proteins involved in the secretion of hemolysin, a known toxin), but because the word "toxin" occurred in the annotation section of the flatfile, the entry was included in the database. The best similarities obtained from the ALLPEPTIDES database were to the Cre recombinase protein, as expected. Not surprisingly, lower scoring hits (*E* scores of less than 1×10^{-5}) included DNA integrase proteins from a variety of organisms. DNA integrases are proteins that catalyze the integration and excision of DNA, like Cre recombinase.

Characterization of Cre Recombinase. Cre recombinase was purified from *Escherichia coli* using an intein fusion expression system as previously described (*16*). N-terminal sequence analysis (15 residues, MSNLLTVHQNLPALP) confirmed that Cre recombinase was cleaved properly from the intein fusion, when isolated from the chitin chromatography media. Total protein concentration, determined using a Bio-Rad protein assay, was 1.3 mg/mL. Purity and molecular weight of Cre recombinase were estimated to be 93% and 36.4 kDa, respectively, using SDS−PAGE under denaturing and reducing conditions. The identity of the purified Cre recombinase was confirmed by three methods: immunoblot analysis with Cre-specific antisera, N-terminal sequence analysis, and MALDI-TOF MS (data not shown). Over 70% of the predicted amino acid sequence was confirmed using MALDI-TOF MS mass map analysis of trypsin-digested Cre recombinase.

To further characterize the protein, a functional activity assay was developed using a linear DNA substrate containing two *lox* sites in direct orientation. As summarized below, assay parameters were tested to demonstrate robustness and were not intended to fully characterize the functional activity of Cre recombinase. Activity was evaluated over a range of Cre recombinase protein concentrations and yielded a value of 31 000 U/mg, consistent with the previously reported values of 40 000 U/mg (*22*) and 16 400 U/mg (*16*). Recombination products were observed to increase linearly between 0 and 7 min, and the reaction appeared to be complete within approximately 10 min. When reactions were terminated at 30 min, the maximum amount of recombination products ranged typically from 25 to 30% of the total amount of substrate used. No appreciable difference was observed in the rate of product formation when the assay pH was varied from 7 to 8. Similarly, varying the temperature from 35 to 39 °C had no appreciable effect on the rate of product formation. The effect of repeated freeze−thaw cycles was evaluated to characterize one aspect of storage stability. A single vial containing Cre recombinase was thawed, assayed, and re-frozen on five consecutive days. No effect on the rate of product formation was observed.

Conformational Instability of Cre Recombinase in Acid. When Cre recombinase was diluted in an acidic solution that approximated gastric conditions, no recombinase activity was observed after neutralization when tested as described in the Materials and Methods. To further investigate the effect of low pH on Cre recombinase structural conformation, circular dichroism (CD) spectroscopy was used (**Figure 3**). CD spectrapolarimeters are often used to monitor conformational changes of proteins under different conditions (*28*). The far-UV CD spectrum (**Figure 3A**) of Cre recombinase in a pH 8 buffer (20 mM HEPES, 50 mM NaCl) showed extensive secondary structure with approximately 30% α-helix (*29*). In contrast, the far-UV spectrum of Cre recombinase in a gastric-like solution (10 mM HCl, 35 mM NaCl, pH 1.2) was reduced to ap-

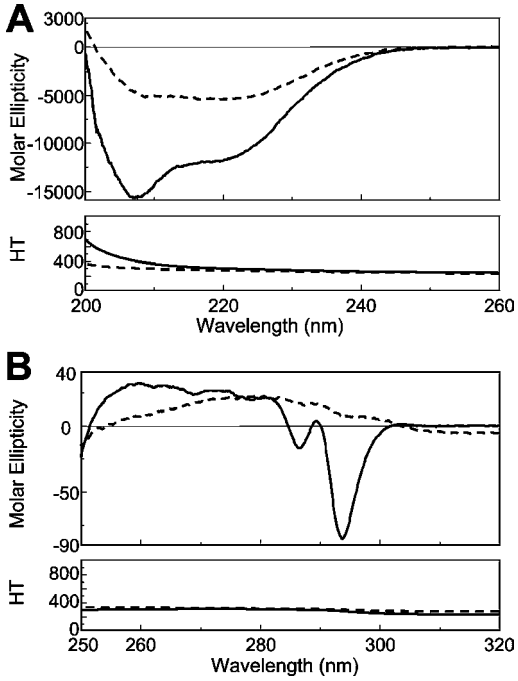

Figure 3. Effect of pH on the structure of Cre recombinase. Panel **A** corresponds to the far UV spectra of Cre recombinase in a neutral pH buffer (solid line) and acid buffer (dashed line). Panel **B** corresponds to the near-UV spectra spectra of Cre recombinase in a neutral pH buffer (solid line) and acid buffer (dashed line).

proximately 10% α-helix. The near-UV CD spectrum (**Figure 3B**) of Cre recombinase in the acidic solution showed no fine structures, whereas sharp peaks at 293 and 286 nm, corresponding to tryptophan and tyrosine residues, were observed for spectra in the pH 8 buffer. These data indicate that Cre recombinase was denatured when placed in an acidic solution, consistent with loss of recombinase activity at low pH.

Digestibility of Cre Recombinase. The digestibility of Cre recombinase in simulated gastric fluid (SGF) containing pepsin was evaluated on Coomassie Brilliant Blue G colloidal stained polyacrylamide gels (**Figure 4**). A separate SDS−polyacrylamide gel was run concurrently to determine the limit of detection (LOD) of Cre recombinase by loading various dilutions of the T0 time point (**Figure 4A**). The lower limit of detection was visually estimated to be 2.7 ng. This represents approximately 0.4% of the total protein loaded based on the pre-digestion concentration of Cre recombinase (700 ng per lane).

Visual examination of **Figure 4B** showed that greater than 99% (100% − 0.4% = 99.6%) of the full-length Cre recombinase (~38 kDa) was digested to a level below the LOD within 40 s (0.67 min) of incubation in SGF (lane T1). Furthermore, all resulting protein fragments were digested to sizes no longer detectable on the stained gel by the 5 min digestion time point (**Figure 4B**, lane T3).

Experimental controls in which Cre recombinase was omitted showed that pepsin was stable throughout the digestion (**Figure 4B**, lane N0 vs lane N7). Experimental controls in which Cre recombinase was incubated in SGF lacking pepsin demonstrated that Cre recombinase underwent some degradation after 60 min (**Figure 4B**, lane P0 vs lane P7). This confirmed that while most of the degradation of Cre recombinase was due to the proteolytic activity of pepsin present in SGF, some degradation was due to instability of Cre recombinase at pH 1.2 and 37 °C.

The faint band that migrated to approximately 76 kDa in some time point samples (e.g., **Figure 4B**, lanes P0 and T0) is likely a Cre recombinase protein dimer because it was detected by the Cre recombinase-specific antibodies used in the Western blot (**Figure 5**). Very faint bands migrating near 25 and 12 kDa were also observed in the same lanes and are likely degradation products of Cre recombinase. These faint bands were also digested within 40 s of incubation in SGF (**Figure 4B**, lane T1).

The digestibility of Cre recombinase was also evaluated using Western blotting with antibodies specific for Cre recombinase (**Figure 5**). A separate Western blot with various dilutions of the T0 time point was run concurrently to determine the LOD (**Figure 5A**). The lower limit of detection was visually estimated to be 0.1 ng, the lowest amount loaded. This value represents 0.2% of the total protein loaded based on the pre-digestion concentration of Cre recombinase (50 ng per lane).

Visual examination of **Figure 5B** showed that greater than 99% (100% − 0.2% = 99.8%) of the full-length immunoreactive Cre recombinase was digested to a level below the LOD within 40 s of incubation in SGF (**Figure 5B**, lane T1). Additionally, no immunoreactive proteolytic fragments were observed at the 40-second digestion time point (T1), including those fragments observed in the stained gel for specimens T1 and T2 (**Figure 4A**, lanes T1 and T2). Consistent with the results of the stained gels, faint immunoreactive bands were observed near 76 and 25 kDa (**Figure 5B**, lanes P0 and T0), which suggested that these bands represent dimers and degradation products of Cre recombinase.

Experimental controls lacking Cre recombinase (**Figure 5B**, lanes N0 and N7) showed that there were no immunoreactive background signals due to SGF. Experimental controls in which Cre recombinase was incubated in SGF lacking pepsin demonstrated that Cre recombinase underwent partial degradation after 60 min (**Figure 5B**, lane P0 vs lane P7), consistent with the previously described results observed with the Coomassie Brilliant Blue G colloidal stained gel.

Assessment of Potential Oral Toxicity of Cre Recombinase in Mice. Because most proteins that are toxic act through acute mechanisms, the acute oral toxicity of Cre recombinase was evaluated in CD-1 mice. Each dosing group was comprised of 10 males and 10 females. The Cre recombinase dosing solution was a suspension; however, testing of samples taken from the solution showed that it was maintained as a homogeneous preparation. The Cre recombinase and BSA dose levels were experimentally determined from the observed total protein concentrations and purities of these proteins.

Because the final isolation step of Cre recombinase purification yielded protein in a high salt buffer that was unsuitable for administration to animals, the protein used for dosing the mice was re-formulated into a low ionic strength buffer to minimize the potential toxic effects of a high salt vehicle buffer. However, this reformulation resulted in some potential degradation of Cre recombinase, because the level of intact protein decreased from 93% to 83%. The amount of apparent intact protein was observed to further decline during the dosing period to approximately 67% of total protein, also most likely attributable to the sub-optimal ionic strength of the vehicle buffer while stirring at room temperature. Nevertheless, the functional activity of Cre recombinase was stable for the duration of the dosing period questioning whether the reduction in apparent intact protein was actual degradation.

Because of the apparent decrease in intact protein during dosing of the mice, the administered level of Cre recombinase to the mice was conservatively calculated to be 53 mg/kg body weight (BW), using the lower value of apparent intact protein

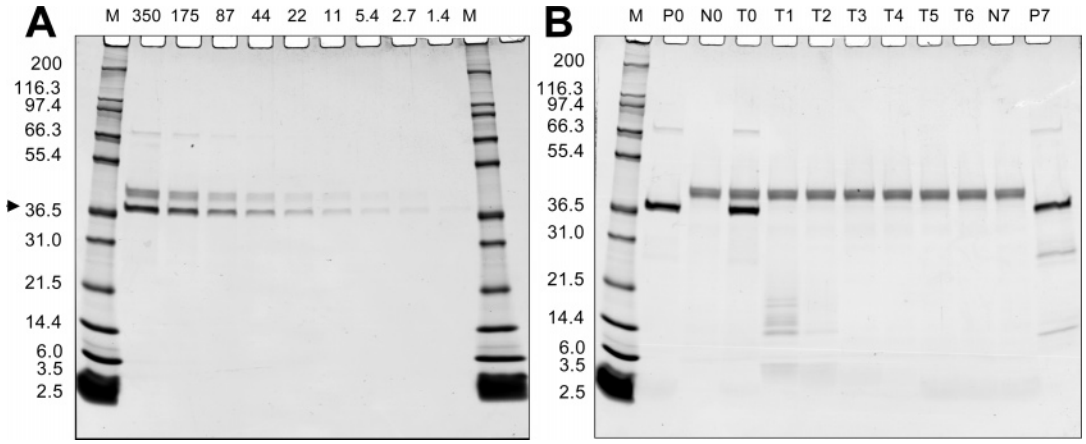

Figure 4. Digestibility of Cre recombinase analyzed using Coomassie Brilliant Blue G colloidal stained SDS–polyacrylamide gels. Panel **A** corresponds to the limit of detection of Cre recombinase. The values above each lane correspond to the approximate amount (ng) loaded. Panel **B** corresponds to Cre recombinase digestion in SGF. Approximately 700 ng (total protein) was loaded in each lane. T0, T1, T2, T3, T4, T5, and T6 correspond to Cre recombinase incubated in SGF for 0, 0.67, 2.08, 5.08, 10.67, 20.00, 30.03, and 60.00 min. P0 and P7 correspond to Cre recombinase incubated in SGF lacking pepsin for 0 and 60 min. N0 and N7 correspond to SGF lacking Cre recombinase and were incubated for 0 and 60 min. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cre recombinase migrated to approximately 38 kDa (indicated by the arrow on the left) and pepsin to approximately 40 kDa.

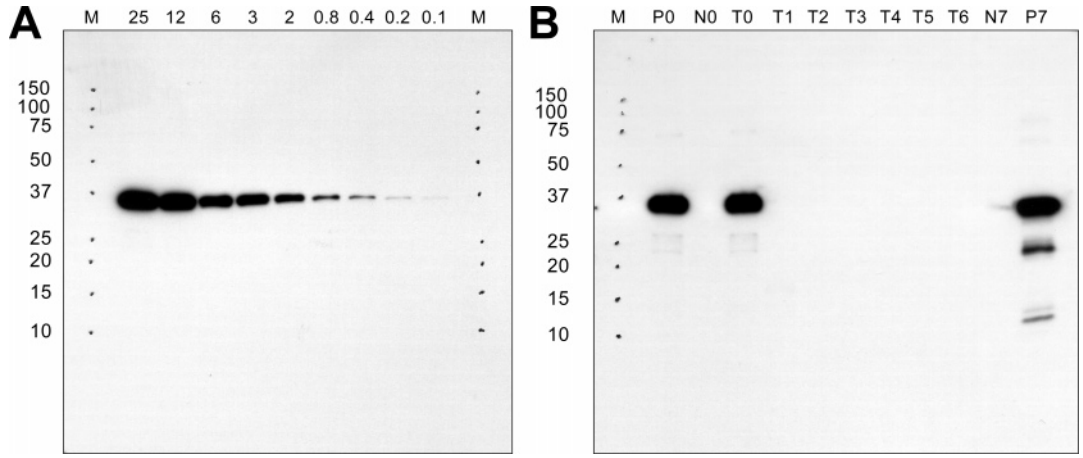

Figure 5. Digestibility of Cre recombinase analyzed using Western blot analysis. Panel **A** corresponds to the limit of detection of Cre recombinase. The values above each lane correspond to the approximate amount (ng) loaded. Panel **B** corresponds to Cre recombinase digestion in SGF. Approximately 50 ng (total protein) was loaded in each lane. T0, T1, T2, T3, T4, T5, and T6 correspond to Cre recombinase incubated in SGF for 0, 0.67, 2.08, 5.08, 10.67, 20.00, 30.03, and 60.00 min. P0 and P7 correspond to Cre recombinase incubated in SGF lacking pepsin for 0 and 60 min. N0 and N7 correspond to SGF lacking Cre recombinase protein and were incubated for 0 and 60 min. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. A 5 min exposure is shown.

Table 1. Summary of Body Weight Data Observed in the Acute Oral Toxicity Study in Mice

	time[a]									
	day 0		day 7		change (day 0 to day 7)		day 14		change (day 7 to day 14)	
group	male	female	male	female	male	female	male	female	male	female
Cre recombinase	27.5 (0.8)	25.6 (0.5)	31.6 (1.5)	27.8 (0.8)	4.1 (1.2)	2.2 (0.5)	33.4 (2.4)	28.8 (0.9)	1.8 (1.2)	1.0 (0.5)
BSA (protein control)	27.9 (0.8)	25.4 (1.0)	31.8 (1.3)	27.7 (1.3)	3.9 (1.1)	2.2 (0.8)	34.1 (2.4)	28.6 (1.4)	2.3 (1.2)	1.0 (0.7)
vehicle (buffer control)	27.6 (1.0)	25.7 (0.8)	32.0 (2.1)	27.6 (1.0)	4.4 (1.4)	1.9 (0.6)	34.4 (2.3)	29.0 (1.4)	2.3 (0.7)	1.4 (0.5)

[a] Values correspond to the mean body weight (and standard deviation) of 10 animals in units of grams.

noted at the end of the dosing period. The level of intact BSA in the protein control article dosing solution was comparable for samples taken before and after administration; the BSA protein was calculated to be 50 mg/kg BW.

No mortality occurred during the study. All animals appeared normal throughout the study based on daily clinical observations. Body weight gain was noted for most animals during the 14 day test period (**Table 1**), with no significant differences ($p < 0.05$) in body weight or body weight changes between the three groups (Cre recombinase, BSA, and vehicle) during the study. All animals appeared normal based on daily clinical observations and postmortem examination of organs and tissues. No significant differences between the three groups ($p < 0.05$) were observed in the food consumption data during the study

Table 2. Summary of Food Consumption Data Observed in the Acute Oral Toxicity Study in Mice

	time[a]							
	day 0 to day 7				day 7 to day 14			
	g kg^{-1} day^{-1}		g animal^{-1} day^{-1}		g kg^{-1} day^{-1}		g animal^{-1} day^{-1}	
group	male	female	male	female	male	female	male	female
Cre recombinase	230.4 (16.5)	238.7 (31.9)	6.6 (0.5)	6.3 (1.0)	186.5 (14.0)	241.5 (72.2)	6.1 (0.6)	6.8 (1.9)
BSA (protein control)	232.3 (19.5)	232.5 (23.6)	6.7 (0.6)	6.1 (0.7)	197.7 (16.9)	240.3 (76.2)	6.5 (0.7)	6.7 (1.9)
vehicle (buffer control)	239.9 (21.4)	238.8 (43.8)	6.9 (0.8)	6.3 (1.2)	198.8 (18.8)	213.7 (19.1)	6.6 (0.9)	6.1 (0.7)

[a] Values correspond to the mean (and standard deviation) of 10 animals per group.

(**Table 2**). No treatment-related gross pathological findings were observed at necropsy on day 14. Details regarding the extent of daily clinical observations, examination of organs, and tissue collection are provided in the Materials and Methods section. Because no toxicity was observed under the conditions of this acute oral toxicity test, the no adverse effect level (NOAEL) in mice was estimated to be greater than the administered dose of 53 mg/kg BW.

DISCUSSION

Targeted excision of DNA using the Cre-*lox* recombination system in biotechnology, followed by managed breeding, results in plants that retain the desired genetic trait(s) but lack the Cre recombinase genetic construct and the excised DNA, such as an antibiotic resistance marker (*8*, *12*). Therefore, no exposure to Cre recombinase is expected in the resulting commercial event. However, studies were performed to assess the safety of Cre recombinase, to provide added assurance of safety of products developed using this technology.

Food and feed safety assessment of proteins introduced into crops using agricultural biotechnology begins by considering the known safety of the gene/source and includes an investigation of the history of dietary consumption of the introduced protein by humans and animals (*30*). In this case, the source of Cre recombinase is the P1 bacteriophage. Numerous studies have shown that virus particles are abundant in aquatic and other ecosystems (*31*−*36*), particularly broad-host-range bacteriophages like P1, from which Cre recombinase was isolated. It has been shown that bacteriophage with a broad host range like P1, are common and readily isolated viruses from complex natural microbial communities by using plaque-forming assays (*37*). *P*1 are "temperate phage", capable of both lytic and lysogenic lifecycles after they infect a sensitive bacterial host. Compared with other bacteriophage, P1 has a broad host-range across Gram-negative bacteria including *Pseudomonadaceae*, *Rhizobiaceae*, and *Enterobacteriaceae* (like *E. coli* strains B, C, and K (*38*), *Shigella dysenteria* (*39*), and *S. flexneri* (*40*)) and in rough forms of *Salmonella typhi* (*41*) and *S. typhimurium* (*41*). The most significant source of human dietary exposure to bacteriophage P1 in nature is likely to be lysogenic P1 bacteriophage in coliform bacteria (the *Enterobacteriaeciae* and *E. coli*). *E. coli* itself constitutes a significant proportion of the normal microbial flora of the large intestine in humans and animals.

Another aspect of the safety assessment included bioinformatic comparisons of Cre recombinase to select protein databases. These bioinformatic analyses are used to detect potential similarities and infer homologies between Cre recombinase and known allergens, toxins, or other pharmacologically relevant proteins. Homologous proteins share secondary structure and common three-dimensional folds (*42*), which may indicate that they share common allergenic, toxic, or pharmacologic effect(s). Because the degree of similarity between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. For example, while related (homologous) proteins may share only 25% amino acid identity in a 200 amino acid overlap (*43*), this is not generally sufficient to indicate allergenic potential through IgE-mediated cross-reactivity (*44*). Indeed, potential allergenic cross-reactivity caused by proteins sharing conformational or linear epitopes is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (*45*). Such high levels of identity are readily detected using the bioinformatic tool, FASTA (*14*).

Short contiguous amino acid sequence identities shared between Cre recombinase and sequences in the allergen database were identified using the ALLERGENSEARCH algorithm (*13*). This analysis was performed to detect potential IgE-binding epitopes in the absence of a comprehensive, validated, and robust IgE-binding epitope database (*46*). For practical (optimal signal-to-noise ratio) and theoretical (approximate average linear epitope length) reasons, the current approach recommends searching for eight or more contiguous amino acid identities.

Cre recombinase was found to share no biologically relevant structural similarities to known allergens or toxins. No linearly contiguous identities of eight amino acids were found to proteins within the allergen sequence database. When compared to all known protein sequences, only the expected hits to Cre recombinase itself and other DNA-binding proteins (integrases) were observed.

Proteins must be stable to pepsin and the acidic conditions of digestive systems if they are to reach and pass through the intestinal mucosa to elicit an allergenic or toxic response (*27*, *47*). Previous studies have shown that in vitro simulated mammalian gastric fluid are useful models of animal digestion to assess the susceptibility of proteins expressed in genetically modified plants to proteolytic digestion (*48*). A similar model has also been used to examine the stability of milk allergens (*49*). These models have been used to investigate the digestibility of plant proteins (*50*, *51*), animal proteins (*52*), and food additives (*53*) to assess protein quality (*54*), to study digestion in pigs and poultry, to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (*54*), and to investigate the controlled-release of experimental pharmaceuticals (*55*). Recently the International Life Science Institute (ILSI) standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (*23*). The SGF formulation, time course, and experimental parameters used in the present study conform to the ILSI multi-laboratory method. In addition, many known protein toxins are stable to digestion and most act through acute mechanisms (*56*−*58*). The exceptions to this rule include certain anti-nutrient proteins (lectins, protease inhibitors) that can exert potential toxicity due to prolonged exposure (*59*).

However, even these anti-nutritional proteins are characteristically poorly digested.

Therefore the stability of Cre recombinase to simulated digestion tract conditions was important to determining its potential allergenicity and/or toxicity. Interestingly, even in the absence of proteolytic enzymes, CD spectroscopy showed that the structural conformation of Cre recombinase was lost upon simple exposure to low pH (1.2). Functional activity of Cre recombinase was also abolished virtually immediately upon exposure of the protein to the low pH solution.

Because Cre recombinase is rapidly and fully degraded to small peptides in simulated gastric fluid containing the proteolytic enzyme pepsin (*23*), it is unlikely to cause allergenic or toxic effects if consumed. The rapid inactivation and digestion of Cre recombinase in simulated gastric conditions is consistent with the previously mentioned observation that humans and animals are constantly exposed to the bacteriophage P1 and its associated Cre recombinase protein without apparent incident.

Short-term (2−4 week) feeding studies are required to evaluate the toxicity of anti-nutrients (*59*). However, Cre recombinase does not belong to any of these families of anti-nutrient proteins and is shown by the present studies to be readily digested, unlike most anti-nutritient proteins. Therefore, doses of Cre recombinase were formulated for oral dosage in mice in an acute study to provide large margins of dietary exposure when compared to hypothetical human dietary exposure. Mice orally dosed once with Cre recombinase (53 mg/kg BW) did not experience any observed adverse effects to the protein.

Collectively, these data are consistent with results expected for proteins that are considered to be safe when consumed in the diet and support the conclusion that the Cre recombinase protein poses no risks to the safety of food or feed. Cre recombinase amino acid sequence shares no relevant similarity to known allergens, toxins, or pharmacologically active proteins. Cre recombinase was observed to unfold and lose enzymatic activity when exposed to a low pH solution of simulated gastric fluid and was rapidly degraded to peptides or smaller when exposed to pepsin in a standardized digestion model. Therefore, if Cre recombinase were part of a diet, it would not survive typical gastric conditions, indicating that there would be negligible dietary exposure of humans and animals to the intact active protein. The lack of toxicity was corroborated when Cre recombinase was tested for acute oral toxicity in mice. No toxicity and no gross pathology findings were observed. Therefore, in the unlikely event that Cre recombinase was detected in the food or feed supply, it is unlikely to pose any meaningful risks.

ABBREVIATIONS USED

aa, amino acid; bp, base pair; BSA, bovine serum albumin; BW, body weight; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ILSI, International Life Science Institute; LOD, limit of detection; NOAEL, no adverse effect level; SGF, simulated gastric fluid; UV, ultraviolet

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