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18 August 2021

Dr. Geoffrey Wong
Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notification of GraINzyme® Glucanase for Use in Swine Feed by Agrivida, Inc.

Dear Dr. Wong,

Under the Final Rule for the notification of self-determination of "Generally Recognized As Safe" (GRAS) for novel animal feed additives (21 CFR Parts 20, 25, 170 et al., Federal Register, vol. 81, No. 159; August 17, 2016) Agrivida, Inc. is hereby submitting a notification of the conclusion by Agrivida, Inc. that the use of the glucanase enzyme, GraINzyme® AC1 Glucanase, in the feed of swine is GRAS. This enzyme hydrolyzes β-D-1,4 glucan bonds in soluble nonstarch polysaccharides that are present in certain feed grains, thereby reducing the viscosity of the digesta and improving the digestibility of nutrients in feeds.

Agrivida's conclusion of the GRAS status of the AC1 Glucanase is based upon scientific procedures and information developed through scientific studies conducted by Agrivida, Inc. and its cooperators. The information upon which this conclusion is based is presented in a document two copies of which are enclosed with this letter. In addition, you will find an electronic file of this GRAS notice in PDF format entitled "AC1 GRASn_Swine_18Aug21" that is present on the compact disc that accompanies this letter. Copies of each of the scientific reports cited in this document to support the information and conclusions of Agrivida, Inc. are contained within a folder that is also present on the enclosed compact disc.

The complete data and original information that are the basis of this GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours at our offices located at 78E Olympia Avenue, Medford, MA 01801.

If you have questions or comments related to this notice, please forward them to me.

Sincerely,

(b)(6)

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

Agrivida

grainzyme®

A thermotolerant β-glucanase feed enzyme expressed in *Zea mays*

SUMMARY of DATA SUPPORTING a NOTIFICATION of GRAS STATUS for USE in SWINE FEED

Submitting Company:

Agrivida, Inc.
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Submitted by:

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August 18, 2021

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Executive Summary

Agrivida, Inc. has developed a new feed enzyme product to improve carbohydrate utilization in swine feeds. This enzyme is a glucanase referred to as the AC1 Glucanase in this document, and it will be marketed under the trade name GraINzyme® AC1 Glucanase. Previously, Agrivida, Inc. submitted to FDA/CVM a GRAS notice (AGRN 31) for the use of this enzyme product in poultry feed. The gene encoding the AC1 Glucanase was derived using Gene Site Saturation Mutagenesis (GSSM; Short, 2001) starting from a sequence that had been isolated from an environmental library and that encoded a protein identical to the Cel5A endoglucanase of *Thermotoga maritima*. The AC1 gene under the control of a monocot-derived seed-specific promoter was transformed into maize (*Zea mays*) using *Agrobacterium*-mediated plant transformation techniques. The resulting transformed maize produces 150 to 300 units of glucanase activity per gram of grain.

The AC1 product is produced using common agronomic practices for the production of maize grain, followed by milling to form a coarse meal. The coarse meal will be added as a feed additive at relatively low inclusion levels to the feed of swine at a dose range of (b) (4) units per kg of feed. The intended effect of the AC1 enzyme in animal feed is to improve the digestibility of feed in the animal's gastrointestinal tract through the solubilization of non-starch polysaccharides (NSP) in the diet, thereby reducing the viscosity of the digesta and improving access of the animal's digestive enzymes to nutrients in the diet.

Agrivida, Inc. has conducted and published studies and reports that demonstrate the AC1 Glucanase product's safety and efficacy and that support a conclusion that the AC1 Glucanase product is generally recognized as safe (GRAS) for its intended use. The details and results of the studies and reports that support the functionality and a conclusion of the GRAS nature of the AC1 Glucanase are presented herein.

The AC1 gene construct that was used in the transformation of maize contained one copy of the AC1 gene, under the control of the *Oryza sativa*-derived glutelin-1 seed specific promoter. The maize AC1 gene transformants were selected using the well-known phosphomannose isomerase (*pmi*) gene whose safety and utility has been well-established.

The maize event that produces the AC1 Glucanase contains a single T-DNA insertion in its genome. The AC1 gene insertion is located on maize chromosome (b) (4) and it contains the complete transfer DNA (T-DNA) with one copy of the AC1 gene. The complete sequence of the insertion including approximately 1.5 kilobase pairs (Kb) of flanking maize DNA on each side of the insert was determined. The plasmid that contains the T-DNA fragment that was used to transform maize contains an antibiotic resistance gene for maintenance in bacterial hosts. The antibiotic resistance gene was not transformed into the maize genome since it is not located within the T-DNA region of the AC1 gene transformation plasmid. The absence in

the maize genome of the antibiotic resistance gene and other elements of the transformation plasmid outside of the T-DNA was confirmed by Southern hybridization techniques. The stability of the AC1 gene insertion in maize over multiple generations was also demonstrated.

The AC1 Glucanase enzyme derived from three representative product batches was fully characterized. The molecular weight, immunoreactivity and glucanase activity were confirmed. The pH and thermal tolerance profile for the AC1 Glucanase were determined and the (b) (4) amino acid sequence of the AC1 Glucanase was confirmed.

It was further demonstrated that the AC1 Glucanase produced by maize is not glycosylated and, consistent with other enzymes in the Glycohydrolase Family 5 group, also possesses endo-cellulase, exo-cellulase and endo-mannanase activities. Three AC1 product batches were demonstrated to meet all JECFA specifications for food enzymes with the exception of number of coliforms and total bacteria. However, the product is within the range for coliforms and total bacteria that are known to be typical for maize grain that is produced by common agricultural practices and widely used in food and feed.

The functionality of the AC1 Glucanase in swine was demonstrated through viscosity measurements of the digesta in the ileum of live pigs in feeding studies (Lessard *et al.*, 2021) and in one *in vitro* feed viscosity study. In addition, a swine performance study that included pigs fed diets treated with the AC1 Glucanase is presented that support the functionality of the product in swine feed.

Viscosities of digesta from pigs consuming a basal diet high in soluble NSPs (containing DDGS) that was supplemented with 0, 150, or 450 units of AC1 Glucanase/kg feed were compared to a negative control group that was fed the same basal diet without AC1 Glucanase supplementation. AC1 supplementation reduced the viscosity of the digesta *in vivo* in a dose dependent manner. The results of thise study were reported by Lessard *et al.* (2021).

The AC1 Glucanase product is concluded to be safe based upon the history of safe use of glucanase enzymes in animal feed and the safety of the maize production host. In addition, a high dose of the AC1 Glucanase (2126 U/kg feed) was included in a swine feeding study to assess the safety of high doses of AC1 in pigs. Assessments of key hematological measurements of the high AC1 dose groups were compared to those of the negative control (NC) and positive control (PC) groups and there were no indications of toxicity or abnormalities in the high AC1 dose groups. Further, post-mortem examinations of animals from the high AC1 dose groups did not reveal any indications of abnormalities or toxicity. The results of this study were described by Lessard *et al.* (2021).

Based on the above information which is supported by the information contained in this document, Agrivida, Inc. concludes that the AC1 Glucanase product is safe and effective, and is GRAS when used as intended in the feed of swine.

Introduction

Cereal grains are broadly classified into two major categories, viscous and non-viscous cereals, depending on their content of soluble non-starch polysaccharides (NSPs). Rye, barley, oats, and wheat contain considerable amounts of soluble NSP and are classified as viscous grains, whereas corn, sorghum, millet and rice contain reduced amounts of soluble NSP and are considered to be non-viscous cereals. Due to the high content of viscous NSP in grain of the former, feeds produced from these grains result in high digesta viscosity in the gastrointestinal (GI) tracts of monogastric animals resulting in reduced nutrient digestibility and availability, negative impacts on the gut microbiome and other negative effects (Burnett, 1966; Choct and Annison, 1992; Bedford and Classen, 1992; Danicke *et al.*, 1999). Since the 1980s glucanase and other enzymes that degrade soluble NSP have been added to the feed of monogastric animals to increase performance of animals fed diets based on grains with a high soluble NSP content (Hesselman and Åman, 1986; Campbell *et al.*, 1989; Broz and Frigg, 1986; Newman and Newman, 1987). A large number of enzymes categorized as NSPase are approved for use in animal feed to depolymerize soluble NSPs and improve the digestion of nutrients in feeds based on grains high in soluble NSP content. These include glucanase as well as galactosidase, mannanase, pectinase, and xylanase. β-glucans are a primary soluble NSP of barley, wheat and other grains high in soluble NSPs and are present at levels of 0.2-0.7% in wheat and 1.9-5.4% in barley (Havrlentová and Kraic, 2006). β-glucan is a glucose polymer containing a mixture of β-1-3 and β-1-4 linkages that make its physicochemical properties different from cellulose that is a straight-chain glucose polymer with only β-1-4 linkages. Four types of endo-acting glucanases, classified according to the type of glycosidic linkage they cleave, are capable of depolymerizing (1,3)-(1,4)-β-D-glucan: endo-(1,3)-(1,4)-β-glucanases, endo-1,3(4)-β-glucanases, endo-1,4-β-glucanases, and to a lesser extent, endo-1,3-β-glucanases (McCarthy *et al.* 2003).

Although glucanases have been widely used in feeds based on grains high in soluble NSPs, their utility in corn-soybean meal based diets has also been demonstrated. NSPs in corn-soybean meal based diets have been shown to decrease the digestibility of nutrients by restricting access of digestive enzymes such as amylase and proteases to nutrients intertwined in fibrous cellular matrices (Cowieson, 2005). In addition, legume (e.g., soybean) NSPs are more complex in structure than those of cereals, containing a mixture of colloidal polysaccharides (galacturonans, galactan and arabinans). Accordingly, the addition of pectinase to a corn-soybean meal diet has been shown to significantly increase the metabolizable energy (ME) value of the diet. This improvement in the ME coincided with increased digestibility of galactose-rich polysaccharides (Kocher *et al.*, 2002).

Agrivida, Inc. is developing animal feed enzyme products that are produced in maize grain. Genes encoding the enzymes, under the regulation of monocot-derived seed-specific promoters, are transformed into maize. The enzyme products produced in this manner will be marketed under the trade name of GraINzyme®. One of the

GraINzyme® products under development by Agrivida, Inc. is a glucanase feed enzyme whose primary activity is endo-1,4-β-glucanase but that also exhibits lesser levels of other NSPase activities, including endocellulase, exocellulase, and endo-mannanase. The AC1 Glucanase was developed using GSSM, resulting in the introduction of (b) (4) amino acid changes into the coding sequence of the Cel5A glucanase sequence (NCBI accession Q9X273) (Nelson *et al.*, 1999) to improve the thermostability of the AC1 Glucanase protein. The AC1 Glucanase consists of (b) (4) amino acids that share 96% identity with the Cel5A cellulase from *Thermotoga maritima* GH5.

Maize plants engineered to express this gene using Agrivida's GraINzyme® technology produce 150 to 300 units of glucanase activity/g of grain. The GraINzyme® Glucanase product referred to herein as AC1 Glucanase will consist of coarsely ground corn meal produced from maize plants expressing the glucanase gene in the grain. It will be included in relatively small amounts as a feed additive in swine diets in order to reduce the viscosity of digesta and improve the digestibility of major feed ingredients.

1.0 Signed statements and certification

1.1. Submission of a GRAS notice

Agrivida, Inc. is hereby submitting a GRAS notice in accordance with §170.225(c) of 21 CFR Parts 20, 25, 170 and 570 *et. al* (Federal Register, Vol. 81, No. 159, August 17, 2016) for an endo-1,4-β-glucanase enzyme for use in the feed of swine to improve the digestibility of feeds containing soluble non-starch polysaccharides (NSP).

1.2. Name and address of notifier

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801 USA
Tel: 781-391-1262

Person responsible for the dossier:

James Ligon, PhD
Agrivida, Inc.
VP, Regulatory Affairs and Stewardship
1023 Christopher Drive
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1.3 Name of the notified substance

The substance that is the subject of this GRAS notice is an endo-1,4-β-glucanase enzyme with high similarity to the *Thermotoga maritima* Cel5A glucanase enzyme (E.C. 3.2.1.6). The glucanase is produced in the grain of *Zea mays*. The trade name of the product is GraINzyme® AC1 Glucanase.

1.4 Conditions of use of the notified substance

This GRAS notice is for the purpose of establishing GRAS status for GraINzyme® AC1 Glucanase in swine feed to increase the digestibility of feed containing soluble NSP. The recommended inclusion rate of the GraINzyme® AC1 Glucanase in swine feed is (b) (4) glucanase activity units (U) per kg of feed.

1.5 Statutory basis for conclusion of GRAS status

The conclusion that the GraINzyme® AC1 Glucanase enzyme is GRAS for use in swine feeds is based on scientific procedures in accordance with 21 CFR Part 570, Subpart E (Federal Register, Vol. 81, No. 159, August 16, 2016).

1.6 Substance is exempt from premarket approval

It is the opinion of Agrivida, Inc. that the GraINzyme® AC1 Glucanase is exempt from the requirement for premarket approval under the Food, Drug and Cosmetic Act based on our conclusion that it is GRAS for its intended use in swine feed.

1.7 Data availability

The data that are the basis for the conclusion that the GraINzyme® AC1 Glucanase is GRAS for its intended use are fully encompassed by this submission, including published manuscripts and reports. In addition, upon request by the FDA, Agrivida, Inc. will produce any additional relevant information either in paper or suitable electronic form and provide these to the FDA.

1.8 Confidential business information in this GRAS notice

Agrivida, Inc. does not consider information contained in this document to be confidential business information.

1.9 Certification

Agrivida, Inc. hereby certifies that to the best of its knowledge, this GRAS notice includes all relevant information, both favorable and unfavorable, that is pertinent to the safety and functionality of the GraINzyme® AC1 Glucanase for its use in the feed of swine.

1.10 Signatory person

(b)(6)

Date: August 18, 2021

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

1.11 Authorization to send trade secrets

If necessary, Agrivida, Inc. authorizes FDA Center for Veterinary Medicine to send information from this notification, including information considered by Agrivida, Inc. to be trade secret or CBI, to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture.

2.0 Identity, method of manufacture, specifications and technical effect

2.1 Identification of the notified substance

The GraINzyme® AC1 Glucanase product developed by Agrivida, Inc. is an endo-1,4-β-glucanase (EC 3.2.1.6) produced by genetically modified *Zea mays* (corn).

The gene encoding the AC1 Glucanase was derived from a gene isolated from an environmental DNA library that is identical to the Cel5A glucanase gene of *Thermotoga maritima* (NCBI accession Q9X273) (Nelson *et al.*, 1999). The gene sequence was modified by GSSM to improve its thermostability. The resulting AC1 glucanase differs from the Cel5A glucanase by (b) (4) amino acids. Expression of the AC1 gene is directed by a monocot-derived seed-specific promoter, such that the GraINzyme® AC1 Glucanase is produced only in the grain of *Z. mays*. Detailed information about the production organism, enzyme, manufacturing process and safety of the GraINzyme® AC1 Glucanase for use in swine nutrition is contained in this submission. The production host organism, *Z. mays*, is well-characterized with respect to safety and toxicology and is considered safe for consumption by humans and animals.

2.2 Method of manufacture

The GraINzyme® AC1 Glucanase product is produced by cultivating *Z. mays* producing GraINzyme® AC1 Glucanase using common agricultural procedures for producing maize grain, and the harvested grain containing GraINzyme® AC1 Glucanase is milled to a coarse meal and packaged in suitably labeled containers for inclusion in swine feed.

3.0 Target animal exposure and safety factor calculation

The AC1 Glucanase product will be marketed according to its label specifications. The product label will provide instructions for the user to include the product in the feed of swine at a rate of (b) (4). It is assumed that the users of this product will follow the product dose recommendations provided by Agrivida, Inc. on the product label and so no other significant exposure to animals other than poultry and swine consuming feed treated with the AC1 Glucanase is expected.

Exposure to other substances as a result of the use of this product is primarily limited to the breakdown products of β-1,4 glucans as would be expected with the use of any other glucanase product or the normal digestion of β-1,4 glucans. Since the AC1 Glucanase also exhibits lesser amounts of endocellulase, exocellulase, and endo-mannanase activity, the products from these activities, including glucose, oligosaccharides and polysaccharides, will also be released from the diet. All of these products are nutritious and are normally released from complex carbohydrates in the diet during normal digestion so the level of exposure to these is not expected to be substantially different.

Since the AC1 Glucanase is contained within the grain of maize and the nutritional composition of the grain is not different from that of conventional maize grain, the only exposure to other products would be those derived from maize grain that are considered safe for food.

4.0 Self-limiting levels of use

The GraINzyme® AC1 Glucanase product is not intended for inclusion in human food and it will be marketed with a label that states that the product is to be used only for inclusion in poultry and swine feeds. Therefore, according to §170.240 of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016) there is no requirement to establish a self-limiting level of use for the GraINzyme® AC1 Glucanase product.

The GraINzyme® AC1 Glucanase is produced by maize genetically engineered with a glucanase gene that is closely related to the *Cel5A* cellulase gene of *Thermotoga maritima*, and produces GraINzyme® AC1 Glucanase in the grain. Typically, grain derived from the maize production host contains between 150-300U per gram of grain. Other than the presence of the GraINzyme® AC1 Glucanase enzyme, the GraINzyme® AC1 Glucanase product contains maize grain that is nutritionally equivalent to conventional maize grain used as a major swine feed ingredient. The presence of the GraINzyme® AC1 Glucanase in maize grain does not appear to affect the taste, palatability, or other organoleptic properties of the grain. Therefore, the maximum amount of GraINzyme® AC1 Glucanase product that might be theoretically consumed by an animal is equal to the total amount of maize meal included in the feed. In the case of swine feed based on a maize/soybean meal diet, the maize component typically comprises between 50% and 70% of the total feed. Accordingly, the maximum amount of GraINzyme® AC1 Glucanase that might be consumed by swine is equivalent to the amount of GraINzyme® AC1 Glucanase contained in the maize component of the diet assuming that all of the maize meal was GraINzyme® AC1 Glucanase product. However, since the GraINzyme® AC1 Glucanase product will be marketed primarily in either (b) (4) bags or (b) (4) totes with a product label that directs the user to add the appropriate amount of the product when mixing the feed, the likelihood that a feed would be prepared using the GraINzyme® AC1 Glucanase product to replace all of the maize meal in the diet is very remote.

Nevertheless, assuming that a (b) (4) tote of GraINzyme® AC1 Glucanase product was used in place of normal maize meal to make a swine feed, the maximum amount of feed that could be produced would be (b) (4) or less. In the unlikely event that this transpired, the resulting feed would not be expected to cause adverse effects on the swine that consume it, even at a maximum dose of approximately 210,000 U/kg as potentially could be expected. Glucanase is an enzyme whose primary enzymatic activity is the depolymerization of 1,3-1,4-β-D-glucan. If large amounts of glucanase were included in a feed it would be expected that most or all of the 1,4-β-D-glucan bonds in the diet would be hydrolyzed to produce simpler carbohydrates with resulting increased levels of energy availability.

Based on the above, it is expected that in the unlikely event that grain from GraINzyme® AC1 Glucanase expressing maize were to be substituted for all of the maize in a typical maize/soybean meal swine diet that it would not adversely affect

the performance of the swine nor would it cause any safety concerns for the animals. Additionally, the AC1 Glucanase protein would not be expected to be present in the meat derived from animals consuming it since it has been well established that ingested proteins are digested and absorbed as small peptides and amino acids (Metcalf *et al.*, 1996; Betz *et al.*, 2000). Therefore, the meat of animals consuming feed treated with GraINzyme® AC1 Glucanase protein would be safe for human consumption.

5.0 Experience based on common use prior to 1958

The GraINzyme® AC1 Glucanase product was not in use prior to 1958 and Agrivida, Inc.'s conclusion of GRAS status for the use of this product in poultry feed is not based on its common use prior to 1958. Agrivida's conclusion that the GraINzyme® AC1 Glucanase product is GRAS for use in swine feed is based on scientific principles. Therefore, the requirement to provide evidence of its use prior to 1958 is not applicable.

6.0 Narrative

6.1 Safety of GraINzyme® AC1 Glucanase

The characterization and safety assessment of feed enzymes produced through the use of recombinant DNA technology involves a number of factors that include the following:

1) The safety of the organism that was the source of the gene encoding the enzyme
2) The safety of the host/recipient organism, in this case *Zea mays*
3) Characterization of the construct used to transform the host/recipient
4) Characterization of the production organism i.e. the transgenic *Zea mays* used to produce the enzyme
5) The characterization and safety of the enzyme itself.

Each of the above factors in relation to the safety of the AC1 Glucanase is discussed in this section.

6.1.1 Source of the gene encoding AC1 Glucanase

The AC1 gene encoding the GraINzyme® AC1 Glucanase enzyme was derived from a gene isolated from an environmental DNA library. The isolated gene encoded an enzyme that is identical to the Cel5A cellulase from *Thermotoga maritima* (NCBI accession Q9X273) (Nelson *et al.*, 1999). This gene was modified by GSSM resulting in (b)(4) amino acids changes in order to improve the thermostability of the AC1 Glucanase. The modified gene encodes a glucanase enzyme that is a 37.7 kDa protein with 96% identity to the Cel5A glucanase of *T. maritima*. Agrivida, Inc. has compared the Cel5A and AC1 glucanase enzymes and has demonstrated that the enzyme kinetics of these two glucanases is nearly identical (Table 1). Expression of the AC1 gene is directed by a monocot-derived seed-specific promoter, such that the GraINzyme® AC1 Glucanase is produced only in the grain of *Z. mays*. Detailed information about the production organism, enzyme, manufacturing process and safety of the GraINzyme® AC1 Glucanase for use in poultry nutrition is contained in this submission. The production host organism, *Z. mays*, is well characterized with respect to safety and toxicology and is considered safe for consumption by humans and animals.

Table 1. Comparison of the enzyme kinetic properties of the Cel5A glucanase of *T. maritima* and the AC1 Glucanase produced by maize Event FG259.

	Vmax µmoles/min/mg	Kcat min^{-1}	Km mg/mL
Cel5A	36.5	1365	0.38
AC1	31.2	1167	0.22

Since only the coding sequence of the *AC1* Glucanase gene and no other DNA derived from the original host is included in the the transformation construct of plasmid (b) (4), the identity of the source organism or its safety profile is not relevant to a discussion of the safety of the AC1 Glucanase protein.

6.1.2 Description of the Host Organism

A detailed description of *Zea mays* (Maize) is presented in AGRN 31, the GRAS notice for the use of AC1 Glucanase in poultry feed (§6.1.2 Description of the Host Organism, pg. 17).

6.1.3 Source of the maize line

The maize line that was used to transform maize with the AC1 Glucanase gene is described in AGRN 31, the GRAS notice for use of the AC1 Glucanase in poultry feed (§ 6.1.3 Source of the maize line, pg. 19).

6.1.4 Characteristics of the AC1 Glucanase gene expression construct

A transformation gene cassette containing a gene encoding the AC1 Glucanase under the control of the (b) (4) signal was constructed in plasmid (b) (4) A complete description of the genetic elements of the T-DNA fragment that was used to transform maize and other attributes of plasmid (b) (4) have been described in the GRAS notice for the use of the AC1 Glucanase product in poutry (AGRN 31, §6.3.2 Characteristics of the AC1 Glucanase gene expression construct, pg. 20).

In addition to containing the AC1 Glucanase coding sequence, (b) (4) Agrivida, Inc. has conducted (b) (4) amino acid sequencing of AC1 (b) (4) The transformation event chosen for commercial development was designated maize Event FG259.

6.1.5 Genetic Characterization of Maize Event FG259

The genetic characterization of maize event FG259 and the T-DNA insertion containing the *AC1* gene was described in detail in the GRAS notice for the use of AC1 Glucanase in poultry feed (AGRN 31, §6.1.5 Characterization of the production

organism: Maize Event FG259, pg. 25). Southern hybridization experiments demonstrated that event FG259 contains one T-DNA insertion (AGRN 31, §6.1.5.1 Determination of number of DNA insertions, pg. 25) and confirmed the absence of DNA fragments from the transformation vector backbone in the genome of Maize Event FG259 (AGRN 31, §6.1.5.2 Screening for the absence of plasmid vector backbone fragments, pg. 32). The complete T-DNA and genomic maize flanking DNA were sequenced and characterized (AGRN 31, §6.1.5.3, Characterization of the T-DNA chromosomal integration site in Event FG259 pg. 35). The genetic stability of the AC1 Glucanase gene containing T-DNA in maize event FG259 was evaluated over multiple backcross generations (AGRN 31, §6.1.5.4 Genetic stability of the insert over multiple generations, pg. 37) and the results demonstrated that the insertion was stable over the four backcross generations that were examined. In addition, it was demonstrated that the AC1 gene was inherited over four backcross generations in the expected Mendelian pattern (AGRN 31, §6.1.5.5 Mendelian inheritance of the AC1 Glucanase gene in Event FG259, pg. 39). The results of the genetic characterization of the AC1 gene containing T-DNA and flanking genomic DNA in maize event FG259 did not reveal any issues or concerns regarding the safety of consumption of grain derived from event FG259.

6.2 Characterization and safety of the AC1 Glucanase

6.2.1 Introduction to β-glucanase enzymes

β-glucanase enzymes are ubiquitous in nature. In most plants they play a role in the opening of plasmodesmata channels that connect adjacent plant cells (Levy *et al.*, 2007; Leubner-Metzger, 2003) and they are produced in the germinating seeds of plants (Vögeli-Lange *et al.*, 1994), including tomato (Chun-Ta *et al.*, 2001), barley (Leah *et al.*, 1995), peanut (Liang *et al.* 2005), wheat (Moravčíková et al., 2016) and many others. In addition, β-1,3-glucanases attack critical components of fungal cell walls and are expressed in many plants as part of a broad generalized defense mechanism against fungal pathogenesis (Boller, 1987; Collinge and Slusarenko, 1987; Cornelissen and Melchers, 1993). Therefore, it can be concluded that glucanase enzymes are present in many different plant-derived food ingredients. β-glucanases from various sources are also widely used as food processing aids in the production of fermented beverages such as beer and wine, and in the production of yeast extract.

Glucanase enzymes have been used for many years in animal feed to increase the digestibility of the feed for monogastric animals (Bedford and Classen, 1992; Burnett, 1966). For the same purpose, glucanase enzymes are components of digestive aids marketed to humans and they appear in many such products, including Veganzyme® (Global Healing Center, 2020), Digestive Enzymes Ultra (Pureformulas, 2020), and βeta-1,3D Glucan (LifeExtension, 2020). Glucanases and other carbohydrase enzymes have been used for many years in the production of

food as processing aids for clarifying fruit and vegetable juices and beer and other alcoholic beverages (FDA, 2018; Table 2). As such, it can be concluded that there is a long history of safe consumption of glucanase enzymes in monogastric animals, including poultry, swine and humans.

Table 2. A partial list of β-glucanases and related carbohydrases that have been approved by FDA as GRAS for use in food production in the past 5 years (FDA, 2018).

GRAS No.	Enzyme	Source Organism	GRAS Use
592	β-glucanase	*Bacillus subtilis*	Beer processing
584	Cellulase	*Penicillium funiculosum*	Processing for beer and baking
566	Mannanase	*Trichoderma reesei*	Oil, fruit, vegetable processing; coffee production
535	β-glucanase	*Streptomyces violaceoruber*	Processing of yeast extract and alcoholic beverages
482	β-glucanase and Xylanase	*Disporotrichum dimorphosporum*	Production of beer and fermented beverages
479	β-glucanase, cellulase and Xylanase	*Talaromyces emersonii*	Production of beer and fermented beverages

6.2.2 AC1 Glucanase product characterization

The AC1 Glucanase is a 37.7 kDa endo-1,4-β-glucanase that possesses 96% identity to the Glycosyl Hydrolase Family 5 Cel5A cellulase from *Thermotoga maritima*, originally isolated from geothermally heated marine sediments (Huber *et al.*, 1986). The gene encoding the AC1 Glucanase was isolated from an environmental DNA library, where it was found to encode a protein that was identical to Cel5A from *T. maritima*. This sequence was further modified by GSSM for improved thermostability to generate the AC1 Glucanase gene.

For the purpose of characterizing the AC1 Glucanase product, protein extracts prepared from grain derived from three representative AC1 Glucanase product batches were assessed as described in AGRN 31 (§6.2.2 AC1 Glucanase product characterization, pg. 40). The specific activity of the AC1 Glucanase in each of the three product batches was determined and it was shown to be similar in each of the three product batches (AGRN 31, §6.2.3 Specific activity of AC1 Glucanase, pg. 41). The glycosylation status of the AC1 Glucanase protein produced in maize Event FG259 was examined and it was determined that the AC1 Glucanase protein is not glycosylated in maize (AGRN 31, §6.2.4 Assessment of the glycosylation status of the

AC1 Glucanase, pg. 42). The expected amino acid sequence of the AC1 Glucanase was confirmed by (b) (4) amino acid sequencing of the purified AC1 Glucanase protein and the site of cleaveage of the maize (b) (4) was confirmed (AGRN 31, §6.2.5 Confirmation of the AC1 Glucanase amino acid sequence, pg. 43). The glucanase activity of the AC1 Glucanase in the three product batches over a range of pH from pH3.0 to pH10 was determined (AGRN 31, §6.2.6 pH optimum of the AC1 Glucanase, pg. 45). Likewise, the glucanase activity of the AC1 Glucanase from three product batches was determined over a range of temperatures and it was demonstrated that the AC1 Glucanase maintains a significant proportion of its activity up to temperatures of 100°C (AGRN 31, §6.2.7 Thermal tolerance of the AC1 Glucanase, pg. 46). The enzymatic side activities of the AC1 Glucanase were investigated and it was demonstrated that it also possesses significant endocellulase and exocellulase activities in addition to detectable levels of β-1,3-glucanase, β-xylanosidase and endomannanase activities (AGRN 31, §6.2.8 Enzymatic activities of the AC1 Glucanase, pg. 47). An examination of the molecular weight and immunoreactivity of the AC1 Glucanase confirmed that it is the expected size of approximately 37 kDa and that it reacts with a mouse monoclonal antibody generated against the AC1 Glucanase protein (AGRN 31, §6.2.9 Molecular weight and Immunoreactivity of AC1 Glucanase, pg. 48).

6.2.3 Assessment of Amino Acid Homology of AC1 Glucanase to Known Protein Toxins

A global sequence similarity search of the AC1 Glucanase amino acid sequence was conducted on August, 6, 2021 against the NCBI Protein dataset using the BLASTP algorithm (Altschul et al., 2005). A sequence file comprising the translation of the *AC1* Glucanase gene was queried using the BLASTP 2.6.1 algorithm against the "nr" dataset, which incorporates non-redundant entries from all GenBank nucleotide translations along with protein sequences from SWISS-PROT, PIR, PRF, and PDB.

A cutoff expectation (E) score of 1.0 was used to generate biologically meaningful similarity between the AC1 Glucanase protein and proteins in the "nr" dataset. Although a statistically significant sequence similarity generally requires a match with an E score of less than 0.01 (Pearson, 2000), a cutoff of E < 1.0 ensures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 5000.

The top 5000 proteins with homology to the AC1 Glucanase protein with an E score of less than 1.0 were examined. A large number of the accessions returned by the search displayed complete significance (E = 0) and represented nearly identical or closely related glucanase proteins from various microbial species. Most of the remaining sequences represented a variety of proteins that were all functionally related bacterial glycoside hydrolase enzymes such as endoglucanases and cellulases. None of the proteins were known toxins or proteins with toxicity related activities. This demonstrates that the AC1 Glucanase protein is unlikely to share

relevant sequence similarities with known protein toxins and is therefore unlikely to be toxic.

6.2.4 Evaluation of the Safety of the AC1 Glucanase

The safety of the AC1 Glucanase is reviewed and evaluated in the GRAS notice for use in poultry (AGRN 31, §6.2.11 Evaluation of the Safety of the AC1 Glucanase, pg. 51 and §6.2.12 Conclusions on the Safety of AC1 Glucanase, pg. 52). Based on the review of the information presented in AGRN 31, Agrivida, Inc. has concluded that the AC1 Glucanase is safe for use as an additive in poultry and swine feed.

6.2.5 Tolerance Study with GraINzyme® AC1 Glucanase in Weaned Piglets

Given the range of possible effects that carbohydrases may have on animal health it is reasonable to assess how well animals tolerate a new enzyme as a feed additive (Schliffka *et al.* 2019). To test this, weaned pigs were fed either a typical corn/soy/DDGS diet supplemented or not supplemented with 2126 U/kg AC1 Glucanase, with the expectation that such a high dose would reveal any adverse effects of the enzyme. Effects on performance (body weight, average daily gain, average daily feed intake, and gain-to-feed ratio), as well as hematology characters and blood chemistry markers, which are sensitive to nutritional malabsorption, disease, and other physiological disorders (Clark and Coffer 2008; Schliffka *et al.* 2019) were all measured. In addition, all animals were monitored during the live phase for gross health effects as well as via necropsy at the end of the trial. The results and conclusions of this study are described by Lessard *et al.* (2021).

The trial was a randomized complete block and double-blinded design in which 40 animals were ranked by weight and blocked by sex, then randomly assigned to two treatment groups (negative control or AC1 Glucanase treatment), each treatment group containing 20 pigs with males and females equally represented. Once assigned to treatment group, the pigs in each treatment group were ranked by weight and sequentially divided into 10 sets of two pigs each of the same sex to ensure relative uniformity of weight of the two pigs in each pen. Each set of two pigs was then randomly assigned to one of 20 pens.

Pigs were fed nutritionally complete corn-soy-DDGS diets in mash form, manufactured by (b) (4) whose composition is shown in Table 3. The two diets were identical except that 12.6 kg of the conventional corn in the treated diet was replaced by 12.6 kg of corn derived from maize Event FG259 that produces the AC1 Glucanase (170 U/g of β-1,4-glucanase activity), that provided the treated diet with approximately 2126 U/kg AC1 activity, based on analysis of the completed feed. Pigs had free access to water and feed. Pigs were observed at least daily for general health from study days 0 to 42. Pigs were individually weighed on study days 0, 21, and 42. Feed consumption was recorded

throughout the study and total feed consumed per pen was calculated on study days 21 and 42.

Two samples of blood were collected from the jugular vein of each pig on study day 42. For the first sample, approximately 6 mL of whole blood was collected into one serum separation tube for each pig. Serum was submitted to the (b) (4) for serum chemistry analysis, including albumin, glucose, phosphorus, alanine transaminase (ALT), and creatine phosphokinase (CK). For the second sample, approximately 5 mL of whole blood was collected into one tube containing EDTA for each pig. Immediately after collection, tubes were gently inverted several times to ensure thorough mixing of the blood and anticoagulant. Samples were submitted on ice packs within 24 hours of collection to (b) (4) for hematology analysis including total white blood cells count (WBC), WBC differential (i.e., neutrophil, lymphocyte, monocyte, eosinophil, basophil), absolute large unstained cells (LUC), red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, and mean platelet volume (MPV). These tests include several markers that are sensitive to malabsorption of nutrients such as carbohydrates, minerals and vitamins, kidney and liver function, disease, infection, stress, hydration, and overall health status (Cooper *et al.* 2014; Onasanya *et al.* 2015). All pigs were euthanized and necropsied on study day 42. Pigs were examined by a board-certified pathologist for visual abnormalities or indications of toxicity in the major organ systems.

Data were analyzed using (b) (4) for Windows, Version 25.0 (b) (4). Pen was used as experimental unit for each analyzed variable, except for hematology and blood chemistry data, in which the pig providing the sample served at the experimental unit. All production data were initially evaluated for both Normality and Homogeneity of Variance by Treatment. The ANOVA model included treatment, block, and their interactions, and P-values < 0.05 were considered significant in all comparisons. Any reference to trends assumes P-values < 0.10.

In the enzyme treatment group, animals ingested on average 930 U/d AC1 from day 0 and day 21, and 2796 U/d from day 21 to day 42 (1866 U/d overall). There were no differences in body weight (BW) and average daily gain (ADG) between pigs fed diets containing 2126 U/kg AC1 or the control diet without enzyme (Table 4). Similarly, there were no significant differences in average daily feed intake (ADFI) or gain-to-feed ratios (G:F) during any interval. These results indicate that inclusion of very high levels of AC1 does not adversely affect the performance of the animals.

Necropsies conducted at the end of the study revealed gross lesions in seven out of the forty pigs (two pigs from the group that were fed the diet containing 2126 U/kg AC1 and five pigs from the group that were fed the control diet). Briefly, the two pigs from the group that were fed AC1 presented thickened gastric mucosa and the

presence of chyle in the mesenteric lymphovascular channels. Of the five pigs from the group that were fed the control diet, two also had thickened gastric mucosa; one had remnants of an umbilical hernia that had resolved on its own along with complications arising from the umbilical hernia; one had a number of lesions accompanied with a general failure to thrive, which were attributed to iron deficiency anemia; and one had suppurative omphalitis, with no evidence of entrance into the peritoneal cavity.

Blood samples from all animals were analyzed for both hematological characters (Table 5) and serum chemistry markers (Table 6). Results from these examinations showed no statistically significant differences between animals consuming the control diet or the diet containing 2126 U/kg of AC1 after 42 days.

Table 3. Composition of Basal Diet for the Tolerance Feeding Study

Ingredients	Control	AC1
Corn	45.87	44.61
Corn expressing AC1		1.26
Soymeal Full Fat	30.896	30.896
Distillers HI Pro 38%	10	10
Soybean Meal 47%	7.654	7.654
Veg Oil	1.891	1.891
Super Cal 38 Gilemore	1.435	1.435
Dical 21 Biofos	1.039	1.039
Salt Mixing	0.519	0.519
Choline Chloride 60%	0.208	0.208
MB Turkey Vtm Pmx	0.2	0.2
Biolys	0.18	0.18
StreesessPlus	0.048	0.048
Magnesium Oxide	0.047	0.047
Vitamin E 50% 227	0.01	0.01
Copper Sulfate 25.2%	0.003	0.003
Total	100	100

Composition	Common basal diet
ME, kcal/kg	3402
Lys, %	1.13
Met + Cys, %	0.63
Trp, %	0.25
Thr, %	0.75
Ca, %	0.84
Available P, %	0.37
DM, %	90.79
NDF, %	13.8
ADF, %	4.9
AC1 activity (U/kg)*	2126

*b-1,4-glucanase activity, detected only in diet B

Table 4. Performance of pigs on untreated (control) and treated (AC1) diets

	Control		AC1		
BW (kg)	**Mean**	SD	**Mean**	SD	***P*-value**
d0	**6.714**	0.666	**6.773**	0.666	0.798
d21	**10.877**	2.203	**11.250**	1.658	0.549
d42	**25.677**	5.287	**26.695**	3.727	0.486
ADG (kg/d)					
d0-21	**0.198**	0.096	**0.213**	0.064	0.564
d21-42	**0.704**	0.156	**0.736**	0.118	0.474
d0-42	**0.451**	0.121	**0.474**	0.085	0.487
ADFI (kg/d)					
d0-21	**0.809**	0.174	**0.795**	0.138	0.848
d21-42	**2.223**	0.276	**2.391**	0.201	0.137
d0-42	**1.509**	0.209	**1.595**	0.135	0.286
G:F (kg/kg)					
d0-21	**0.488**	0.098	**0.536**	0.066	0.214
d21-42	**0.638**	0.038	**0.614**	0.042	0.196
d0-42	**0.598**	0.049	**0.594**	0.032	0.832

Table 5. Hematological characters in pigs after 42 days on the untreated (control) diet or the treated (AC1) diet.

		*normal range**		Control		AC1		
Hematology	**unit**	**Low**	**High**	**Mean**	SD	**Mean**	SD	***P*-Value**
WBC	$\times 10^3$/ul	11.0	22.0	**15.407**	3.089	**15.783**	3.020	0.707
RBC	$\times 10^6$/ul	4.87	8.19	**6.570**	0.460	**6.605**	0.514	0.822
Hemoglobin	gm/dl	8.1	14.7	**11.311**	1.285	**11.779**	0.518	0.149
Hematocrit	%	28.2	42.6	**38.753**	3.808	**39.821**	2.130	0.293
MCV	fl	43.4	64.5	**59.195**	5.985	**60.495**	3.900	0.433
MCH	pg	12.4	20.6	**17.290**	2.084	**17.911**	1.234	0.271
MCHC	gm/dl	27.4	35.8	**29.158**	1.243	**29.595**	0.721	0.194
RDW	%	14.9	32.4	**22.116**	3.612	**20.505**	1.831	0.092
Platelet	$\times 10^3$/ul	119	1000	**415.950**	117.605	**449.210**	98.733	0.351
MPV	fl	6.5	11.9	**9.542**	1.590	**9.479**	1.043	0.886
Neutrophil	$\times 10^3$/ul	2.0	11.9	**4.400**	1.383	**4.961**	1.635	0.261
Lymphocyte	$\times 10^3$/ul	4.0	17.9	**9.653**	2.757	**9.562**	1.871	0.906
Monocyte	$\times 10^3$/ul	0.0	3.7	**0.752**	0.365	**0.726**	0.200	0.788
Eosinophil	$\times 10^3$/ul	0.0	1.3	**0.320**	0.167	**0.301**	0.118	0.689
Basophils	$\times 10^3$/ul	0.0	1.6	**0.128**	0.051	**0.122**	0.044	0.660
Absolute LUC	$\times 10^3$/ul			**0.153**	0.092	**0.111**	0.080	0.137

**Normal range corresponds to reference intervals established by the* (b) (4)

Table 6. Serum chemistry markers in pigs after 42 days on the untreated (control) diet or the treated (AC1) diet

		*normal range**		Control		AC1		
Chemistry	**unit**	**Low**	**High**	**Mean**	SD	**Mean**	SD	***P*-Value**
ALT	IU/L	25.0	90.0	**34.79**	6.61	**37.20**	6.64	0.263
Sodium	mEq/L	135.0	150.0	**139.53**	1.98	**139.65**	1.63	0.832
Potassium	mEq/L	4.0	7.0	**5.93**	0.47	**5.77**	0.35	0.220
Chloride	mEq/L	95.0	110.0	**100.53**	1.39	**101.05**	1.73	0.306
Bicarbonate	mEq/L			**31.37**	2.03	**30.45**	1.79	0.142
Calcium	mg/dl	8.0	12.0	**10.98**	0.41	**10.79**	0.48	0.185
Phosphorus	mg/dl	4.5	9.0	**9.94**	0.70	**10.30**	0.89	0.177
Magnesium	mg/dl	1.82	3.65	**2.36**	0.28	**2.40**	0.16	0.631
BUN	mg/dl	6	30	**9.11**	2.40	**9.45**	1.79	0.613
Creat	mg/dl	0.5	2.7	**0.90**	0.12	**0.86**	0.11	0.248
Glucose	mg/dl	65.0	150.0	**109.79**	7.54	**111.45**	6.25	0.458
Total Protein	gm/dl	7.0	8.9	**4.78**	0.24	**4.70**	0.29	0.326
Albumin	gm/dl	3.0	4.5	**3.42**	0.34	**3.33**	0.33	0.379
AST	IU/L	10.0	100.0	**46.00**	23.90	**55.70**	45.77	0.416
Creatine Kinase	IU/L	100.0	2500.0	**2368.42**	2702.54	**2327.68**	2930.74	0.965
Alk Phos	IU/L	25.0	130.0	**231.89**	45.22	**245.45**	101.01	0.595
GGT	IU/L	10	100	**52.42**	15.31	**49.20**	13.84	0.495
Total Bilirubin	mg/dl	0.0	1.0	**		**		
Anion Gap				**13.42**	2.09	**13.95**	2.39	0.468
Lipemic Indice				**20**	0	**20**	0	
Hemolytic Indice				**26.68**	19.36	**21.80**	14.30	0.374
Icteric Indice				**2**	0	**2**	0	

**Normal range corresponds to reference intervals established by the* (b) (4)

***Total bilirubin was below the LOD (0.10 mg/dl) in all samples at d42*

Discussion of Results of the Tolerance Study

Effects on performance (body weight, average daily gain, average daily feed intake, and gain-to-feed ratio), as well as hematology characters and blood chemistry markers, which are sensitive to nutritional malabsorption, disease, and other physiological disorders (Clark and Coffer 2008; Schliffka *et al.* 2019) were all measured, and no significant differences were observed, indicating that the physiology of these animals was not altered by consuming the enzyme. In addition, all animals were monitored during the live phase for gross health effects as well as via necropsy at the end of the trial. While a small number of pigs displayed gross lesions during the necropsies, more of the affected animals were in the control group than in the enzyme-treated group, and none of the findings could be attributed to consumption of the enzyme. These results confirm that AC1 is well

tolerated in the diets of young pigs and complement the results that demonstrated the tolerance of this enzyme in broiler diets (Broomhead *et al.* 2019).

6.2.6 Conclusions of the Safety of GraINzyme® AC1 Glucanase

The biological activity of the GraINzyme® AC1 Glucanase has been well characterized (AGRN 31, §6.2.8 Enzymatic activities of the AC1 Glucanase, pg. 47) and was demonstrated to be primarily a β-1-4 glucanase with minor levels of other carbohydrase activities. These activities are known to be safe, and similar enzymes are present in commonly consumed food and in human nutritional supplement products. A decision tree designed to assess the safety of enzymes used in food preparation (Pariza and Johnson, 2001) was applied to the AC1 Glucanase produced by Event FG259 and the results indicated that the AC1 Glucanase should be considered safe for food use (AGRN 31, §6.2.11 Evaluation of the Safety of the AC1 Glucanase, pg. 51). A bioinformatic assessment of the amino acid sequence of the AC1 Glucanase protein revealed that it does not share significant homology with known protein toxins. A tolerance study in which swine were grown for 42 days on diets containing 2126 U GraINzyme® AC1 Glucanase/kg of feed described by Lessard *et al.* (2021) demonstrates that the growth and weight gain of the treated swine was not different from that of the control group whose feed did not contain the AC1 Glucanase. Furthermore, the results of hematological analyses of the swine at the end of the study (42 days) confirms that there were no differences between the GraINzyme® AC1 Glucanase treated and control groups that would indicate that the high dose of GraINzyme® AC1 Glucanase in the treated group resulted in any negative safety issues. In the course of the necropsies performed on the swine from both the GraINzyme® AC1 Glucanase treated and control groups there were no indications of health or safety issues in tissues of the treated group that are considered to be related to the AC1 Glucanase in the treated feed. The results of studies conducted by Agrivida, Inc. described herein support a conclusion that the inclusion of GraINzyme® AC1 Glucanase in the feed of swine at up to 2126 U/kg is safe and effective and does not impede the growth or normal development of swine.

6.3 Enzyme Functionality in Swine

Feed grains such as rye, barley, oats and wheat contain high amounts of soluble NSP that result in increased viscosity of digesta in animals that consume feeds based on these grains. The increased viscosity of digesta in the GI tracts of monograstric animals results in reduced nutrient digestibility and availability and negative impacts on the gut microbiome (Burnett, 1966; Choct and Annison, 1992; Bedford and Classen, 1992; Danicke et al., 1999). Since the 1980s glucanase and other carbohydrase enzymes that degrade soluble NSP and reduce the viscosity of digesta in the GI tract have been added to the feed of monogastric animals to increase performance of animals fed diets based on grains with a high soluble NSP content (Hesselman and Åman, 1986; Campbell *et al.*, 1989; Broz and Frigg, 1986; Newman and Newman, 1987). The functionality of the AC1 Glucanase in swine has been

demonstrated to cause a reduction in the viscosity of the digesta of pigs in a swine feeding trial that was conducted at (b) (4) (Lessard *et al.*, 2021). The AC1 Glucanase was also shown to reduce the viscosity of feed that included wheat and dried distiller's grain solids (DDGS) in *in vitro* feed slurries (AGRN 31, §6.3.2 Reduction of the viscosity of feed, pg. 63). The positive effects on the performance of swine provide further support of the functionality of the AC1 Glucanase when it was included in the feed (Lessard *et al.*, 2021).

6.3.1 Functionality results from a swine study

A feeding trial (Lessard *et al.*, 2021) was conducted at (b) (4) and all animal experimental procedures were approved by the (b) (4). Sixty pigs (10.2 ± 1.3 kg) at 5 weeks of age were individually housed and blocked based on initial body weight and sex. Within each block, pigs were randomly allotted to 6 dietary treatments based on a 2 × 3 factorial arrangement. The first factor was distiller's dried grains with solubles (DDGS) inclusion (15 or 30%), and the second factor was AC1 Glucanase supplementation (0, 150, or 450 U/kg feed). Two different basal diets (99.1%) were mixed depending on the inclusion of DDGS, and then AC1 corn was added at the rates of 0%, 0.3%, and 0.9% premixed with normal corn to reach the target glucanase activities of 0, 150, and 450 U/kg feed in each dietary treatment, respectively. The diet composition was summarized in Table 7, and nutrient levels of all diets met the requirement suggested by NRC (NRC, 2012). Feed intake was recorded at weekly intervals during the trial.

The experimental period was 21 d. Pens (1.50 × 0.74 m) with slatted floors were equipped with 1 nipple drinker and 1 self-feeder. Pigs had free access to water and feed. On d 14, titanium dioxide (0.3%) was blended into experimental diets as an indigestible marker to measure apparent ileal digestibility (AID). On d 21, all pigs were euthanized via captive-bolt stunning. Digesta (40 mL) was collected from the ileum (30 cm of small intestine from the ileo-cecal junction) to measure viscosity immediately after euthanasia. Ileal digesta (100 mL) was collected and frozen at -20°C.

The method to measure digesta viscosity was described by Passos *et al.* (2015) with a viscometer (b) (4)). The digesta samples were centrifuged at 3,000 × g for 5 min and then the supernatant was pipetted out to a 2 mL tube and centrifuged at 12,500 × g for 5 min. The viscometer was set at 25°C, and 0.5 mL of digesta supernatant was placed into the viscometer. The final result was calculated as the average of viscosity at 45.0 sec^{-1} and 22.5 sec^{-1} shear rates.

Before analysis, ileal digesta was freeze-dried (24D x 48 (b) (4)). Feed and ileal digesta samples were ground and analyzed for dry matter (DM; AOAC Method 934.01). Titanium concentration was measured according to the previously described protocol (Myers *et al.*, 2004). The gross energy (GE) was determined

using a calorimeter ((b) (4) . Crude fat was measured using a modified ether extract method (AOAC Method 920.39). Nitrogen in the feed and digesta samples was measured using a (b) (4) N Nitrogen Determinator ((b) (4)) to calculate crude protein (CP; 6.25 × N). Samples of feed and ileal digesta were analyzed sequentially for neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the method of (Van Soest *et al.*, 1991) in a batch processor ((b) (4)). Apparent ileal digestibility of DM, GE, Crude fat, CP, NDF, and ADF were calculated as a function of the titanium concentration in the feed and digesta as follows:

$$AID, \% = \left(1 - \frac{Ti_{feed} \times Nutr_{digesta}}{Ti_{digesta} \times Nutr_{feed}}\right) \times 100\%,$$

where Ti_{feed} represents the titanium concentration in the feed, $Ti_{digesta}$ is the titanium concentration in the ileal digesta, $Nutr_{feed}$ represents the nutrient concentration in the feed, and $Nutr_{digesta}$ is the nutrient concentration in the ileal digesta.

No interactions were observed between DDGS and enzyme inclusion levels for viscosity of the digesta or AID of nutrients when pigs were fed corn-soy diets containing two different levels of DDGS and three different levels of AC1 (Table 8; $P > 0.10$). There was also no effect on viscosity associated with increasing DDGS inclusion from 15% to 30% (Table 8). However, supplemental AC1 resulted in a dose dependent decrease in the viscosity of distal jejunal digesta (Table 8). Increasing DDGS in the diet increased AID of crude fat ($P = 0.002$) and tended to decrease AID of NDF ($P = 0.075$) and ADF ($P = 0.054$). Supplemental AC1 tended to increase ($P = 0.076$) AID of NDF. There was no significant difference in feed intake across the treatments (data not shown), so enzyme consumption was a function of the inclusion rate in the diet.

Table 7. Composition of diets for swine feeding trial

DDGS, %		**15**			**30**	
Glucanase, U/kg	**0**	**150**	**450**	**0**	**150**	**450**
Ingredient, %						
Yellow corn	49.765	(b) (4)		37.955	(b) (4)	
Poultry fat	2.00			2.00		
DDGS	15.00			30.00		
SBM	23.00			20.00		
Whey permeate	5.00			5.00		
Blood plasma	2.00			2.00		
Supplement	0.00			0.00		
L-Lys HCl	0.43			0.45		
DL-Met	0.10			0.05		
L-Thr	0.08			0.05		
Salt	0.22			0.22		
Vitamin premix	0.03			0.03		
Mineral premix	0.15			0.15		
Dical Phosphate	0.55			0.32		
Limestone	1.30			1.40		
Zinc oxide	0.25			0.25		
Mecadox 10	0.125			0.125		
Total	100.00			100.00		
Composition[1]						
ME, kcal/kg	3386	(b) (4)		3394	(b) (4)	
Lys, %	1.24			1.24		
Met + Cys, %	0.7			0.7		
Trp, %	0.21			0.21		
Thr, %	0.73			0.73		
Ca, %	0.71			0.7		
Available P, %	0.33			0.33		
DM, %	88.98			88.88		
NDF, %	13.95			18.6		
ADF, %	3.94			5.26		
AC1 Activity (U/kg)	2			13		

[1]*ME, Ca, available P: calculated values; DM, NDF, ADF and AC1 b-1,4 -glucanase activity: analyzed values.*

Table 8. Viscosity of distal jejunal digesta (cP[1]) and apparent ileal of digestibility (AID) of DM, GE, crude fat, CP, NDF, and ADF in pigs supplemented with AC1 (based on units of b-1,4-glucanase activity) and distillers dried grains and solubles (DDGS), along with P-values to assess the effects of the DDGS inclusion, the enzyme inclusion (Enz) or the interaction of the two (DDGS × Enz).

DDGS[2], %	**15**			**30**			**SEM**	***P* value**		
Glucanase, U/kg	**0**	**150**	**450**	**0**	**150**	**450**		**DDGS**	**Enz**	**DDGS×Enz**
Viscosity, cP	2.64	(b)	(4)	2.57	(b)	(4)	0.17	0.233	0.092	0.527
AID, %										
DM	73.67			73.21			1.13	0.730	0.266	0.994
GE	78.48			79.79			0.97	0.710	0.288	0.651
Crude fat	91.37			93.86			0.58	0.002	0.490	0.442
CP	84.27			85.39			0.93	0.176	0.387	0.854
NDF	44.17			43.30			2.87	0.075	0.076	0.597
ADF	42.87			32.98			3.46	0.054	0.181	0.546

[1]cP=centipoise (1 cP=1/100 dyne s/cm^2)
[2]DDGS: distiller's dried grains with solubles

6.3.2 Conclusions on the Functionality of GraINzyme® AC1 Glucanase

Agrivida, Inc. has demonstrated that the inclusion of AC1 Glucanase in feed results in a reduction of viscosity of feed slurries in an *in vitro* enviornment (AGRN 31, §6.3.2 Reduction of the viscosity of feed, pg. 63). Additional support of the functionality of the AC1 Glucanse in swine feed comes from a study by Lessard *et al.* (2021) in which the inclusion of the AC1 Glucanase in swine feed was demonstrated to decrease the viscosity of ileal digesta in a dose dependent manner. The ability of the AC1 Glucanase to decrease the viscosity of the digesta in the GI tract of poultry when included in feeds that contain wheat and DDGS has been demonstrated (Ayres *et al.*, 2018), and therefore it is not surprising that it has the same functionality in swine feed. Taken together, these studies provide clear confirmation of the functionality of the AC1 GraINzyme® Glucanase in the feed of swine.

6.3.3 Safety of human consumption of meat produced by animals treated with GraINzyme® AC1 Glucanase

The meat derived from animals that consume feed treated with GraINzyme® AC1 Glucanase is safe for human consumption and does not present any human safety concerns. The GraINzyme® AC1 Glucanase is an enzyme and enzymes are proteins. The dietary fate of the GraINzyme® AC1 Glucanase in animals that consume feed treated with it is the same as that of all other proteins in the animal's diet that are digested into the constituent amino acids of the dietary proteins (Metcalf *et al.*, 1996; Betz *et al.*, 2000). As part of an Early Food Safety Evaluation for the GraINzyme® AC1 Glucanase that was submitted to the U.S. FDA Center for Food Safety and Nutrition, Agrivida, Inc. demonstrated that the GraINzyme® AC1 Glucanase enzyme is sensitive to digestion in a simulated gastric environment (Agrivida, 2018). Therefore, the GraINzyme® AC1 Glucanase is expected to be digested in the gastrointestinal tracts of animals and is not expected to be absorbed intact into the blood of animals that consume it, nor to be deposited into the tissues of the animals, including the meat. The safety of glucanase feed additives for humans that consume meat from animals that consume feed treated with glucanases is further supported by the fact that glucanases have been included in the feed of poultry and swine for decades without any adverse effects on human health or nutrition.

6.4 JECFA Specifications

Each of three representative AC1 Glucanase product batches were analyzed to demonstrate that they meet the purity, chemical and microbial specifications established for enzyme preparations, as outlined in the specifications established for enzymes used in food processing, as proposed by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2006). Physical, chemical, and microbial characteristics were determined for each of the AC1 Glucanase product batches by (b) (4) Three AC1 product batches were demonstrated to meet all JECFA specifications for food enzymes with the exception of number of coliforms and total bacteria. However, the product is within the range for coliforms and total bacteria that are known to be typical for maize grain that is produced by common agricultural practices and widely used in food and feed. The results of these analyses are presented in AGRN 31 (§6.4 JECFA Specifications, pg.66).

6.5 Stability and Homogeneity of the AC1 Glucanase Product

The stability of AC1 activity in three representative AC1 product batches over time and under different storage conditions was examined. The results demonstrate that AC1 Glucanase in feed mixtures retains its activity when stored for up to 12 weeks under refrigerated or ambient conditions. The stability of AC1 Glucanase in feed mixtures at different storage conditions was also assessed as described in AGRN 31 (§6.5.2 Stability of the AC1 Glucanase in feed mixtures, pg. 73).

A study on the homogeneity of the AC1 Glucanase product in standard corn/soybean meal based diets was conducted at (b) (4) and is reported by Ayres *et al.* (2018). A 1,225 kg batch of feed was mixed in a vertical mixer with 4.232 kg of AC1 Glucanase product that had an activity of 145 unit/g to make a diet with a target dose of 500 unit/kg diet. Ten 0.5 kg samples were collected randomly from the mixed feed and the β-glucanase activity was measured. The average glucanase activity of all samples was 425 unit/kg with a coefficient of variation (CV) of approximately 6.22%. A second study of the homogeneity of AC1 Glucanase product in poultry diets made with corn/soybean was conducted at (b) (4) and was reported by Jasek *et al.* (2018). AC1 Glucanase product with an activity of 170 U/g was mixed into 1500 lbs of feed to achieve a 100 U/kg target dose. Ten 500g samples were collected randomly from the mixed feed and were subjected to glucanase activity analysis. The average glucanase activity in the feed samples was 97.24 U/kg with a CV of approximately 9.98%. In summary, enzyme activity analysis of AC1 Glucanase product in two independent studies has demonstrated that AC1 Glucanase product is homogeneously distributed in typical corn/soybean meal based feeds (Ayres *et al.*, 2018 and Jasek *et al.*, 2018).

6.6 Stability of the AC1 Glucanase During Pelleting

The stability of the glucanase activity of the AC1 Glucanase product in feed mixtures during pelleting was investigated and reported by Ayres *et al.* (2018) and is described in the GRAS notice for use of the product in poultry (AGRN 31, §6.5.4 Stability of the AC1 Glucanase During Pelleting, pg. 76). In summary, the results of two independent pelleting stability studies with corn/soybean meal based feed treated with AC1 Glucanase have demonstrated the thermostability of AC1 Glucanase in high temperature pelleting processes. These studies demonstrated that AC1 Glucanase treated feed retains at least 80% activity after pelleting at 90°C.

6.7 GraINzyme® AC1 Glucanase Product Label

GraINzyme® AC1 Glucanase

Description: This product consists of corn meal produced from a genetically engineered variety of corn that produces AC1 Glucanase in the grain. AC1 Glucanase is a thermostable enzyme that improves the digestibility of feedstuffs through reducing the viscosity of the feed in the animal's digestive tract.

Lot Number:

Guaranteed Analysis: This product contains a minimum of β-glucanase activity of 150 units/gram grain. One unit is determined on β-glucan substrate at 80°C and pH 6.5.

Ingredients: Corn meal containing GraINzyme® AC1 Glucanase.

Directions for use:
1) In poultry: Add sufficient amount of GraINzyme® AC1 Glucanase per ton of complete feed to deliver (b) (4) U/kg of feed;

2) In swine: Add sufficient amount of GraINzyme® AC1 Glucanase per ton of complete feed to deliver (b) (4) U/kg of feed.

If pelleting feed do not exceed 90°C. Store dry at room temperature.

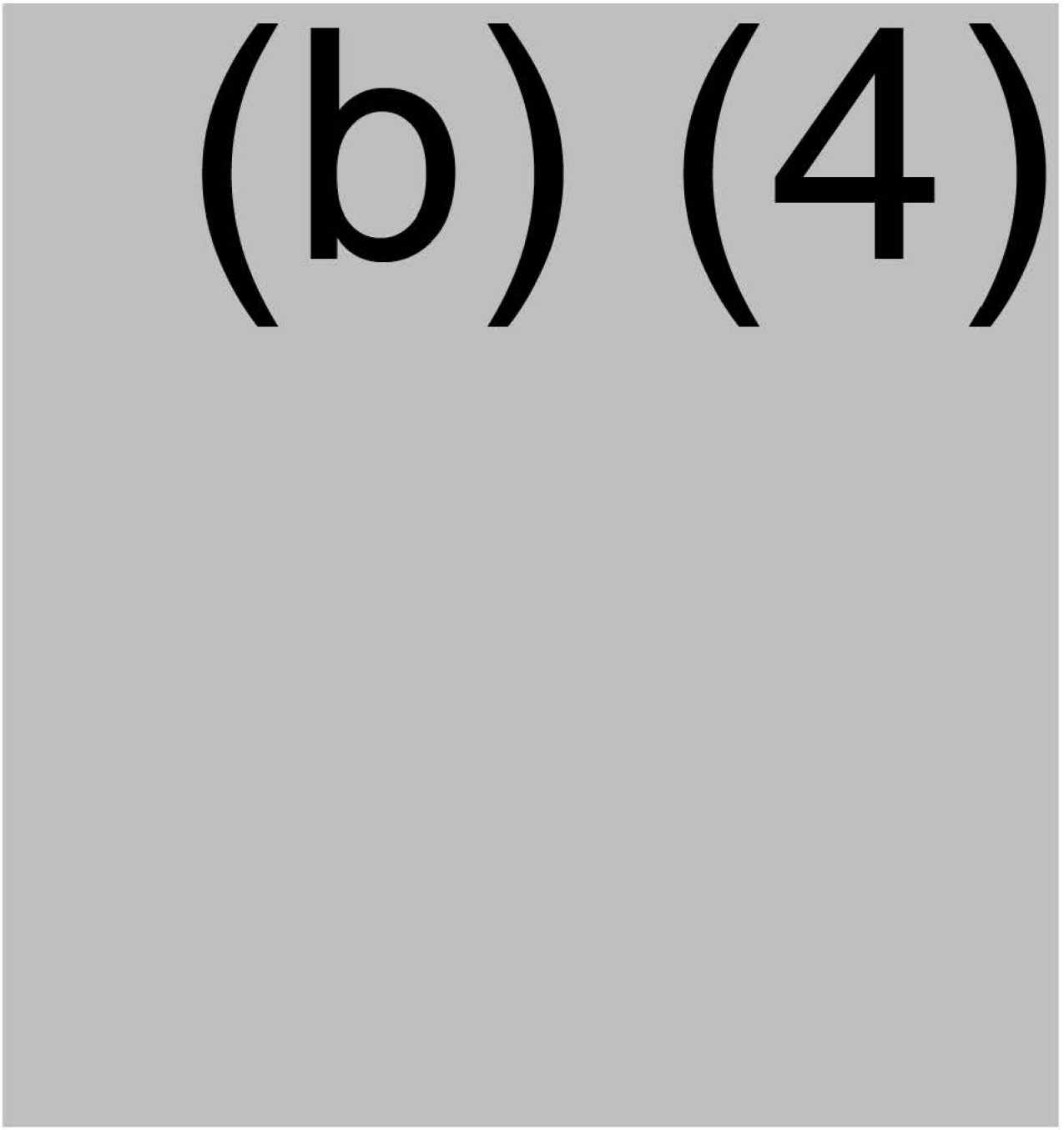
Expiration date: use within 24 months of the date of manufacture

Produced by: Agrivida, Inc., 78E Olympia Ave., Woburn, MA 01801, USA

Net weight: 50 lbs

See Material Safety Data Sheet for further information

6.8 Manufacturing Process



6.9 Data inconsistent with a conclusion of GRAS

In this GRAS notification, Agrivida, Inc. has presented all information in its possession and of which it is aware that is relevant and pertinent to its conclusion that the use of the GraINzyme® AC1 Glucanase in swine is GRAS. Agrivida, Inc. has no information nor is it aware of any information that is inconsistent with, or

contradicts, this conclusion of GRAS status for the use of GraINzyme® AC1 Glucanase in the feed of swine.

6.10 Summary Conclusions

The data and information described in §6.0 above that is the basis for the conclusion by Agrivida, Inc. that the GraINzyme® AC1 Glucanase product is GRAS for use in swine includes the following:

1. The GraINzyme® AC1 Glucanase is an enzyme and enzymes generally are known to be non-toxic. In all cases of proteins that are toxic, toxicity is derived from the biological mode of action of the protein. The biological mode of action of glucanases is generally recognized to be safe.

2. The history of safe use of glucanases as animal feed additives and in human nutritional supplements is well established and generally recognized. In addition, glucanase enzymes are present in many plant based foods that have been consumed safely by humans for millennia.

3. The safety of the AC1 Glucanase was thoroughly assessed in an Early Food Safety Evaluation conducted by Agrivida, Inc. for this product and this evaluation was reviewed by the U.S. FDA Center for Food Safety and Applied Nutrition. (Agrivida, 2018).

4. The safety of GraINzyme® AC1 Glucanase is supported by results of a tolerance study described in §6.2.5 in which weaned piglets were fed feed supplemented with 2126 U glucanase/kg feed without any indications of toxicity (Lessard *et al.*, 2021).

5. The application of a well used decision tree for evaluating the safety of enzymes used in food and feed (Pariza and Johnson, 2001) to the GraINzyme® AC1 Glucanase product indicates that this product is unlikely to be toxic (AGRN 31, §6.2.11 Evaluation of the Safety of the AC1 Glucanase, pg 51).

6. The results of swine feeding trials in which the GraINzyme® AC1 Glucanase was included in the feed at a range of doses and *in vitro* dietary viscosity assays support a conclusion that the GraINzyme® AC1 Glucanase product is safe and functional when included in swine feed at the doses described (Lessard *et al.*, 2021).

Based on the publicly and generally available information described above, Agrivida, Inc. believes that experts in the fields of animal nutrition, toxicology or related fields would agree with the conclusion of Agrivida, Inc. that the GraINzyme® AC1 Glucanase is safe and effective when included in the feed of swine in the dose ranges described herein.

7.0 References

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From:	Animalfood-premarket
To:	jim.ligon@agrivida.com
Cc:	Animalfood-premarket
Subject:	GRAS Notice AGRN #50
Date:	Thursday, March 17, 2022 1:41:14 PM
Attachments:	image009.png image010.png image011.png image012.png image013.png image014.png

RECEIVED DATE:
MARCH 30, 2022

Dr. Ligon,

In regards to Agrivida's, GRAS notice for ground grain obtained from a corn (Zea mays) variety that expresses an altered AC1 beta-glucanase gene obtained from an environmental DNA library (transformation event FG259) to increase digestibility of swine feeds containing soluble non-starch polysaccharides, designated as GRAS Notice No. AGRN 50. Which was filed on October 25, 2021. CVM is requesting an amendment to address molecular biology and utility questions. Below are the items that CVM is requesting be addressed.

Molecular Biology

1. On page 5 of the current notice, the notifier states "The plasmid that contains the T-DNA fragment that was used to transform maize contains an antibiotic resistance gene for maintenance in bacterial hosts. The antibiotic resistance gene was not transformed into the maize genome". However, based on the information included in AGRN 31 Table 2, page 25, our understanding is that the plasmid (b) (4) contains two antibiotic resistance genes (b) (4) (b) (4) Please clarify how many antibiotic resistance genes are present in the plasmid (b) (4) and confirm the absence of all antibiotic resistance genes in the maize genome.
2. On page 18 of the current notice, the notifier incorrectly cites "AGRN 31, §6.3.2 Characteristics of the ACl Glucanase gene expression construct, pg. 20" to reference the description of the genetic elements used in maize event FG259. The section number should be §6.1.4. Please correct or clarify this apparent error.

Utility

1. The intended use of the notified substance as stated in section 1.4 of the GRAS notice is "to increase the digestibility of feed containing soluble non-starch polysaccharides (NSP)." Additionally, reduction in digesta viscosity is also indicated elsewhere in the notice as a proposed intended use. The notifier should clarify this discrepancy and clearly state the intended use. The intended use should be consistently addressed in the notice because in accordance with the GRAS final rule, substances are not GRAS, but the intended use of the substance is what is evaluated to be GRAS by qualified experts.
2. The data provided by the notifier does not support the functionality of the notified substance in swine diets for either of the aforementioned intended uses. In the Lessard et al. (2021) publication cited by the notifier to support the intended use, there were no statistically significant differences ($p<0.05$) in reported parameters that would support the notified substance's functionality regardless if the intended use was to reduce digesta viscosity or increase the digestibility of feed containing soluble NSP. We note that the parameter (ileal digesta viscosity) selected, in the study reported in Lessard et al. (2021), to support reduction

in digesta viscosity claim is acceptable. However, lack of differences between the treated (enzyme supplemented diets) groups and the control group do not support this intended use. We note that the parameters selected, in the Lessard et al. (2021) study, to support increase in digestibility of feed containing soluble NSP claim are not adequate. The notifier should clarify the intended use of the notified substance and provide publicly available information to support such intended use along with clearly stated minimum amount of enzymatic activity/amount of feed required to achieve the intended use.

3. Alternatively, if the notifier concludes that no safety concerns are identified when the notified enzyme does not achieve its intended use, the notifier could provide an argument addressing why there are no adverse effects on the animal or its nutrition when the notified enzyme lacks functionality. The notifier's narrative would need to clearly explain how safety is not affected if the notified glucanase does not accomplish the stated intended use in swine diets, which should be supported by publicly available information and the scientific literature.

The notifier may provide an amendment to address the questions and comments in this email. The notifier should send this amendment to animalfood-premarket@fda.hhs.gov within the next two weeks, which is no later than April 1, 2022. Alternatively, the notifier may send a letter asking CVM to cease to evaluate the GRAS Notice. If no response is received, CVM will proceed with evaluation of the notice.

Sincerely,

Carissa Adams, MPH
Animal Scientist

Office of Surveillance and Compliance
Center for Veterinary Medicine
U.S. Food and Drug Administration
Tel: 240-402-6283
Personal e-mail address: carissa.adams@fda.hhs.gov
To schedule a meeting with DAF, please e-mail: animalfood-premarket@fda.hhs.gov

From: jim.ligon@agrivida.com
To: Animalfood-premarket
Subject: [EXTERNAL] Amendment to AGRN #50
Date: Wednesday, March 30, 2022 9:49:03 AM
Attachments: Amendment to AGRN50_30Mar22.docx

Dear Sir/Madam,

Agrivida, Inc. is submitting an amendment to AGRN #50 in response to issues identified by CVM in their review of this GRAS notice (email of 17 March 2022 from C. Adams). Please find the amendment in the attached document. If there are further issues that need to be addressed in relation to this notice, please do not hesitate to contact me.

Sincerely,

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
www.agrivida.com

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919-675-6666

1023 Christopher Drive
Chapel Hill, NC 27517

Amendments and Supplementary Information to GRAS Notice 50 for the Use of AC1 Glucanase in Swine Feed

The information herein is in response to issues raised by CVM (received March 17, 2022) in the review of a GRAS notice for the use of AC1 Glucanase in swine feed.

The comments from CVM are presented below along with responses from Agrivida, Inc.:

Molecular Biology

1. On page 5 of the current notice, the notifier states "The plasmid that contains the T-DNA fragment that was used to transform maize contains an antibiotic resistance gene for maintenance in bacterial hosts. The antibiotic resistance gene was not transformed into the maize genome". However, based on the information included in AGRN 31 Table 2, page 25, our understanding is that the plasmid (b) (4) contains two antibiotic resistance genes: (b) (4) Please clarify how many antibiotic resistance genes are present in the plasmid (b) (4) and confirm the absence of all antibiotic resistance genes in the maize genome.

Response: It is correct that the transformation plasmid (b) (4) that was used to transform maize and create Event FG259 contains two antibiotic resistance genes for maintenance in bacterial hosts. The two antibiotic resistance genes include the (b) (4) and (b) (4) genes as noted. Both antibiotic resistance genes are listed and described in Table 2 (pg. 23) of GRASN 31 that describes the genetic elements present in plasmid (b) (4). Therefore, the statement referenced on page 5 of the notice is incorrect. It should read as follows:

"The plasmid that contains the T-DNA fragment that was used to transform maize contains two antibiotic resistance genes for maintenance in bacterial hosts. The antibiotic resistance genes were not transformed into the maize genome".

The absence of both antibiotic resistance genes from the genome of maize Event FG259 was demonstrated by Southern hybridization using two different DNA fragments containing the coding sequences of the two antibiotic resistance genes as probes. This is described in detail in §6.1.5.2 (Screening for the absence of plasmid vector backbone fragments; pg. 32) of GRASN 31.

2. On page 18 of the current notice, the notifier incorrectly cites "AGRN 31, §6.3.2 Characteristics of the AC1 Glucanase gene expression construct, pg. 20" to reference the description of the genetic elements used in maize event FG259. The section number should be §6.1.4. Please correct or clarify this apparent error.

Response: As noted, the reference to §6.3.2 of GRASN 31 to describe the genetic characteristics of the AC1 Glucanase gene expression construct in maize Event FG259 is

incorrect. Also as noted, the correct reference for this information should be to §6.1.4 on pg. 20 of GRASN 31. Therefore, Agrivida, Inc. requests to replace the errant reference in the notice with the following sentence:

"A complete description of the genetic elements of the T-DNA fragment that was used to transform maize and other attributes of plasmid (b) (4) have been described in the GRAS notice for the use of the AC1 Glucanase product in poultry (AGRN 31, §6.1.4; Characteristics of the AC1 Glucanase gene expression construct, pg. 20)."

Utility

1. The intended use of the notified substance as stated in section 1.4 of the GRAS notice is "to increase the digestibility of feed containing soluble non-starch polysaccharides (NSP)." Additionally, reduction in digesta viscosity is also indicated elsewhere in the notice as a proposed intended use. The notifier should clarify this discrepancy and clearly state the intended use. The intended use should be consistently addressed in the notice because in accordance with the GRAS final rule, substances are not GRAS, but the intended use of the substance is what is evaluated to be GRAS by qualified experts.

Response: It is well established in the scientific literature that the inclusion of NSPs in animal feed results in higher viscosity of the digesta in the intestinal tract. The increased viscosity of the digesta in such feeds is a direct result of the soluble NSPs in the diet. As explained in the current GRAS notice (AGRN 50) and supported by citations therein, highly viscous digesta may lead to digestion issues and poor animal gut health and performance. Glucanase enzymes have been added to animal feeds for decades to help digest soluble NSPs in the diet that are the cause of viscous digesta. In the view of Agrivida, Inc., increased digestibility of NSPs from the addition of glucanase results in decreased digesta viscosity and so the two phenomena are essentially equivalent (*i.e.*, increased digestion of NSPs in the digesta results in decreased digesta viscosity). A similar situation is found in statements of intended use for phytase enzymes in other reviewed GRAS notices. For example, a common claim for phytase enzymes is that they are intended to increase the availability of phytin bound phosphorous in the feed. However, in order to demonstrate functionality in the GRAS process, the indicator of functionality proposed by CVM is an increase in bone ash or bone breaking strength, both of which are indicators of the increased availability of phosphorus. In order to clarify this point in the statement in question in section 1.4 of the GRAS notice, Agrivida, Inc. proposes to amend the statement as follows:

1.4 Conditions of use of the notified substance

This GRAS notice is for the purpose of establishing GRAS status for GraINzyme® AC1 Glucanase in swine feed to increase the digestibility of soluble NSP in feed thereby reducing the viscosity of intestinal digesta associated with feed containing soluble NSPs. The recommended inclusion rate of the GraINzyme® AC1 Glucanase in swine feed is (b) (4) glucanase activity units (U) per kg of feed.

2. The data provided by the notifier does not support the functionality of the notified substance in swine diets for either of the aforementioned intended uses. In the Lessard *et al.* (2021) publication cited by the notifier to support the intended use, there were no statistically significant differences ($p<0.05$) in reported parameters that would support the notified substance's functionality regardless if the intended use was to reduce digesta viscosity or increase the digestibility of feed containing soluble NSP. We note that the parameter (ileal digesta viscosity) selected, in the study reported in Lessard *et al.* (2021), to support reduction in digesta viscosity claim is acceptable. However, lack of differences between the treated (enzyme supplemented diets) groups and the control group do not support this intended use. We note that the parameters selected, in the Lessard *et al*. (2021) study, to support increase in digestibility of feed containing soluble NSP claim are not adequate. The notifier should clarify the intended use of the notified substance and provide publicly available information to support such intended use along with clearly stated minimum amount of enzymatic activity/amount of feed required to achieve the intended use.

Response: Representatives from Agrivida, Inc. and CVM met on September 13, 2016, at the CVM offices in College Park, MD to discuss requirements to achieve GRAS status of the AC1 Glucanase product. At that meeting the requirements to demonstrate functionality of a glucanase product were thoroughly discussed. CVM instructed Agrivida, Inc. that the requirements for demonstrating functionality of a glucanase enzyme were different from those of other enzymes (*e.g*., phytase) since diets formulated to contain glucanase enzymes are typically not otherwise changed or adjusted from normal, healthy diets and contain a complete range of nutrients to ensure healthy growth and development. Further, CVM informed Agrivida, Inc. that a single functionality trial demonstrating the desired activity in either *in vitro* feed slurries or in the intestinal tract of animals would be sufficient to support functionality of the enzyme. Further, Dr. Mika Alewynse noted that the data in functionality trials for glucanase enzymes do not need to be statistically significant between the dose treatments and that a numerical progression of the data in a dose dependent manner is sufficient to support functionality. Since demonstration of functionality of a glucanase in feed slurries *in vitro* is independent of animal species and since the functionality of the AC1 Glucanase in such an assay was demonstrated and described in the GRAS notice for the use of AC1 Glucanase in poultry (AGRN 31, §6.3.2 Reduction of the viscosity of feed, pg. 63; Ayres *et al*., 2018), Agrivida, Inc. proposes that no further studies to demonstrate functionality are required to support the use of AC1 Glucanase in swine feed. This is supported by the fact that the composition of the poultry feed used in the above referenced *in vitro* feed slurry study is not significantly different from a typical swine feed since both feeds are based primarily on a corn and soybean meal base diet. However, in order to provide further support for the functionality of the AC1 Glucanase, Agrivida, Inc. included in the current GRAS notice for swine the results of a study that examined the effect of the inclusion of AC1 Glucanase in feed on the viscosity of jejunal digesta (Lessard *et al*., 2021). The results from this study that are presented in §6.3.1 (pg. 28) of the current notice demonstrate a numerical decrease in the viscosity of jejunal digesta in swine consuming feeds containing 15 and 30% DDGS. Furthermore, the

viscosity in the different dose treatments decreases in a dose dependent manner and all AC1 treatment levels reduced the viscosity compared to the control group. The data in both the *in vitro* feed slurry study referenced from AGRN 31 and the swine feeding study described in the current GRAS notice are not different at statistically significant levels ($p<0.05$). However, both studies showed numerical, dose dependent reductions of viscosity relative to the control groups receiving no glucanase and according to the guidance provided to Agrivida, Inc. at the meeting with CVM, this data is sufficient to demonstrate functionality of the AC1 Glucanase in swine.

In section 1.4 of the GRAS notice, Agrivida, Inc. states that in order to achieve the intended effect, the recommended inclusion rate of the AC1 Glucanase in swine feed is (b) (4) glucanase activity units (U) per kg of feed. The efficacy of the product when included in swine feed at the lower limit of this dose range ((b) (4) feed) is supported by the study described in AGRN 31 in which AC1 Glucanase at this dose demonstrated a clear reduction in the viscosity of a feed slurry *in vitro* that contained 10% wheat and 10% DDGS. Since CVM has informed Agrivida, Inc. that the demonstration of the reduction of viscosity of feed slurries *in vitro* is acceptable to support the functionality of a glucanase product and since such assays are species independent, this study provides support for the functionality of the AC1 Glucanase at a dose of (b) (4) feed in swine.

3. Alternatively, if the notifier concludes that no safety concerns are identified when the notified enzyme does not achieve its intended use, the notifier could provide an argument addressing why there are no adverse effects on the animal or its nutrition when the notified enzyme lacks functionality. The notifier's narrative would need to clearly explain how safety is not affected if the notified glucanase does not accomplish the stated intended use in swine diets, which should be supported by publicly available information and the scientific literature.

Response: Glucanase enzymes are typically added to poultry and swine feeds without any other amendment to the typical diet formulation. This is unlike the use of phytase enzymes in which the addition of phytase to feeds is combined with the reduction of inorganic phosphorus in the diet since the phytase activity is expected to make phytin bound phosphorus nutritionally available for the animals and replace the amount of inorganic phosphorus that was reduced. In this case, if the phytase fails to be functional, the animals may suffer from insufficient available phosphorus in the diet. However, in the case of glucanase enzymes, since the composition of the feed is not changed due to the addition of glucanase, if the glucanase is not functional, the animal still receives a normal, healthy diet that will support healthy growth and development. In the meeting between Agrivida, Inc. and CVM noted above, CVM cited this rationale to support the requirement for less rigorous data to demonstrate functionality of a glucanase enzyme compared to a phytase. In the current GRAS notice, Agrivida, Inc. presents data from a tolerance study in section 6.2.5 (pg. 21) that demonstrates that high doses of the AC1 Glucanase added to swine diets do not result in any indications of toxicity or abnormal growth and development. This study supports a conclusion that the AC1 Glucanase is safe and nontoxic if added to swine feed. In the unexpected event that the AC1 Glucanase fails to function in the feed, for the reasons

cited above, the swine diets will still be nutritious and support healthy growth and development.