SUMMARY

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STUDY TITLE

Human and Livestock Exposure Assessment for AAD-1 Protein in DAS-40278-9 Maize

DATA REQUIREMENTS

21 CFR 192.25

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STUDY COMPLETED ON

15 JUL 2009

PERFORMING LABORATORY

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LABORATORY STUDY ID

091115

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This report presents a summary of the assessment for toxic or allergenic potential to the AAD-1 protein as well as a dietary exposure assessment for humans and livestock. Low level expression of the AAD-1 protein in grain and forage of DAS-40278-9 corn plants across environments indicates a low exposure risk to humans and animals. Results of the overall safety assessment of the AAD-1 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-1 protein

Title: Human and Livestock Exposure Assessment for AAD-1 Protein in DAS-40278-9 Maize

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Company: Dow AgroSciences LLC

Company Agent: L. A. Tagliani

Title: Regulatory Manager

Signature: L A. Tagliani

Date: 15-July-2009

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

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This study does not meet requirements of 40 CFR part 160.

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Human and Livestock Exposure Assessment for AAD-1 Protein in DAS-40278-9 Maize

ABSTRACT

This report presents a summary of the assessment for toxic or allergenic potential to the AAD-1 protein as well as a dietary exposure assessment for humans and livestock. Low level expression of the AAD-1 protein in grain and forage of event DAS-40278-9 corn plants across environments indicates a low exposure risk to humans and animals. There was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-1 protein. A dietary exposure assessment for both humans and livestock reveals large margins of exposure (MOE) values for AAD-1 protein in DAS-40278-9 maize, indicating no concern for adverse effects from acute dietary exposure through corn.

A weight-of-evidence approach was used to assess the potential for allergenic effects from the AAD-1 protein. The AAD-1 protein is present at very low levels in DAS-40278-9 plants. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-1 amino acid sequence. The AAD-1 protein hydrolyzes rapidly in simulated gastric fluid. Glycosylation analysis of the plant-derived AAD-1 protein revealed no detectable covalently linked carbohydrates. Results indicate the AAD-1 protein is unlikely to cause allergy.

BACKGROUND

Dow AgroSciences has produced a novel transgenic corn product that provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) acetyl coenzyme A carboxylase (ACCase) inhibitors ("fop" herbicides). This herbicide-tolerance trait is commonly known as DHT-1 and is accomplished via corn plants which have been genetically modified to express the aryloxyalkanoate dioxygenase (AAD-1) protein. The *aad-1* gene, which expresses the AAD-1 protein, was derived from *Sphingobium herbicidovorans,* a gram-negative soil bacterium. *Sphingobium* spp. are widespread in the environment, therefore, animals and humans are regularly exposed without adverse consequences to the organism and its components. *Sphingobium* spp. degrade a number of chemicals in the environment which include aromatic and chloroaromatic compounds, phenols, herbicides and polycyclic hydrocarbons.

This report presents a summary of the assessment for toxic or allergenic potential to the AAD-1 protein as well as a dietary exposure assessment of DAS-40278-9 corn for humans and livestock. Results of the overall safety assessment of the AAD-1 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

MAMMALIAN TOXICITY ASSESSMENT

Mammalian Acute Toxicity

An acute oral toxicity study with AAD-1 protein was conducted in mice at a level of 2000 mg AAD-1/kg after adjustment for purity (1). All animals survived and no clinical signs were observed during the study. All animals gained weight by study termination on day 15. There were no treatment-related gross pathological observations. The report concludes that under the conditions of this study, the acute oral LD_{50} of AAD-1 in male and female mice was greater than

2000 mg/kg. In the US, based on this LD_{50} value, EPA would classify this substance as a category III for acute oral toxicity, indicating very low toxicity has been observed. Upon review of the report, it is assumed that the NOEL is also >2000 mg/kg based on fact that no mortality was observed and there were no observeable effects (adverse or non-adverse effects) with the AAD-1 treated animals. **AAD-1 protein has been shown to display very low acute toxicity potential.**

Lack of Homology to Known Toxins

The AAD-1 protein does not share meaningful amino acid sequence similarities with known toxins (2). Amino acid homologies were evaluated using a global sequence similarity search against the GenBank non-redundant protein dataset (posted on February 10, 2007 containing 4,554,902 sequences with 1,568,234,006 amino acids). The only significant homologies identified were with other alpha-ketoglutarate-dependent dioxygenases, the same class of enzymes as AAD-1. None of the similar proteins returned by the search identified any safety concerns that might arise from the expression of AAD-1 protein in plants.

ALLERGENIC POTENTIAL ASSESSMENT

History of Safe Use

The donor organism, *Sphingobium herbicidovorans* (formerly designated *Sphingomonas herbicidovorans*) is a soil dwelling bacterium carrying genes which encode enzymes that facilitate the breakdown of phenoxy auxin and AOPP herbicides to compounds that can be used as carbon sources for the bacterium (3). *Sphingomonas herbicidovorans* is a member of the sphingomonads, a widely distributed bacterial group in nature which has been isolated from land and water habitats, as well as from plant root systems. Due to their biodegradative and biosynthetic capabilities, the sphingomonads have been used for a wide range of

biotechnological applications such as bioremediation of environmental contaminants and production of extracellular polymers such as sphingans which are used extensively in the food industry (4,5,6,7).

Lack of Allergenic Potential

The step-wise, weight-of-evidence approach (8) was used to assess the allergenic potential of the AAD-1 protein. The AAD-1 protein is present in plants at very low concentrations in DAS-40278-9 corn plants. The AAD-1 protein does not share meaningful amino acid sequence similarities with known allergens. No significant homology was identified when the AAD-1 protein sequence was compared to known allergens in the FARRP (Food Allergy Research and Resource Program) version 7.00 allergen database, using the search criteria of either a match of eight or more contiguous identical amino acids, or 35% identity over 80 amino acid residues (9).

The AAD-1 protein is rapidly degraded below the level of detection in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestions. The AAD-1 protein was readily digested, i.e., not detectable after 30 seconds, under *in vitro* SGF conditions (0.32% pepsin, pH 1.2; 37 ^{o}C) as demonstrated by both SDS-PAGE and western blot analyses (10). When tested under *in vitro* SIF conditions (1% pancreatin, pH 7.5, 37 ^{o}C), after 1 minute, the AAD-1 protein was digested and no longer detectable by SDS-PAGE or western blot analyses (11).

The AAD-1 protein is not present in a glycosylated state. No glycosylation of the AAD-1 protein was detected using SDS-PAGE and a glycosylation detection system (12).

Together this information indicates the AAD-1 protein is unlikely to cause allergenic effects in humans or animals.

HUMAN DIETARY EXPOSURE ASSESSMENT

Per the FDA Proposed Rules for 21 CFR 192.25 (13), a notifier must provide a dietary exposure estimate for the substance (or a justification for why it is unnecessary). This requirement is fulfilled here by coupling field expression information for AAD-1 protein with conservative (i.e. protective) human dietary consumption data for corn. In addition, the relevance of the exposure estimate is placed into context, based on a the known mammalian toxicity information.

Potential Human Exposure to AAD-1 Protein via Corn

The field expression of AAD-1 protein in Event DAS-40278-9 corn has been measured using an AAD-1 specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of corn (14). Protein expression was analyzed in leaf, root, pollen, whole plant and grain tissues collected throughout the growing season from DAS 40278 corn plants treated with 2,4-D, quizalofop, both 2,4-D and quizalofop, or not treated with either herbicide. In general, the results showed low level expression of the AAD-1 protein with or without 2,4-D or quizalofop herbicide treatments and across environments, indicating a low exposure risk to humans and animals. In corn grain collected at growth stage R6 to maturity, the average value of AAD-1 protein (across treatments) was **4.81 ng/mg tissue on a dry weight basis**. The full range of values was 1.07 to 9.10 ng/mg tissue, but the use of an average expression value is most appropriate, because corn grain is a highly blended commodity (making consumption of single-servings of grain at the maximum expression-level highly unlikely). Use of this value is a conservative and protective estimate for exposure to the AAD-1 protein from corn; actual dietary exposure to the protein will be lower because: 1) there will be protein degradation during transport and storage, 2) grain containing AAD-1 will be mixed with non-AAD-1 grain, 3) for humans, consumption of corn products is often in foodforms which are cooked and heat is known to denature this protein (15) and 4) a portion of the consumer dietary exposure to corn is in forms where the protein concentrations will be reduced by processing, such as in corn syrup. It is also known that oils contain very little protein.

A conservative acute consumption (i.e. exposure) estimate is made based on global data published by the World Health Organization (WHO). WHO has established a maximum consumption of each food commodity for acute exposures for the entire world, based on maximum inputs from multiple countries (16). Table 1 includes 97.5th percentile values for all possible commodities associated with maize and corn. **For AAD-1 maize/corn, the appropriate maximum consumption value is associated with the "GC 645 Maize" group with an upper limit for maize reported by France**. Other information for sweet corn and popcorn are presented here for completeness as well, however there are no plans for introduction of the AAD-1 trait into these related commodities. Information for maize oil is presented here for completeness, but it is known that the oils and other highly refined fractions do not contain significant amounts of protein. Moreover, total acute consumption across all these entities cannot be calculated, because it is not appropriate to add 97.5th percentile values for individual commodities for survey results from different countries.

Table 1. Estimates of Acute Maize Consumption from the GEMS/Food Highest 97.5th Percentile "Eater-Only" Worldwide

		Consumption[a] (g/kg/day)	
Commodity	Country with Reported Maximum	General Population	Children ≤6 years
GC 645 maize	**France**	**4.06**	**6.17**
VO 447 sweet corn[b] (corn on the cob)	Thailand	7.16	11.52
GC 656 popcorn	Japan	3.33	3.33
OR 645 maize oil, refined	Netherlands	0.89	0.68

[a] Total acute consumption across these entities cannot be calculated because, it is not appropriate to add 97.5th percentile values for individual commodities survey results from different countries; REF (16).

When the WHO "GC 645 maize" acute consumption information is coupled to the AAD-1 **field expression level of 4.81 ng/mg tissue, an upper limit for acute exposure to AAD-1 protein** via corn is estimated as:

- **0.0195 mg protein/kg bw/day, for general population (i.e. adults)**
- **0.0297 mg protein/kg bw/day, for children of 6 years or younger**

Margin of Exposure Calculation

Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds which have no associated mortalities below 1000 mg/kg bw in single dose studies (17). Nonetheless, to place the AAD-1 protein exposure estimate in context, a comparison of the exposure information to the lower limit NOEL has been made to provide Margins of Exposure (MOE) in Table 2 for AAD-1 protein where:

$$MOE = \frac{NOEL}{Exposure}$$

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOEL threshold. The **calculated MOE values for AAD-1 protein in maize are extremely large, indicating no concern for adverse effects from acute dietary exposure through corn**.

Table 2. Margins of Exposure for AAD-1 Protein in Masize Based on WHO 97.5th Percentile Consumption

	Exposure[a] (mg AAD-1 /kg bw/day)	**NOEL (mg/kg bw)**	**MOE**
General Population	0.0195	>2000	102564
Children <6 year	0.0297	>2000	67340

[a] Based on WHO 97.5th percentile consumption of maize under commodity GC 645.

LIVESTOCK DIETARY ASSESSMENT

Some countries require a dietary exposure estimate for novel feed in livestock diets based on traditional use of the unmodified feeds. This requirement is fulfilled here by coupling field expression information for AAD-1 protein from DAS-40278-9 corn plants with livestock dietary consumption assumptions for corn and corn forage. In addition, the relevance of the exposure estimate is placed into context, based on the mammalian toxicity information.

Animal Feed Exposure

Corn grain and forage is used for animal feeds. An assessment for livestock exposure is presented here based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures of US EPA (18). The MRBD guidance has been used to construct a maximum corn grain contribution for swine, poultry and cattle based on the average value for AAD-1 of 4.81 ng/mg (or ppm) in DAS-40278-9 corn grain. For cattle, the field expression levels of AAD-1 in forage (collected at R4) are also applicable. The average value of AAD-1 protein in corn forage plants (across treatments) was 7.05 ng/mg tissue (dry weight basis) and the maximum value observed was 11.6 ng/mg tissue. The presence of AAD-1 protein in maize is not anticipated to have impact for feed ration formulation, because nutrient composition analyses have shown that DAS-40278-9 corn is substantially equivalent to conventional corn (14).

These livestock diets have been built based on the traditional use of the unmodified counterpart per US EPA procedures; and estimates of dietary exposure are conservative (and protective) in that they have assumed 100% replacement of the unmodified counterpart. US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (18):

Beef: Finishing or feedlot beef (body weight at slaughter, 1200 lb or **544 kg,** daily feed intake of 20 lb or **9 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% CC), forages (15% R), and protein sources (5% PC) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

Dairy: Mature lactating cow (body weight, 1350 lb or **612 kg**, daily feed intake of 53 lb or **24 kg dry matter feed,** and producing average of 90 lb of milk a day). Feed rations include forages (45% R), grain or grain supplements (45% CC), and protein source (10% PC). Dairy cows generally calve at **24 to 28 months of age**. The usual length of lactation is 250 to 450 days, with a 305 day lactation being the standard. Dairy cows are usually slaughtered after 2 or 3 calves. The average productive life span of the mature lactating dairy cow is 3 to 4 years.

Poultry: Chicken: Laying hen (body weight, 4.2 lb or **1.9 kg**, average daily intake of 52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% PC.

Swine: Finishing or Market hog (body weight, up to 250 lb or **113 kg**, average daily intake of 6.8 lb or **3.1 kg of feed**). Hogs are slaughtered in **5 to 8 months**. In general, daily ration consists of high grain or grain supplement (85% CC) and oilseed meal (15% PC).

The above assumptions apply for finishing animals in US feedlots. For cattle, a younger animal would receive a higher percentage of forage than grain, but analysis of younger animals would not result in substantially different overall conclusion given the low toxicity of the AAD-1 protein. In addition, the worse case value of 11.6 ppm for forage has been assumed here at 100% AAD-1 event DAS-40278-9 corn. In reality, exposure via forage will be lower, given the average of 7.05 ppm in forage and market adoption of DAS-40278-9 corn will not be 100 percent. The resulting intake dietary burden for animal feeds is totaled in Table 3:

Table 3. Intake Animal Dietary Burdens for Livestock

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	AAD-1 (ppm)	Beef	Dairy	Poultry	Pig
Corn, grain	CC	88	80	45	75	85	**4.81**	4.37	2.46	3.61	4.09
Corn, forage/silage	R	40	15	45	Nu	Nu	**11.6**	4.35	13.05		
							Total	**8.72**	**15.53**	**3.61**	**4.09**

Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal in Table 4:

Table 4. Livestock Daily Dose Estimates of AAD-1 Protein from Corn Feeds

	Chicken	Dairy	Beef	Pig
Body weight (kg)	1.9	612	544	113
Daily Maximum Feed (kg)	0.052	24	9	3.1
Maximum AAD-1 intake (mg/kg feed)	3.61	15.51	8.72	4.09
Maximum intake (mg/kg bw)	0.10	0.61	0.15	0.11

The highest exposed animal is the dairy cow with 0.61 mg AAD-1/kg bw. When this value is compared to the acute NOEL of >2000 mg/kg bw, there is an adequate margin of safety for livestock. Variations in livestock feed diets elsewhere in the world could results in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for AAD-1 protein in DAS-40278-9 maize.

CONCLUSION

Results of the overall safety assessment of the AAD-1 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

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