SUMMARY

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STUDY TITLE

Human and Livestock Exposure Assessment for AAD-12 Protein in DAS 68416-4 Soybeans

DATA REQUIREMENTS

21 CFR 192.25

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STUDY COMPLETED ON

16 OCT 2009

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LABORATORY STUDY ID

091141

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This report presents a summary of the assessment for toxic or allergenic potential to the AAD-12 protein as well as a dietary exposure assessment for humans and livestock. Expression levels of the AAD-12 protein in plant tissues of DAS-68416-4 soybean across environments demonstrate a low exposure risk to humans and animals. Results of the overall safety assessment of the AAD-12 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-12 protein

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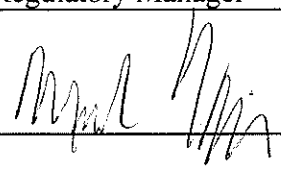
Signature: 

Date: 19 October 2009

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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Human and Livestock Exposure Assessment for AAD-12 Protein in DAS 68416-4 Soybeans

Study Initiation Date: 15 SEPT 2009
Experimental Start Date: NA Experiment Termination Date: NA

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

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

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Study Completion Date

QUALITY ASSURANCE STATEMENT

Compound: AAD-12 protein

Title: Human and Livestock Exposure Assessment for AAD-12 Protein in DAS 68416-4 Soybeans

Study Initiation Date: 15 SEPT 2009 Study Completion Date: 16 OCT 2009

NON-GLP STUDY

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TABLE OF CONTENTS

Human and Livestock Exposure Assessment for AAD-12 Protein in DAS 68416-4 Soybeans

ABSTRACT

This report presents a summary of the assessment for toxic or allergenic potential to the AAD-12 protein as well as a dietary exposure assessment for humans and livestock. Expression levels of the AAD-12 protein in plant tissues of DAS-68416-4 soybean across environments demonstrate 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-12 protein. A dietary exposure assessment for both humans and livestock reveals large margins of exposure (MOE) values for AAD-12 protein in DAS-68416-4 soybean, indicating no concern for adverse effects from acute dietary exposure through soybean.

A weight-of-evidence approach was used to assess the potential for allergenic effects from the AAD-12 protein. The AAD-12 protein is present at ng/mg levels in DAS-68416-4 plants. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 protein hydrolyzes rapidly in simulated gastric fluid. Glycosylation analysis indicated the plant-derived AAD-12 protein is not present in a glycosylated state. Results indicate the AAD-12 protein is unlikely to cause allergy.

BACKGROUND

Dow AgroSciences has produced a transgenic soybean that provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) as well as the herbicide glufosinate. Tolerance to 2,-4-D is obtained from expression of the aryloxyalkanoate dioxygenase-12 (AAD-12) protein The *aad-12* gene, which expresses the AAD-12 protein, was derived from *Delftia acidovorans,* a common soil bacterium. *D. acidovorans* is found in several environmental matrices and also has a history of safe use in the food processing industry. Therefore, animals and humans are regularly exposed without adverse consequences to the *D. acidovorans* organism and its components.

DAS-68416-4 soybean also contains the *pat* gene from *Streptomyces viridochromogenes* which encodes a phosphinothriein acetyltransferase (PAT) protein which detoxifies L-phosphonitricin, the active ingredient in the herbicide glufosinate ammonium. This report does not focus further on the PAT protein, because the PAT protein is a well-undersood protein and has a history of regulatory approvals and use in transgenic crops.

This report presents a summary of the assessment for toxic or allergenic potential to the AAD-12 protein as well as a dietary exposure assessment of transformed soybean DAS-68416-4 for humans and livestock. Results of the overall safety assessment of the AAD-12 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-12 protein was conducted in mice at a level of 2000 mg AAD-12 protien/kg (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-12 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-12 treated animals. **AAD-12 protein has been shown to display very low acute toxicity potential.**

Lack of Homology to Known Toxins

The AAD-12 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 17, 2007 containing 4,626,804 sequences with 1,596,079,149 amino acids). The AAD-12 similarity search identified 618 proteins. These proteins were annotated with an enzyme function, because the GenPept page for this protein contained a well characterized enzyme activity in its Features annotation under product or region_name. These proteins can be broken down into a few major subclasses. The largest subclass, containing 474 proteins, was identified as tauD or taurine dioxygenases. These are proteins involved in the degradation of taurine. (3). The next largest class, with 138 members, was clavaminic acid synthetases or "CAS-like"(4). There were 2 tolC proteins which are known efflux pumps (5). The last four proteins were: 1) a (S)-2-(2,4-dichlorophenoxy)propionate, 2-oxoglutarate dioxygenase (6); 2) a pvcB protein which is a known CAS like protein (see accession page of NP_968348); 3) an inosine-uridine preferring nucleoside hydrolase (7); and 4) a hypothetical protein with no functional annotation. Inspection of the BLink results accessible from the GenPept page of this protein reveals significant homology (9e-150) to both CAS and tauD. None of the similar proteins returned by the search identified any safety concerns that might arise from the expression of AAD-12 protein in plants.

ALLERGENIC POTENTIAL ASSESSMENT

History of Safe Use

The source organism for the *aad-12* gene, *Delftia acidovorans* (previously identified as *Pseudomonas acidovorans* and *Comamonas acidovorans*) is a strictly aerobic, gram-negative, bacteria that is present in soil, fresh water, activated sludge, and clinical specimens (8, 9, 10). *Delftia acidovorans*, like many other soil dwelling bacteria, has evolved the ability to use herbicides as one of many carbon sources for growth, affording the bacteria a competitive advantage in soil.

D. acidovorans can be used to transform ferulic acid into vanillin and related flavor metabolites (11,12). This utility has led to a history of safe use for *D. acidovorans* in the food processing industry. For example, US Patent 5,128,253 "Bioconversion process for the production of vanillin" was issued on July 7, 1992 to Kraft General Foods. This patent demonstrates the use of a variety of microorganisms, including *Pseudomonas acidovorans*, for the transformation of ferulic acid to vanillin.

Lack of Allergenic Potential

The step-wise, weight-of-evidence approach (13) was used to assess the allergenic potential of the AAD-12 protein. The AAD-12 protein is present in plants at low concentrations in DAS-68416-4 soybean plants. The AAD-12 protein does not share meaningful amino acid sequence similarities with known allergens. No significant homology was identified when the AAD-12 protein sequence was compared with known allergens in the FARRP (Food Allergy Research and Resource Program) version 9.00 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.

The AAD-12 protein is rapidly degraded below the level of detection in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestions. The AAD-12 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 (14).

The AAD-12 protein is not present in a glycosylated state. No glycosylation of the AAD-12 protein was detected using SDS-PAGE and a glycosylation detection system (15).

Together this information indicates the AAD-12 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 (16), 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-12 protein with conservative (i.e. protective) human dietary consumption data for soybean. In addition, the relevance of the exposure estimate is placed into context, based on the known mammalian toxicity information.

Potential Human Exposure to AAD-12 Protein via Soybean

The field expression of AAD-12 protein in DAS-68416-4 soybean has been measured using a specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (17). Protein expression was analyzed in leaf, root, forage and grain tissues collected throughout the growing season (V5 to R8 growth stages) from DAS-68416-4 soybean which was a transformation of a “Maverick” soybeen with plasmid pDAB4468. Within the six North American field trials, transformed soybean plants were treated either with 2,4-D, glufosinate, both 2,4-D and glufosinate, or not treated with either herbicide; in addition an untreated group of non-transformed Maverick variety served as a control. In general, the results

showed low level expression of the AAD-12 protein with or without 2,4-D or glufosinate herbicide treatments and across environments, indicating a low exposure risk to humans and animals. Only the protein expression in the soybean grain is applicable for human dietary consideration.

In soybean grain collected at growth stage R8, the average value of AAD-12 protein (across treatments) was **16.52 ng/mg tissue on a dry weight basis**. The full range of values was narrow with observations from16.21 to 16.94 ng/mg tissue. Use of the average expression values will be used in the following human dietary assessment, because grains are a blended commodity, making consumption of single-servings of soybean at the maximum expression-level highly unlikely. Use of these values are conservative and protective estimates for exposure to the AAD-12 protein from soybean; actual dietary exposure to the proteins will be lower because: 1) there will be protein degradation during transport and storage, 2) soybean containing AAD-12 will be mixed with non-transformed soybean, 3) for humans, consumption of soybean products is often in foodforms which are cooked and heat is known to denature this protein (18) and 4) a portion of the consumer dietary exposure to soybeans is in forms where the protein concentrations will be reduced by processing, such as in soybean oil which 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 (19). Table 1 includes 97.5^{th} percentile values for all possible commodities associated with soybean. **For DAS-68416-4 soybean, the appropriate maximum consumption value is associated with the "VD541" group with an upper limit for dry soybean reported by Japan**. Consumption information for immature seeds is presented here for completeness, but the immature consumption value is lower than for mature seeds and more importantly the seeds cannot be consumed more than once, so the consumption of the mature seeds alone represents a conservative estimate of exposure. Information for soybean oil is presented here for completeness as well, but soybean oil does not contain significant amounts

of protein as the protein remains in the meal fraction during processing (20). 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 Soybean Consumption from the GEMS/Food Highest 97.5th Percentile "Eater-Only" Worldwide

		Consumption[a] (g/kg/day)	
Commodity[a]	Country with Reported Maximum	General Population	Children ≤6 years
VP 541 soya bean (immature seeds)	Thailand	2.41	3.86
VD 541 soya bean (dry)	**Japan**	**3.03**	**5.55**
OR 541 soya bean oil, refined	USA	1.51	2.36

[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 (19).

When the WHO "VD 541 soya bean (dry)" acute consumption information is coupled to the AAD-12 field expression level of 16.52 ng/mg tissue, an upper limit for acute exposure to the proteins via soybean are estimated as:

- **0.0500 mg AAD-12 protein/kg bw/day, for general population (i.e. adults)**
- **0.0917 mg AAD-12 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 (21). Nonetheless, to place the AAD-12 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-12 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-12 protein in soybean are extremely large, indicating no concern for adverse effects from acute dietary exposure through soybean**.

Table 2. Margins of Exposure for AAD-12 Protein in Soybean Based on WHO 97.5th Percentile Consumption

	Protein Exposure[a] (mg/kg bw/day)	**NOEL (mg/kg bw)**	**MOE**
General Population	0.0500	>2000	39960
Children <6 year	0.0917	>2000	21810

[a] Based on WHO 97.5th percentile consumption of soybean under commodity VD 541.

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-12 protein from DAS-68416-4 soybean plants with livestock dietary consumption assumptions for soybean animal feeds. In addition, the relevance of the exposure estimate is placed into context, based on the mammalian toxicity information.

Animal Feed Exposure

An assessment for livestock exposure is presented here based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures of US EPA (22). Accordingly, several soybean commodity forms are considered potential animal feeds: seed, forage, hay meal, hulls and aspirated grain fractions. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average values of 16.52 ng/mg (or ppm) for AAD-12 protein in DAS-68416-4 soybean seed. This value for soybean seed has also been used to estimate exposure to soybean feeds for which there was no

direct expression measurement: the value for the seed is substituted for the meal and hull feeds and a 20X concentration of the seed residue has been assumed for potential aspirated grain exposure. Note however given meal and seed are both protein concentrates and are not simultaneously used in a diet. In addition, for cattle, the field expression level of AAD-12 protein in forage (collected at R3) is applicable. The average value of AAD-12 protein in soybean forage (across treatments) was 40.17 ng/mg tissue (dry weight basis) and the maximum value observed was 41.11 ng/mg tissue. This maximum value in forage will be used in the conservative calculation of acute dairy animal feed exposure. No direct measurement for hay was available; moreover exposure from hay is assumed to be covered by the forage value and if a treated commodity is used as forage it can not be reeaten as hay.

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. The presence of AAD-12 protein in soybean tissue is not anticipated to have impact for feed ration formulation, because nutrient composition analyses have shown that DAS-68416-4 soybean is substantially equivalent to conventional soybean (23) per the general OECD and ILIS guidance (20, 24). US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (22):

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-12 protein. In addition, the higher values of 41.11 ppm of AAD-12 protien for forage are assumed at 100% DAS transgenic soybean. In reality, exposure via forage will be lower, given the average values in forage are slightly lower and more importantly market adoption of DAS-68416-4 soybean 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 for AAD-12

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	AAD-12 (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls*	R	90	15	20	Nu	Nu	**16.52**	2.75	3.76	-	-
Aspriated grain**	CC	85	5	Nu	Nu	Nu	**330.4**	19.44	-	-	-
Soybean seed	PC	89	5	10	*Meal used*	15	**16.52**	0.93	1.86	-	2.48
Soybean Forage	R	35	Nu	20	Nu	Nu	**41.11**	-	23.49	-	-
Soybean meal*	PC	NA	*Seed used*	*Seed used*	25	*Seed used*	**16.52**	-	-	4.13	-
							Total	**23.12**	**29.02**	**4.13**	**2.48**

* estimate based on measured value for seed

**based on theorectical estimate of 20X the value in soybean seed

Because only soybean feeds are considered he nutritional balance of the diets are assumed to be comprised of unmodified feeds. 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-12 Protein from Soybean 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-12 intake (mg/kg feed)	4.13	29.02	23.12	2.48
Maximum intake (mg/kg bw)	0.11	1.14	0.39	0.07

The highest exposed animal is the dairy cow with 1.14 mg AAD-12/kg bw estimate. 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 result 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-12 protein in DAS-68416-4 soybean.

CONCLUSION

Results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

ARCHIVING

The final version of this report along with the associated data analysis will be filed in the Dow AgroSciences facility archives, Indianapolis, Indiana upon issuing the final report.

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