FOOD DERIVED FROM INSECT-PROTECTED CORN LINE MON 810

A SAFETY ASSESSMENT

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SUMMARY

Food derived from the insect-protected corn line MON 810 has been evaluated to determine its suitability for human consumption. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, stability of the introduced genes, evaluation of intended and unintended changes and assessment of the potential allergenicity or toxicity of any newly expressed proteins.

Nature of the modification

The genetically modified corn line MON 810 was generated by the transfer of the *cry1*A(b) gene into the parental line (genotype Hi-II) which confers protection against attack from insects. The protein product is an insecticidal crystal protein, whose toxic effect is specific to Lepidopteran insects, in this case the European corn borer. No other genes were transferred to the corn plant. The introduced gene for *cry1*A(b) was found to be stably integrated into the corn plant genome and is phenotypically and genetically stable over multiple generations.

General safety issues

This *Bacillis thuringiensis* (Bt) insecticidal protein has a long history of use in agriculture as a biopesticide and no evidence of adverse health effects has emerged. The newly expressed Cry1A(b) protein in corn was detected in leaves, kernels, whole plant and pollen in very small amounts (>0.001% total protein).

The insect-protected corn line MON 810 does not contain any antibiotic resistance genes and therefore poses no risk to the development of antibiotic resistant pathogenic bacteria.

Toxicological issues

Data for the newly expressed Cry1A(b) endotoxin in the insect-protected corn line MON 810 has been evaluated for its potential toxicity to humans. No signs of toxicity were observed among mice following acute oral doses up to 4000 mg/kg Cry1A(b) of the endotoxin and no significant similarity to the amino acid sequence of known toxins was identified.

An examination of the digestion of the proteins in simulated mammalian digestive systems resulted in rapid digestion of the proteins. Additionally, the protein does not have chemical or physical characteristics that are typical of known food allergens. Amino acid sequence analysis did not reveal any similarities to known allergens.

Therefore, the evidence does not indicate that there is any potential for the protein to be toxic or allergenic to humans.

Nutritional Issues

Comprehensive nutrient analyses did not indicate any significant differences in the levels of major constituents, nutrients or anti-nutritional factors between insect-protected corn line MON 810 and the control corn lines. The major components assessed on corn kernels were proximate (protein, fat, moisture, calories, carbohydrates and ash), amino acids, fatty acids, inorganic components, carbohydrate and tocopherols. The level of trypsin inhibitors were also analysed.

These analyses confirm that insect protected corn line MON 810 is nutritionally and compositionally comparable to other corn lines and that no health or safety risks are posed by consuming food derived from the genetically modified corn.

Conclusion

No public health and safety concerns have been identified in the assessment of insect protected corn line MON 810. Based on currently available data, food derived from corn line MON810 is comparable to food derived from conventional corn in terms of their safety and nutritional adequacy.

FOOD DERIVED FROM INSECT-PROTECTED CORN LINE MON 810

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on corn that has been genetically modified to be protected from Lepidopteran insects, particularly, the European corn borer. This corn is referred to as corn line MON 810.

The corn was developed for cultivation in the United States. Products derived from corn harvested from these plants may have been imported into Australia and New Zealand.

Domestic production of corn in both countries is supplemented by a small amount of imported corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Other products include maize starch which is used by the food industry for the manufacture of dessert mixes and canned foods and corn-based ingredients processed into breakfast cereals, baking products, extruded confectionary and corn chips.

DESCRIPTION OF THE MODIFICATION

Studies evaluated:

J. Kania, P. Keck, E. Levine and P.R. Sanders. 1995. Molecular analysis of insect-protected maize line MON 810. Monsanto Company, USA 63198.

Methods used in the genetic modification

Using particle bombardment, the parental corn line (genotype Hi-II) was simultaneously transformed with two plasmids:

i) *PV–ZMBKO7* which contains:
- the *cry1*A(b) gene for insect resistance; and
- the *npt*II gene for antibiotic resistance;

ii) *PV-ZMGT10* which contains:
- the *gox* gene for glyphosate tolerance;
- the CP4 EPSPS gene for glyphosate tolerance; and
- the *nptII* gene for antibiotic resistance.

Both the gox and CP4 EPSPS genes allow the selection of transformed plants under application of glyphosate (Barry et al, 1992). The bacterial *nptII* gene is a marker used to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells (Bevan et al, 1983).

Transformation with and selection for these plasmids resulted in line MON 810 which is the subject of this assessment. The transformations resulted in the transfer of only one gene - the *cry1*A(b) gene from *Bacillus thuringiensis* subsp. kurstaki strain HD-1.

No genes conferring glyphosate tolerance or antibiotic resistance were transferred to line MON 810.

Function and regulation of the novel gene

Although there was the potential for the transfer of four genes into the corn lines, only one gene, the *cry1*A(b) gene was transferred into line MON 810.

All genes require regulatory sequences that allow them to be transcribed into RNA and then translated into a protein product which are outline in Table 1. These sequences are termed promoter, polyadenylation signal sequence and enhancer sequence. A promoter is the key control element that enables a gene to be transcribed into messenger RNA (mRNA) and a terminator is a DNA (polyadenylation) sequence which stops the transcription of mRNA. These sequences can be unique in each organism and thus regulatory elements that already exist in plants are often used in gene constructs to enable functioning in the plant.

Table 1. Description of the gene transferred to corn Line MON 810

	Gene	**Promoter**	**3' untranslated region**	**Enhancer**
	cry1A(b)	E35S	NOS 3'	*hsp70*
Source of sequence	*Bacillus thuringiensis*	Cauliflower Mosaic Virus	*Agrobacterium tumefaciens*	Maize

The *cry*1A(b) gene includes the following regulatory elements:

(i) the 35S promoter region of the cauliflower mosaic virus (CaMV);

(ii) the intron from the maize *hsp70* gene (heat shock protein); and

(iii) the 3' untranslated region of the nopaline synthase gene (NOS 3') from the Ti plasmid of *Agrobacterium tumefaciens*. The NOS 3' sequence provides the polyadenylation signal for stable expression.

The CaMV E35S promoter enables the constitutive, high-level expression of the *cry*1A(b)gene. It is widespread in nature and is often present in many plants (Odell *et al*, 1985). The enhancer region of a maize intron for the *hsp70* gene is present to increase the levels of gene transcription (Rochester *et al*, 1986). The NOS 3' sequence is present to stop the transcription of the gene by providing a mRNA polyadenylation signal (Fraley *et al*, 1983).

The Cry1A(b) gene

Bacillus thuringiensis (Bt) is the accepted name for a range of soil dwelling, aerobic spore-forming bacteria that forms crystal proteins during sporulation that are toxic to

insects. Studies of the physical properties of the crystal protein structure and screening of many Bt strains have revealed that there are a multitude of crystal types and subsequent spectrum of activity. It is widely accepted that there are four major classes of the crystal protein genes (cry) that are Lepidoptera specific (cry1), Lepidoptera and Diptera specific (cry2), Coleoptera specific (cry3) and Diptera specific (cry4) (Hofte and Whitely, 1989; Drummond and Pinnock, 1991; Cooper 1991; Noteborn *et al*, 1995).

The *cry*1A(b) gene relevant to this application, encodes a nature identical Cry1A(b) insecticidal crystal protein, whose toxic effect is specific to Lepidopteran insects. During sporulation, *B. thuringiensis* produces cytoplasmic inclusions containing one or more of the insecticidal crystal proteins or delta–endotoxins. Most crystal proteins are synthesised intracellularly as inactive protoxins that spontaneously form small crystals, approximately 1 µm in size. Upon ingestion by susceptible insects, the highly alkaline pH of the midgut promotes solubilisation of the protoxin–containing crystals. The protoxin is then activated by trypsin–like gut proteases which cleave off domains from the carboxy– and amino– termini leaving a protease–resistant core which is the active toxin. The now active toxin binds to a highly specific glycoprotein receptor on the surface of midgut epithelial cells in the insect. When about eight of these core proteins aggregate together, they form a pore through the cell membrane. These cells eventually swell and burst, causing loss of gut integrity and resulting in larval death within 1 to 2 days (Cooper, 1991; Hofte and Whitely, 1989).

The *cry*1A(b) gene sequence was modified to increase the levels of expression in corn (Perlak et al, 1991). The native gene contained A+T rich regions that could be potential polyadenylation sites and codons that are not frequently used in plant genes thus impairing its expression in the plant. The *cry*1A(b) gene sequence was modified to reflect plant codon usage therefore allowing efficient expression in the plant.

Characterisation of the genes in the plant

Southern blot analysis is used to detect the presence of specific DNA sequences and to establish the mode, number and stability of inserted DNA (Lewin, 1997). In line MON 810, Southern blot analysis was used to demonstrate that there was a single DNA copy of the *cry1*A(b) gene, of approximately 5.5 Kb inserted into the corn line. The presence of the *nptII* gene or any DNA from plasmid PV-ZMGT10 was not detected (ie. the CP4 EPSPS, *gox* and *nptII* genes) suggesting that only the *cry1*A(b) gene had been inserted in corn line MON 810.

Stability of the genetic changes

The stability of inserted DNA was demonstrated using ELISA analysis and insect feeding assays. Segregation analysis for line MON 810 is consistent with a stable, single dominant gene segregating according to Mendelian genetics. The insect-protected phenotype and inheritance pattern are consistent over multiple generations.

Conclusion

Insect-protected corn line MON 810 was generated using the particle bombardment transformation system to transfer the *cry*1A(b) gene to corn. No other genes were

transferred during transformation. The DNA has transferred into the corn genome as a single and stable DNA insert.

GENERAL SAFETY ISSUES

The corn used in compositional analyses to assess the safety of the inserted DNA was grown at six locations throughout the USA. The insect-protected corn has been evaluated against the safety assessment guidelines developed by ANZFA (ANZFA, 1999). As the data presented is for the whole kernel, the safety assessment issues relate to Group D foods – food ingredients.

Studies evaluated:

K.A. Croon *et al* , 1995. Safety, compositional and nutritional aspects of insect-protected corn line MON 801: conclusion based on studies and information evaluated according to FDA's policy on foods from new plant varieties. Submitted to FDA on September 15, 1995

K.A. Croon, P.R. Saunders and R.L. Fuchs. 1996. Safety, compositional and nutritional aspects of insect-protected corn line MON 809 and MON 810: Conclusion based on studies and information evaluated according to FDA's policy on foods from new plant varieties. Monsanto # 96-102F

The insect-protected corn is largely imported for processing into a diverse range of products including breakfast cereals, baking products, extruded confectionary and corn chips. Maize starch is used by the food industry for the manufacture of dessert mixes and canned foods. The corn products that Australia and New Zealand currently import are largely highly processed products, particularly high fructose corn syrup. It is noted that the import of corn products may significantly increase in the future.

History of the use of corn as a food source

Corn is widely cultivated on nearly every continent and represents a staple food for a significant portion of the world's population. Most of the corn consumed by humans are corn-based food items rather than whole kernel or processed corn. These products are routinely used in food and have a long history of safe use.

The largest use of corn in the USA is as animal feed for cattle, chickens and pigs.

Nature of the novel protein

The *cry1A*(b) gene is derived from the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*) strain HD-1 (Fischoff *et al*, 1987). It encodes a full length *B.t.k* DH-1 (ie Cry1A(b)) protein of 1156 amino acids (131 kDa). Upon digestion by trypsin, an active trypsin-resistant protein of approximately 600 amino acids is produced (63 kDa).

The *cry*1A(b) gene sequence in insect protected corn line MON 810, has been modified to improve the expression in the plant. Expression of the *cry1A*(b) results in a full length delta–endotoxin that is identical to the one produced by *B. thuringiensis* subsp *kurstaki*, strain HD-1 (*B.t.k* HD-1). Under digestive conditions, the full length delta-endotoxin is cleaved to produce the active trypsin-resistant core protein. Both the full length delta–endotoxin and smaller trypsin resistant core protein produced in

line MON 810 are identical to the naturally occurring full length Cry1A(b) delta–endotoxin and the cleaved active core protein produced by *B. thuringiensis* subsp *kurstaki*.

This trypsin resistant core protein is equivalent to the *E. coli* produced *B.t.k.* HD-1 (trypsin resistant core protein) which is widely used as a biopesticide.

Expression of novel protein in the plant

Two techniques are widely used to detect and quantify the products of genes (ie proteins) that are present in the tissue analysed. Enzyme linked immuno-sorbent assay (ELISA) is a highly sensitive technique that can detect the presence of a protein approximately to a sensitivity of 10-100 ρg. Western blot analysis is a highly specific technique also used for the detection of proteins (Lewin, 1997). Both techniques were used to analyse protein expression in leaf, kernel, whole plant tissue and pollen from the insect-protected corn line.

Cry1A(b)

ELISA and western blot analyses of leaf, kernel, whole plant tissue and pollen from the insect-protected corn line MON 810 demonstrated that the Cry1A(b) delta–endotoxin protein is expressed at very low levels in all tissues tested (Table 2) and constitutes less than 0.001% of the total protein in each tissue. The *cry1*A(b) gene is the only gene expressed in line MON 810 and was expressed highest in the leaves (Table 2).

Table 2: Protein expression levels in the insect-protected corn lines as determined by ELISA analysis

Corn Line MON 810	**Mean expression levels and ranges (µg/g fresh weight)** [1]							
	Leaf		**Grain**		**Whole Plant**[2]		**Pollen**[3]	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Cry1A(b)	9.35	7.93-10.34	0.31	0.19-0.39	4.15	3.65-4.65	0.09	na
CP4 EPSPS	nd[4]	-	nd	-	nd		na	-
GOX	nd	-	nd	-	nd		na	-
nptII	na[4]	-	na	-	na		na	-

[1]Values are means from six plant samples ie. n=6. One plant is taken from each site unless otherwise noted.
[2]Values are means from sample(s) from replicate plant samples.
[3]Values are means from sample(s) from one site only (n=6).
[4]na: not assayed; nd: not detected; -: not applicable.

CP4 EPSPS, GOX and nptII Proteins

CP4 EPSPS and GOX proteins were not detected by ELISA or western blot analysis in leaf, kernel and whole plant tissue from corn line MON 810, confirming the results from the Southern blots which indicated that these genes had not been transferred.

Given that the *nptII* gene was not detected by Southern blots and that it is under the control of a bacterial promoter, it is not expected to be expressed in the transformed plant cells (WHO, 1993) and no analysis were done to detect this protein.

Impact on human health from potential transfer of novel genetic material to cells in the human digestive tract

The human health considerations in this regard depend on the nature of the novel genes and must be assessed on a case-by case basis.

In 1991, the World Health Organization (WHO) issued a report of a Joint FAO[1]/WHO Expert Consultation which looked at strategies for assessing the safety of foods produced by biotechnology (WHO 1991). It was concluded by that consultation that as DNA from all living organisms is structurally similar, the presence of transferred DNA in food products, in itself, poses no health risk to consumers.

The major concern in relation to the transfer of novel genetic material to gut microorganisms is with antibiotic resistance genes.

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993). There are concerns, however, that there could be horizontal gene transfer of the antibiotic resistance gene from ingested food to gut microorganisms and that if the microorganisms are able to express the transferred resistance gene this could lead to an increase, in the gastrointestinal tract, of microorganisms resistant to a specific antibiotic. This, in turn, might lead to an increased potential for the transfer of the antibiotic resistance gene to pathogenic microorganisms, thus compromising the therapeutic use of such antibiotics. There are further concerns that, if the antibiotic resistance gene is expressed in the plant, the expressed protein, when ingested, could inactivate oral doses of the antibiotic to which it confers resistance.

The insect-protected corn line MON 810 does not contain an antibiotic resistance gene as indicated by the Southern blot experiments and therefore no protein product from this gene is possible. The only gene transferred is the insect-protection cry1A(b) gene which is not considered to pose any health risk. Additionally, the products from insect-protected corn are largely consumed as processed corn products and the processing is likely to destroy the function of any DNA present in the food.

As discussed above, it is extremely unlikely that novel genetic material will transfer from GM foods to bacteria in the human digestive tract because of the number of complex and unlikely steps that would need to take place consecutively.

It is equally unlikely that novel genetic material will transfer from GM foods to human cells via the digestive tract. In considering the potential impact on human health, it is important to note that humans have always consumed large amounts of DNA as a normal component of food and there is no evidence that this consumption has had any adverse effect on human health. Furthermore, current scientific knowledge has not revealed any DNA sequences from ingested foods that have been incorporated into human DNA. Novel DNA sequences in GM foods comprise only a minute fraction of the total DNA in the food (generally less than 0.01%) and are

[1] Food and Agriculture Organization.

therefore unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods.

Conclusion

The experiments show that the *cry*1A(b) gene is expressed in insect-protected corn line MON 810 and is expressed at relatively low levels in leaves, kernels and at a virtually negligible level in pollen. Given the history of safe use of Bt in agriculture and that it has been considered non-toxic to humans and other non-target organisms, the transfer of the *cry*1A(b) to corn is not considered to risk public health and safety.

TOXICOLOGICAL ISSUES

Studies evaluated:

T.C. Lee and M. Bailey. 1995. Assessment of the equivalence of *B.t.k.* HD-1 Protein produced in several insect-protected corn lines and *Escherichia coli*. Monsanto Company, USA 63198. 95-040E

T.C. Lee, M. Bailey, S. Sims, J. Zeng, C.E. Smith, A. Shariff, L.R. Holden and P.R. Sanders. 1995. Assessment of the equivalence of *Bacillus thuringiensis* susp. kurstakis HD-1 Protein produced in *Escherichia coli* and European corn borer resistant corn. Monsanto Company, USA 63198.

Levels of naturally-occurring toxins

There are no naturally occurring toxins known to occur at biologically significant levels in corn (Wright, 1987).

Potential toxicity of newly-expressed protein

The crystal protein produced by insect-protected corn line MON 810 is identical to the protein produced by the *B. thuringiensis* formulations that have been used commercially for many years to control insect pests. There is no evidence from this history of use that there is any associated toxicity to humans. The toxicity of these proteins is very specific to Lepidoptera and there is no evidence that they are active against non–target insects, birds, fish or mammals (Drummond and Pinnock, 1991). This lack of activity against non–target species appears to be due to a number of factors including physical differences in the gut environment and an absence of specific gut receptors (Frick 1995) in other organisms. The binding of the delta–endotoxin to specific gut receptors appears to be a pre–requisite for toxicity (Cooper, 1991).

Studies evaluated:

J. Astwood. 1995. *Bacillus thuringiensis* susp. kurstakis HD-1 insecticidal protein (*B.t.k.* HD-1 protein) is homologous to proteins of the *Bacillus thuringiensis* insecticidal crystal protein gene family, but not to protein toxins found in public domain sequence databases. Monsanto Company, USA 63198. MSL-14283

M.W. Naylor. 1992. Acute oral toxicity study of *B.t.k.* HD-1 tryptic core protein in albino mice. Monsanto Company, USA 63198. MSL-11985

The potential for toxicity of the newly expressed proteins, Cry1A(b), were evaluated based on:

. the amino acid sequence similarity with known toxins
. acute toxicity testing in mice.
. the resistance to digestion by proteases and acids in the model digestive/gastric system
. their presence as a major protein component in a specified food.

An amino acid sequence comparison of the Cry1A(b) to a database of known allergens detected significant similarities only to other *B. thuringiensis* insecticidal crystal proteins.

An acute oral toxicity study was done to assess the potential mammalian toxicity of the Cry1A(b) protein. The test protein was produced by fermentation in *E. coli* because the plant lines did not express enough protein for purification of large quantities for toxicity testing. Data was presented to indicate that the bacterially produced Cry1A(b) protein is equivalent to the plant produced Cry1A(b) protein in terms of its molecular mass and immunological cross-reactivity. Therefore the *E. coli* produced Cry1A(b) protein is a suitable substitute for plant produced Cry1A(b) in toxicity testing.

The Cry1A(b) core protein (B.t.k. HD-1 trypsin resistant core protein) was administered to groups of ten CD-1 mice/sex using doses up to 4000 mg/kg body weight. These doses are well above the level of expression found in insect-protected corn plants (refer to Table 2) and represent a test using 200-1000 fold increase in amount of protein that would be expected by consuming the genetically modified plants. A group of mice (vehicle control group) were administered 4000 mg/kg bovine serum albumin and another control group (also termed vehicle control group) were administered 66.66 mg/kg carbonate buffer.

Clinical observations were performed and body weights and food consumption were determined. One female mouse belonging to the vehicle control died during the test — on day 1. The death of the control female was considered a result of the intubation procedure. As there were no deaths in other treated mice, or at higher exposure levels, the death is not considered to be treatment related. All surviving animals were necropsied at study termination (8-9 days). Mice were observed up to 9 days after dosing and no treatment related effects on body weight, food consumption, survival, or gross pathology were observed for mice administered the B.t.k HD-1 core protein.

Levels of naturally occurring allergenic proteins

There are no naturally occurring allergenic proteins known to occur in corn (Wright, 1987).

Potential allergenicity of novel proteins

The Cry1A(b) protein expressed in insect-protected corn plants is identical to the protein contained in microbial formulations that have been used on crops for 30 years (Milner, 1991). The protein assessed in this application is a very safe form of insect control from the human or animal consumption view point, as the acidic pH in the

animal digestive system does not permit processing of the delta-endotoxins to an active form (Vandemark, 1999). This effectively provides a mechanism for a specificity of action against only Lepidopteran insects. Also, in its long history of use, there have been no reported allergenic responses for this protein.

Studies evaluated:

J. Astwood. 1995. *Bacillus thuringiensis* susp. kurstakis HD-1 insecticidal protein (*B.t.k.* HD-1 protein) shares no significant sequence similarity with proteins associated with allergy or Coeliac disease. Monsanto Company, USA 63198. MSL-14172

J.E. Ream and R.L. Fuchs. 1994. Assessment of the in vitro digestive fate of *Bacillus thuringiensis* susp. kurstakis HD-1 protein. Monsanto Company, USA 63198.

Although there are no simple predictive assays available to assess the allergic potential of proteins, a number of characteristics are common among many of the allergens that have been characterised. For instance, amino acid sequence similarity with known allergens may be a useful gauge of allergenic potential. A string of 8-12 consecutive amino acid residues in common with known allergens could be an indicator for allergenicity given that many T-cell epitopes of allergenic proteins are that length (Taylor and Lehrer, 1996). In terms of the chemical and physical nature of proteins, known allergens tend to be glycosylated proteins with a molecular weight of 10–70 KDa (Lehrer et al, 1996). Allergens also tend to be heat stable as well as resistant to peptic and tryptic digestion and the acidic conditions of the stomach. Consequently, many allergenic factors tend to be resistant to proteolytic digestion (Taylor and Lehrer, 1996). The Cry1A(b) protein is evaluated for potential allergenicity against these criteria: size, digestive degradation and sequence similarity to known allergens.

The Cry1A(b) core protein has a molecular weight of 63 kDa, which is in the size range of known allergens.

The amino acid sequence of the Cry1A(b) protein was compared to the amino acid sequences of 219 known allergens present in public domain databases (eg GenBank, EMBL, Swissprot, PIR). No biologically significant homology was found with any of these known allergens.

The digestibility of Cry1A(b) *B.t.k* HD-1 protein was determined experimentally using *in vitro* mammalian digestion models. Purified Cry1A(b) trypsin-resistant core protein (63 kDa) was added to simulated gastric and intestinal fluids and incubated at 37ºC. The protein used was from the same batch that had been produced in *E. coli* for acute toxicity testing in mice. The degradation of the protein in the digestion fluid was assessed over time by Western blot analysis. An insect bioassay was used as an additional means of monitoring *B.t.k* HD-1 degradation in the digestion fluids. The simulated digestion fluids were prepared according to procedures outlined in the United States Pharmacopeia (1990).

The 63 kDa *B.t.k* HD-1 core protein was shown to be rapidly degraded in the simulated gastric system. It was 90% degraded after 2 minutes incubation in the simulated gastric fluid as determined by Western blot analysis. Bioactivity of the

B.t.k HD-1 protein also dissipated readily with up to 90% dissipated after 2 minutes incubation in the simulated gastric fluid.

In the simulated intestinal fluid, the *B.t.k* HD-1 protein did not degrade substantially after approximately 19.5 hours incubations as assessed by both Western blot analysis and insect bioassay. The stability of the trypsin-resistant core protein in the intestinal system is expected as other *Bacillus* insecticidal proteins have been shown to be resistant to digestion by serine proteases (Hofte and Whitely 1989), like trypsin which is the predominant proteolytic component in the intestinal fluid.

The *B.t.k* HD-1 Cry1A(b) protein does not possess the characteristics typical of many known allergens nor does it show significant homology to known allergens. Furthermore, the Cry1A(b) protein is rapidly digested in conditions that mimic human digestion.

Conclusions

The evidence does not indicate that there is any potential for the Cry1A(b) protein to be either toxic or allergenic to humans. The source of the cry1A(b) gene has a long history of use on food crops as a biopesticide and no evidence of adverse effects. The Cry1A(b) protein has no amino acid similarity to known allergens or toxins. Additionally, the protein is expressed at a relatively low level in the corn and is rapidly digested in model digestive systems.

NUTRITIONAL ISSUES

A range of analyses were performed on the insect protected corn line MON 810. The proximate analysis, amino acid composition and fatty acid profiles of the genetically modified and control corn tissue and kernels were analysed under GLP at Corning Hazelton Inc (Madison, Wisconsin) using recognised published methods in accordance with the Association of Official Analytical Chemists (AOAC, 1990).

Studies evaluated:

P.R. Sanders. 1995. Compositional analyses of insect-protected corn line MON 810 from the 1994 field trials. Monsanto Company, USA 63198. MSL-14384

P.R. Sanders, E.N. Elswick, M.E. Groth and B.E. Ledesma. 1995. Evaluation of insect-protected corn line MON 810 in 1994 US field trials. Monsanto Company, USA 63198.

P.R. Sanders, D.M. Henning and M.E. Groth. 1996. Compositional analyses of insect-protected and insect-protected Roundup Ready corn lines from the 1994 US Field Trials. Monsanto Company, USA 63198.

Sanders and Patzer, 1995. Compositional analyses of MON 801 grain and silage from the 1993 and 1994 US field test locations. Study no. 94-01-39-08, an unpublished study conducted by Monsanto Company.

Nutrient analysis

Compositional analyses were done on the insect-protected corn line and comparisons were made to the control line (818 which is derived from the Hi-II) and lines of a

similar genetic background (MON 800/801) that have been previously reported by the applicant and are listed in the reports above (Sanders and Patzer, 1995). Line MON 810 was grown in six field locations in 1994 according to quality assurance guidelines. Seed grown from each of the six sites was analysed and the data subject to statistical analyses. The corn kernels were analysed for compositional quality characteristics according to GLP using standardised analytical methods.

Proximate analysis for major constituents

Proximate analyses were done on corn kernels. Components measured were protein, fat, moisture, calories, carbohydrates and ash and these values are found in Table 3.

As a percentage of dry weight, the component analyses for line MON 810, are approximately: protein 13.1%; fat 3.0%; moisture 12.4%; calories 408 Kcal/100g; ash 1.6%; and carbohydrate 82.4%. In all of the component analyses of line MON 810, there were no significant differences between the insect-protected corn and the control line.

Table 3: Mean values and ranges of Proximate Analyses for corn trials

	Control[2]		**MON 810[2]**	
	mean	**range**	**mean**	**range**
Protein[1]	12.8	11.7-13.6	13.1	12.7-13.6
Fat[1]	2.9	2.6-3.2	3.0	2.6-3.3
Ash[1]	1.5	1.5-1.6	1.6	1.5-1.7
Carbohydrate[1]	82.7	81.7-83.8	82.4	81.8-82.9
Calories Kcal/100g[1]	409	406-410	408	407-410
Moisture	12.0	10.6-14.2	12.4	11.0-14.4

[1]Data as a percentage of dry weight.
[2]Value is the mean of six samples (n=6), one from each of six sites.

Amino acid analysis

Amino acid analyses were done on insect-protected corn kernels. Of the 18 amino acids analysed, the values were comparable for the insect-protected corn and control line, with few exceptions (Table 4).

In line MON 810, the mean values for eight amino acids were significantly different from the values for the control line ($p>0.05$) but were within the values reported in the literature (Watson, 1982; Watson, 1987) or for a corn line with a similar genetic background (Table 4) and thus were not considered to represent a meaningful difference.

Table 4: Profile of the amino acid levels that were significantly different to control

Amino Acid	**MON 810[1]**	**Control (818)**	**Literature Range[2]**	**Lines 800/801**
cysteine	2.0	1.9	1.2-1.6	1.9-2.3
tryptophan	0.6	0.6	0.5-1.2	0.5-0.6
histidine	3.1	2.9	2.0-2.8	2.8-3.3
phenylalanine	5.6	5.4	2.9-5.7	5.2-5.6

alanine	8.2	7.8	6.4-9.9	7.8-8.2
proline	9.9	9.6	6.6-10.3	9.0-9.4
serine	5.5	5.2	4.2-5.5	5.5-6.1
tyrosine	4.4	4.0	2.9-4.7	3.8-4.3

[1]All values shown are percentage of total protein present
[2]Watson, 1982; Watson 1987.
[3]Lines of a similar genetic background evaluated by the applicant (Sanders and Patzer, 1995)

Fatty acid analysis

Corn oil is an excellent source of polyunsaturated fatty acids, with a high level of the essential fatty acid linoleic acid (18:2). In addition, it has naturally low levels of the saturated fatty acids, palmitic acid (16:0, 11%) and stearic acid (18:0, 2%). Corn kernels from insect protected and control corn lines were subject to analysis to determine the fatty acid profile. The components measured that were within the detectable limits of the assay were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 cis), linoleic (C18:2), and linolenic (C18:3). The fatty acids which were not detectable in the assay were: caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, heptadecanoic, eicosadienoic, eicosatrienoic and arachidonic. There were no statistically significant differences between line MON 810 and the control line (Table 5).

Table 5: Fatty acid composition of corn kernels[1]

Fatty Acid	**Control**		**MON 810**		**Literature Range[2]**
	Mean	Range	Mean	Range	
Linoleic (18:2)	63.0	61.8-64.6	62.6	59.5-64.7	35-70
Oleic (18:1)	22.8	21.6-23.9	23.2	21.5-25.4	20-46
Palmitic (16:0)	10.5	10.2-10.7	10.5	10.2-11.1	7-19
Stearic (18:0)	1.8	1.8-1.9	1.9	1.7-2.1	1-3
Linolenic (18:3)	0.9	0.8-0.9	0.8	0.7-0.9	0.8-2

[1]Value of fatty acid is % of total lipid. n=6
[2]Watson, 1982; Watson 1987.

Inorganic components analysis

Inorganic component analysis was done on corn kernels. Like other cereal grains, corn is very low in calcium, and low in other minerals including phosphorus, potassium and magnesium. The components measured were percentage calcium and phosphorus. The value for phosphorus for the insect-protected corn line was not significantly different to the control line (Table 6). The value for calcium in insect-protected corn line MON 810 (0.0036%) was significantly different to the control corn line (0.0033%) but was not considered to represent a biologically meaningful difference as the value was within the range reported by the applicant for control corn lines with a similar genetic background (0.0030 – 0.0040%) (Sanders and Patzer, 1995).

Table 6: Analysis of Carbohydrates, Tocopherols and Inorganic components of corn kernels[1]

Component	Control		MON 810		Literature Range[2]
Inorganic	**Mean**	**Range**	**Mean**	**Range**	
% Phosphorus	0.348	0.327-0.363	0.358	0.334-0.377	0.26-0.75
% Calcium	0.0033	0.0029-0.0037	0.0036	0.0033-0.0039	0.01-0.1
Carbohydrates					
% Starch	66.9	64.6-69.0	67.6	65.3-69.7	64-78.0
% Crude Fibre	2.4	2.3-2.5	2.6	2.5-2.8	2.0-5.5
Sugars g/100g					
Fructose	0.27	0.22-0.40	0.32	0.23-0.35	
Glucose	0.41	0.34-0.46	0.44	0.34-0.47	
Sucrose	0.93	0.68-1.11	0.93	0.79-1.12	
Phytic Acid	0.84	0.79-0.91	0.86	0.81-0.91	0.7-1.0
Tocopherols					
alpha	10.9	9.9-12.1	10.4	9.7-11.3	3.0-12.1
beta	7.5	7.0-7.9	8.5	8.1-9.2	
gamma	21.6	18.8-27.8	20.2	15.3-24.8	

[1]Values on a dry weight basis. n=6, one sample from each field site.
[2]Watson, 1982; Watson 1987. Literature ranges provided if available.

Carbohydrate analysis

The carbohydrate components starch, crude fibre, sugars and phytic acid were evaluated in corn kernels (Table 6). The values for all components in the insect-protected corn line except crude fibre value were not significantly different to the control line. The value for crude fibre in line MON 810 (2.6%) was significantly different to the control line (2.4%) but is not considered to represent a biologically meaningful difference as it is within the range reported in the literature (2.0-5.5%) (Watson, 1982).

Tocopherol analysis

Tocopherols are naturally present in corn oil and have vitamin E potency (Watson, 1987). The values for alpha and gamma tocopherol levels in line MON 810 were not significantly different to the control line. The value for beta tocopherols in line MON 810 (8.5%) was significantly different to the control line (7.5%) but was within the range reported for corn lines with a similar genetic background (beta: 7.9-10.7%). There is no published literature for beta tocopherol levels in corn.

Levels of anti-nutrients

Corn contains few natural toxins or anti-nutrients. The anti-nutrients trypsin and chymotrypsin inhibitors are present in corn at very low levels that are not considered nutritionally significant (Wright 1987). As there are no routine analytical methods for the assessment of trypsin inhibitor activity in corn, the method developed for their study in soybeans was used (AOCS method Ba 12-75, 1997 modified).

The data for trypsin inhibitors was generated from a different set of field trials.

Kernel samples were collected from seven hybrid MON 810 corn corn lines from seven field trials in the USA and untransformed control corn samples taken from the seven USA field trials and thirteen commercial hybrid corn plants in Italy and France. The analyses were independently conducted at the Covance Laboratories, Inc., Madison, Wisconsin. The range of values measured for corn from line MON 810 fell within the ranges reported for the control line.

Table 7: Analysis of trypsin inhibitor levels[1]

	Control[2]	**MON 810[2]**
Trypsin Inhibitor	1.63-5.28	2.35-5.54

[1]Values are Trypsin Inhibitor Units/ milligram dry weight basis.
[2]Control n=20; MON 810 n=7, one sample from each of 7 USA field sites.

Analytical methods for analysis of chymotrypsin inhibitors in corn are not known. There was no evidence in the literature searches conducted to indicate that chymotrypsin inhibitors could be a significant anti-nutrient component of the corn grain.

Ability to support typical growth and well-being

Study submitted:
Draft version of Comparison of Broiler Performance when fed diets containing Yieldgard® Corn, Yieldgard® and Roundup Ready®, parental line or commercial corn. 2001. Colorado Quality Research Inc., Wellington, Colorado. Sponsored by Monsanto Co. Monsanto Study No. 00-01-39-01.

In assessing the safety of a genetically modified food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

In the case of insect-protected corn line MON 810, animal feeding studies were not considered necessary given the comprehensive analysis on the composition of the kernel. The nutritional profile of kernels from line MON 810 indicates that the parameters were essentially comparable to the parental line, as well as to published ranges for other commercial corn varieties. Additionally, studies on the specific novel protein sequence found that no significant similarities to known allergens or toxins exist for the CRY1Ab protein. An acute oral toxicity study in mice using variable amounts of the novel protein, found no evidence to indicate that the protein produces toxic effects in animals. Furthermore, the novel protein has been shown to be rapidly degraded in model digestive systems.

However, the applicant recently provided, an evaluation of corn kernels derived from insect-protected corn line MON 810 as a feed ingredient for commercial broiler chicks (*Gallus domesticus*). The general performance, carcass yield and meat

composition of chicks fed diets containing corn from line MON 810 was assessed and compared to chicks fed non-genetically modified corn feed.

During this study, day-old chicks were fed formulated treatment diets containing between 54-66% corn as the primary source of protein. All diets conformed with the industry standards and met and/or exceeded the nutritional recommendations set forth in the "Nutritional Requirements of Poultry, 9th Revision" by the National Research Council, 1994. Feed was prepared using eight corn varieties: Yieldgard corn, Yieldgard x Roundup Ready Corn, the parental control lines for the two transformed lines and four non-genetically modified commercial corn varieties.

The formulated diets were pelleted and the feed provided *ad libitum* to a total of 800 chicks with 100 chicks (50 females and 50 males) per treatment group (i.e. 10 replicates with 10 chicks each – 5 male and 5 female) for 42 days. The birds and pens were observed twice daily for general flock condition, as well as to check physical environment (lighting, water, feed, ventilation and unanticipated events). Birds were weighed at the start (day 1) and study end (day 42). Any mortalities were recorded and necropsied to determine probable cause of death.

After the final weights were obtained and after an approximate 12-hour feed withdrawal period, several measurements were taken on all of the birds from each pen. The following data was collected: live weight, fat pad weight, chill weight, breast meat weight –skinless and boneless, wings, thighs and drums. Additionally meat samples from two birds per pen were analysed and tissue retained. Meat from the breast and both thighs were collected for protein, fat and moisture analysis. All feed added and removed from pens was weighed and recorded and used to determine total feed consumption and feed conversion efficiency. The data were statistically analysed to determine if significant differences were noted between birds fed the test and the parental or commercial corn varieties.

Starting on day 1, any bird that was removed, found dead or was sacrificed was weighted and recorded. Expected chick mortality related to starve-outs or dehydration was observed during the first 7 days of the study. This mortality was randomly distributed among all groups without any relationship to treatment and occurs commonly in chicks in commercial feeding trials. During the remainder of the study, pen sizes were normalised to a maximum of 10 birds/pen (i.e. starting number was 12 chicks/pen). The birds that died from day 7 to study termination were randomly distributed among different groups without any specific relationship to treatment (deaths per treatment group averaged 6% and ranged from 2-11% across all treatment groups). The greatest number of mortalities occurred with the males (36 males versus 12 females); this is expected since the males grow faster and are heavier. Most of the apparent causes of death were identified at necropsy and occur commonly in chickens (sudden death syndrome and ascites). The birds in all groups were in good health based on twice daily pen observations. The starting and final body weights of the chicks were normal and the average pen body weights were comparable between treatments.

There were no abnormal conditions or abnormal behaviour observed throughout the study period. All performance parameters measured were similar ($p>0.05$) across the

broilers fed diets of MON 810 corn and the MON 810 x Roundup Ready corn, the parental corn lines and reference corn lines.

These data indicate that corn from line MON 810 can be used as a feed ingredient in broiler chicken diets without adverse effects on growth, feed conversion efficiency, survival, behaviour or body composition.

These results are consistent with results from other studies, that found feed containing corn from line MON 810 to be as nutritious and wholesome as other commercially available corn lines (Mireles, et al 2000; Weber et al, 2000; Hendrix et al, 2000; Russell et al, 2000a&b; Faust & Spangler, 2000). In these studies, the test animals included chickens, pigs and gestating and grazing beef cows.

Conclusion

Analysis of the compositional data of the kernel indicates that there were few significant differences in the levels of major constituents, nutrients, anti–nutritional factors or natural toxicants between insect-protected corn line MON 810 and the control corn line. The differences that were noted were not considered to represent a meaningful difference because the values were consistent with the values reported in the literature or for a control corn line with a similar genetic background and thus were considered to represent the natural variability that exists within corn.

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REFERENCES

AOCS Method Ba 12-75 1997. In Official methods and recommended practices of the American Oil Chemists Society. American oil Chemists Society, Champaign, Illinois.

Australia New Zealand Food Authority (ANZFA). 1999. Guidelines for the safety assessment of foods to be included in Standard A18 – Food Produced Using Gene Technology.

Barry G., *et al* . 1992. Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. In *Biosynthesis and Molecular Regulation of Amino Acids in Plants.* Singh (Ed). American Society of Plant Physiologists.

Bevan M.W., R.B. Flavell and M.-D. Chilton. 1983. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184-187.

Cooper, D.. 1991. *Bacillus thuringiensis* toxins and mode of action. In *The Proceedings from the Workshop on* Bacillus thuringiensis*.* Editors R. Milner and C. Chandler. CSIRO, Canberra.

Drummond, J. and D. Pinnock. 1991. Host spectrum of *Bacillus thuringiensis.* In *The Proceedings from the Workshop on* Bacillus thuringiensis. Editors R. Milner and C. Chandler. CSIRO, Canberra.

Faust MA and SM Spangler. 2000. Nutritive value of silage from MON810 Bt and non-Bt near isogenic corn hybrids. Abstract 301 presented at the Midwestern Section ASAS and Midwest Branch ADSA 2000 Meeting, Des Moines, IA.

Fischoff, D.A., K.S. Bowdish, F.J. Perlak, P.G. Marrone, S.M. McCormick, J.G. Niedermeyer, D.A. Dean, K. Kusano-Kretzmer, E.J. Meyer, D.E. Rochester, S.G. Rodgers and R.T. Fraley. 1987. Insect tolerant transgenic tomato plants. Biotechnology 5:807-813.

Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, N.L. Hoffman and S.C. Woo. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80:4803-4807.

Frick, O.L. 1995. The potential for allergenicity in transgenic foods. In *Genetically Modified Foods: Safety Aspects*. Eds. K.-H. Engel, G.R. Takeoka and R. Teranishi. American Chemical Society, Washington DC.

Hendrix, KS, AT Petty and DL Lofgren. 2000. Feeding value of whole plant silage and crop residues from Bt or normal corns. J. Anim. Sci. 78(Suppl. 1):273 Abstract 1146.

Hofte, H. and Whitely, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiol. Rev.* **53**: 242-255.

Lehrer, S.B., W.E. Horner and G. Reese. 1996. Why are some proteins allergenic? Implications for biotechnology. Critical Reviews in Food Science and Nutrition 36(6):553-564.

Lewin, B. 1997. *Genes VI*. Oxford University Press. Oxford.

Milner, R.. 1991. History of *Bacillus thuringiensis.* . In *The Proceedings from the Workshop on* Bacillus thuringiensis. Editors R. Milner and C. Chandler. CSIRO, Canberra.

Mireles Jr, A, S Kim, R Thompson and B Amundsen. 2000. GMO (Bt) corn is similar in composition and nutrient availability to broilers as non-genetically modified corn. Poultry Sci. 79(Suppl. 1):65-66. Abstract 285.

Noteborn, H.P.J.M., M.E. Bienenmann-Ploum, J.H.J. van den Berg, G.M. Alink, L. Zolla, A.Raynaerts, M. Pensa and H.A. Kuiper. 1995. Safety assessment of the *Bacillus thuringiensis* insecticidal crystal protein CRY1A(b) expressed in transgenic tomatoes. In *Genetically Modified Foods: Safety Aspects*. Editors K.-H. Engel, G.R. Takeoka and R. Teranishi. American Chemical Society, Washington DC.

Odell, J.T., Nagy, F., and Chua, N-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**: 810-812.

Perlak, F.J. *et al* . 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA 88:3324-3328

Rochester D.E., J.A. Winer and D.M. Shah. 1986. The structure and expression of maize genes encoding the major heat shock protein hsp70. EMBO J. 5:451-458.

Russell, JR, MJ Hersom, A Pugh, K Barrett and D Farnham. 2000a. Effects of grazing crop residues from bt-corn hybrids on the performance of gestating beef cows. Abstract 244 presented at the Midwestern Section ASAS and Midwest Branch ASAS and Midwest Branch ASDA 2000 Meeting, Des Moines, IA.

Russell, JR, D Farnham, RK Berryman, MJ Hersom, A Pugh, and K Barrett. 2000b. Nutritive value of the crop residues from bt-corn hybrids and their effects on performance of grazing beef cows. 2000 Beef Research Report-Iowa State University. P 56-61.

Taylor S.L. and S.B. Lehrer. 1996. Principles and characteristics of food allergens. Critical Reviews in Food Science and Nutrition 36(S):S91-S118.

The United States Pharmacopeia, Vol. XXII. (1990). United States Pharmacopeial Convention, Inc., Rockville, MD, pp 1788-1789.

Vandemark G. Transgenic Plants for the improvement of field characteristics limiting crop production. In *Molecular Biotechnology for Plant Food Production*. Paredes-Lopez (Ed) Technomic Publishing Co. Lancaster, Penn.

Watson S.A. 1982. Corn: Amazing Maize. General Properties. In *CRC Handbook of Processing and Utilisation in Agriculture, Volume II: Part 1 Plant Products*. I.A. Wolffe (Ed). CRC Press, Inc. Florida.

Watson S.A. 1987. Structure and composition. In: *Corn: Chemistry and Technology*. S.A.Watson and P.E. Ramsted (Eds.), American Association of Cereal Chemists, Inc. St. Paul, MN., USA.

Weber, TE, BT Richert, DC Kendall, KA Bowers and CT Herr. 2000. Grower-finisher performance and carcass characteristics of pigs fed genetically modified "Bt" corn. Purdue University 2000 Swine Day Report. http://www.ansc.purdue.edu/swine/swineday/sda00/psd07-2000.html

Wright, K.N. 1987. Nutritional properties and feeding value of corn and its by-products. In: *Corn: Chemistry and Technology*. S.A.Watson and P.E. Ramsted (Eds.), American Association of Cereal Chemists, Inc. St. Paul, MN., USA.

WHO. 1991. Strategies for assessing the safety of foods produced by biotechnology. Report of a joint FAO/WHO Consultation. World Health Organization, Geneva, 59 pp.

WHO. 1993.Health aspects of marker genes in genetically modified plants. Report of a WHO workshop. World Health Organization, Geneva.