Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000811	Aug 27, 2018
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

RECEIVED
AUG 27 2018
OFFICE OF FOOD ADDITIVE SAFETY

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Janet Oesterling	Regulatory Affairs

Company *(if applicable)*: Novozymes NA

Mailing Address *(number and street)*: 77 Perrys Chapel Church Rd

City	State or Province	Zip Code/Postal Code	Country
Franklinton	North Carolina	27525	United States of America

Telephone Number	Fax Number	E-Mail Address
2529151444		jao@novozymes.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position

Company *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Phospholipase A1 enzyme produced by Aspergillus oryzae

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Paper

☐ Electronic files on physical media with paper signature page

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

The active enzyme is phospholipase A1 (EC 3.1.1.32). Phospholipases specifically act on the fatty acid in position 1 in phospholipid substrates. It catalyzes the hydrolysis of sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and free fatty acid.

Phospholipases are used as processing aids during baked goods manufacturing. During the baked goods manufacturing process, flour lipids (including polar lipids such as lysophospholipids, phospholipids and galacto-lipids) provide functional properties in dough. The maximum intended use is 20 PLA(B) per kg of flour.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	phospholipase A1	EC	3.1.1.32		
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

Stystemic Name: Phosphatidylcholine 1-acylhydrolas, kDa: 31. Liquid formula: water, sorbitol, glycerol, sucrose, sodium benzoate and potassium sorbate. Granulate formula: wheat flour.
Phospholipase A1 is produced by a genetically modified Aspergillus oryzae.

3. Synonyms

Provide as available or relevant:

1	Phosphatidylcholine 1-acylhydrolase
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☐ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Novozymes North America *(name of notifier)*

has concluded that the intended use*(s)* of Phospholipase A1 enzyme produced by Aspergillus oryzae *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Novozymes North America *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Novozymes North America *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

77 Perrys Chapel Church Rd, Franklinton, NC 27525 *(address of notifier or other location)*

Novozymes North America *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
janet oesterling Digitally signed by janet oesterling Date: 2018.08.23 16:35:25 -04'00'	Janet Oesterling, Regulatory Affairs	08/23/2018

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	DecisionTree_Phospholipase A1_2018-08-22.pdf	Administrative
	GRASNotification_Phospholipase A1_180823.pdf	Submission
	Part 1_Phospholipase A1_2018-08-23.pdf	Submission
	Sewalt etal_GRAS Process for Industrial Microbial enzymes.pdf	Administrative
	SummaryofToxicityData_Asparaginase_2014-08-14.pdf	Administrative

PART 1: Signed statement of the conclusion of GRAS (Generally Recognized as Safe) and certification of conformity to 21 CFR §170.205-170.260.

§170.225(c)(1) – Submission of GRAS notice:

Novozymes North America Inc. is hereby submitting a GRAS (Generally Recognized as Safe) notice in accordance with subpart E of part 170.

§170.225(c)(2) - The name and address of the notifier:

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

§170.225(c)(3) – Appropriately descriptive term:

The appropriately descriptive term for this notified substance is: Phospholipase A1 enzyme produced by a genetically modified strain of *Aspergillus oryzae.*

§170.225(b) – Trade secret or confidential:

This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:

The phospholipase enzyme will be used as a processing aid during the manufacturing of baked goods. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices. The “general” population is the target population for consumption.

§170.225(c)(5) - Statutory basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures.

§170.225(c)(6) – Premarket approval:

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

§170.225(c)(7) – Availability of information:

This notification package provides a summary of the information which supports our GRAS conclusion of the notified substance. Complete data and information that are the basis for this GRAS conclusion is available to the Food and Drug Administration for review and copying during customary business hours at Novozymes North America, Inc. or will be sent to FDA upon request.

§170.225(c)(8) - FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Novozymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

(b) (6)

08/23/18

Janet Oesterling
Regulatory Affairs Specialist III

Date

A Phospholipase A1 preparation produced by a genetically modified strain of *Aspergillus oryzae*

Janet Oesterling, Regulatory Affairs, Novozymes North America, Inc., USA

August 2018

PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a Phospholipase A1 enzyme (hereby known as phospholipase) produced by submerged fermentation of a genetically modified of *Aspergillus oryzae* microorganism carrying the gene coding for phospholipase from *Valsaria rubricosa.*

Key enzyme and protein chemical characteristics of the phospholipase are given below:

Systemic Name:	Phosphatidylcholine 1-acylhydrolase
Accepted Name:	Phosphatidylcholine 1-acylhydrolase
EC No.:	3.1.1.32
CAS No.:	9043-29-2
kDA:	31
Specificity:	hydrolyses the fatty acyl ester bond at the sn-1 position of the glycerol moiety resulting in the formation of 2-acyl-1-lysophospholipids and free fatty acids.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Aspergillus oryzae* production strain, AT969, was derived from a recipient strain descending from Jal731, through a series of modifications. The parental strain is a natural isolate of *Aspergillus oryzae* strain A1560.

This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large-Scale Practice) microorganisms (1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (3) and several expert groups (4) (5) (1) (6) (7) (8).

The expression plasmid, used in the strain construction, pAT652, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *lip182* sequence encoding phospholipase A1 from *Valsaria rubricosa*.

2.2(b) Recipient Strain

The recipient strain used in the construction of the *Aspergillus oryzae* production strain was modified at several chromosomal loci during strain development to inactivate genes encoding several amylases and proteases. Also, the strain has impaired kojic acid production. Furthermore, the aflatoxin gene cluster and a region including a gene involved in cyclopiazonic acid biosynthesis were deleted. The lack of the above compounds and proteins represents improvements in the product purity, safety and stability.

2.2(c) Phospholipase Expression Plasmid

The expression plasmid used to introduce the *lip182* gene in the recipient strain is based on the replication origin of *E. coli*. The plasmid contains the expression cassette consisting of an *A. niger* promotor, the *lip182* sequence encoding the phospholipase, a transcriptional terminator from *A. niger* and two selective markers, *pyrG (*orotidine 5'-phosphate carboxylase) and *niaD* (nitrate reductase). Integration restores the native *niaD* gene.

2.2(d) Construction of the Recombinant Microorganism

The resulting phospholipase production strain containing multiple copies of the *lip182* gene in one target locus was named AT969.

The insertion of the expression cassettes in the target locus of the production strain was confirmed by Southern blot and PCR analysis followed by DNA sequencing.

2.2(e) Stability of the Introduced Genetic Sequences

The transforming DNA is stably integrated into the *Aspergillus oryzae* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable. The genetic stability of the introduced DNA sequences was determined by Southern blot hybridization.

2.2(f) Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by genome sequence analysis.

2.2(g) Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore

the first step in the safety assessment as described by the International Food Biotechnology Council (IFBC) is satisfactorily addressed (4).

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the phospholipase which follows standard industry practices (9) (10) (11). The enzyme preparation is also produced in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The quality management system used in the manufacturing process for the phospholipase enzyme preparation complies with the requirements of ISO 9001. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the enzyme concentrate are standard ingredients used in the enzyme industry (11) (10) (9). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes, the raw materials are reviewed by the Quality Control Department to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams and/or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The phospholipase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Aspergillus oryzae* as described in Part 2. During fermentation, the enzyme produced by *Aspergillus oryzae* is secreted into the fermentation media. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Aspergillus oryzae,* described in this Part. Each new batch of

the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48-hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1) Contamination is observed in 2 or more samples by microscopy

2) Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

2.3(f) Purification Process

Enzymes are recovered from the culture broth by the following series of operations:

1) Pretreatment - pH adjustment and flocculation

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5) Final concentration – evaporation and/or ultrafiltration.

6) Preservation and Stabilization of the liquid enzyme concentrate

For liquid products, the enzyme concentrate is stabilized and standardized with sucrose, sorbitol or glycerol and preserved with potassium sorbate and sodium benzoate. For granulate formulations, after the final concentration of the liquid, the unstandardized concentrate is spray dried which results in a highly concentrated granulate and further formulated by the addition of wheat flour. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

The final products are analyzed according to the specifications given below.

2.4(a) Quantitative Composition

The phospholipase enzyme preparation is sold in both a liquid and granulate form. Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. The enzyme preparation, the subject of this notification, does not contain any major food allergens from the fermentation media.

Table 1. Typical composition raw materials of the enzyme preparations

Substance	Approximate Percentage
Enzyme (TOS*)	11%
Liquid Formulation	
Water	45 – 50%
Sorbitol	20 – 30%
Glycerol	20 – 30%
Sucrose	20-30%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%
Granulate Formulation	
Wheat Flour	<80%

*Total Organic Solids, define as: 100% - water – ash – diluents.

2.4(b) Specifications

The phospholipase enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (14). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (13).

This is demonstrated by analytical test results of three representative enzyme batches. See Table 2 below.

Table 2. Analytical data for three food enzyme batches.

Parameter	Specification	PPW51703	PPW51949	PPW52251
Activity unit	PLA(B)/g	386	337	417
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Total Coliforms	Not more than 30/g	<4	<4	<4

Salmonella	Absent in 25g	ND	ND	ND
Escherichia coli	Absent in 25g	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Production Organism	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action and Application

The active enzyme is phospholipase A1 (EC 3.1.1.32). Phospholipases specifically act on the fatty acid in position 1 in phospholipid substrates. It catalyzes the hydrolysis of sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and free fatty acid.

Phospholipases are used as processing aids during baked goods manufacturing. During the baked goods manufacturing process, flour lipids (including polar lipids such as lysophospholipids, phospholipids and galacto-lipids) provide functional properties in dough.

Phospholipases hydrolyze lipids, thereby improving their functional properties by improving their water solubility and enhancing their emulsifying properties (15). This results in an increased dough strength, uniform and improved crumb structure of the bakery product.

2.5(b) Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

The dosage applied in practice by a food manufacturer depends on the particular process. It is based on an initial recommendation by the enzyme manufacturer and optimised to fit the process conditions.

The maximum recommended use level is 20 PLA(B) per kg of flour.

2.5(c) Enzymes Residues in the Final Food

During the baking process, the high temperatures of the oven cause the inactivation of the enzyme activity.

Consequently, it can be concluded that the phospholipase does not exert any technological function in the final foods.

PART 3 - DIETARY EXPOSURE

This Part provides a “worst case” scenario for the calculation of the possible daily human dietary exposure. The enzyme preparation is used as a processing aid and at very low dosages.

The general population is the target population for consumption. There is no specific subpopulation.

3(a) Assumptions in Dietary Exposure

It is assumed that the enzyme product is used in the production of all processed foods and always at the maximum recommended dosage in the production of baked products.

The assumptions are highly exaggerated since not all processed foods are produced with the enzyme nor at the maximum recommended dosage. Overall, the human exposure to the enzyme will be negligible.

3(b) Food Consumption Data

The exposure assessment for adults was performed using the Budget Method (16) (17). The Budget Method assumptions represent a "maximum worst case" scenario of human consumption.

Assumptions in the Budget Method

To demonstrate a worst-case calculation, an exaggerated human intake is estimated using the following assumptions.

a) According to the Budget method, a conservative estimate for the food intake is 25 g per kg body weight per day, of which processed food is 50% of the food intake or 12.5 g processed food per kg body weight per day.
b) It is assumed that all processed food is produced using phospholipase as a processing aid and all processed food is *bread.* Thus, 12.5 g bread.
c) The calculation is made assuming that all enzyme TOS remains in the final product.

The maximum recommended dosage is 20 PLA(B) per kg of flour.

This equates to: 5.8 mg TOS per kg flour.

1 kg of flour will result in 1.4 kg of bread

12.5 g bread corresponds to 8.9 g flour [(1 ÷ 1.4) x 12.5]

Using the Budget Method assumptions:

5.8 mg TOS per kg flour = 0.0058 mg TOS per gram flour

8.9 g of flour x 0.0058 mg TOS = 0.052 mg TOS per kg body weight per day.

Theoretical Maximum Daily Intake (TMDI)

The margin of safety is calculated as dose level with no adverse effect (NOAEL) obtained from the 90-day oral (gavage) study in rats divided by the estimated human exposure. See Table 3 below.

The NOAEL chosen for the calculation of the margin of safety for phospholipase produced by *Aspergillus oryzae* (the subject of this notification) is taken from the 90-day oral (gavage) study on the enzyme concentrate produced by a closely related *Aspergillus oryzae*, which is the subject of the safety studies in Part 7.

The 90-day oral (gavage) study in rats, concluded a NOAEL of 1182 mg/TOS/kg bw/day.

As is required by the FDA, the margin of safety is greater than 100 suggesting that the toxicology data support the intended uses and application doses (18) (19).

Table 3. Margin of Safety Calculation

NOAEL (mg TOS/kg bw/day)	1182
*TMDI (mg TOS/kg bw/day)	0.052
Safety margin	22730

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following sections is the basis for our determination of the general recognition of safety for the phospholipase enzyme preparation. The evaluation follows the generally recognized methodology by Pariza and Johnson, includes both published and unpublished safety information, the common knowledge element and the publication outlining the GRAS process for microbial enzyme by Sewalt, et.al. (3) (20). Our safety evaluation in Part 6 includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6(a) Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (2) (3). The production organism for the phospholipase, *Aspergillus oryzae,* is discussed in Part 2 and in this Part.

If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (4). Pariza and Foster (2) define a non-toxigenic organism as “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a non-pathogenic organism as “one that is very unlikely to produce disease under ordinary circumstances”.

Aspergillus oryzae has been used for decades in the food industry. It is widely distributed in nature, commonly used for production of food grade enzymes (21) and is accepted as a constituent of foods (22).

Enzymes produced by *Aspergillus oryzae,* have been used for many years in the manufacture of Koji and Miso. Furthermore, enzymes from *Aspergillus oryzae* have been used extensively for decades in the Western part of the world in the production of a variety of foods (including syrups, alcohol, fruit juices, meat tendering, brewing and baking products) (23).

Aspergillus oryzae has been used to produce soy sauce in the United States since before 1958. Therefore, *Aspergillus oryzae* meets the criteria for “common use in foods in the US before 1958” and can be considered “generally recognized as safe” (GRAS) (4). Carbohydrase, protease, and lipase enzyme preparations from *Aspergillus oryzae* are included in GRAS petition 3G0016. This petition was converted in to separate Notices by the FDA, on request from the Enzyme Technical Association (ETA) (24) (25).

Since that time, several enzyme preparations from *Aspergillus oryzae,* have been reviewed by the FDA and are listed in the FDA's GRAS registry as having "no questions" regarding the GRAS assessment of that enzyme preparation (24).

An evaluation of the genetically modified production microorganism for the phospholipase, embodying the concepts initially outlined by Pariza and Foster (2) and further developed by IFBC (4), the EU SCF (5), the OECD (1), ILSI Europe Novel Food Task Force (8), FAO/WHO (7), JECFA (13), Pariza and Johnson (3) demonstrates the safety of this genetically modified production organism. The components of this evaluation: the identity of the host strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from this strain, are given in Parts 2 and 3.

The genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances.

Furthermore, the aflatoxin gene cluster and the gene involved in cyclopiazonic acid biosynthesis were deleted. Thereby, eliminating the potential for the production strain to produce unwanted secondary metabolites.

Novozymes' used the decision tree (Appendix 1) suggested in Pariza and Johnson as a basis for our safety assessment (3) . The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed phospholipase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, have been met (3).

Overall, it can be concluded that *Aspergillus oryzae* is widely accepted as a non-pathogenic organism and has a long history of safe use in food and food enzyme production.

6.1(a) Safe Strain Lineage

Aspergillus oryzae has been used for decades in the food industry. Strains within the safe strain lineage below are used to manufacture many food and feed enzymes. The enzyme products from the production strains within the *Aspergillus oryzae* lineage have been subjected to toxicology testing and through safety evaluations as

recommended in the Pariza and Johnson decision tree and following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4).

In accordance with this published criteria, the recipient strain, has been thoroughly characterized, the introduced DNA is well-known and characterized and the introduced genetic material does not encode or express any known harmful or toxic substances, as shown in Part 2.

As is mentioned in Part 2, the *Aspergillus oryzae* production strain, AT969, was derived from a recipient strain descending from Jal731, through a series of modifications. The parental strain is a natural isolate of *Aspergillus oryzae* strain A1560.

This lineage was used in the construction of several Novozymes' production strains for food enzymes which are subjects of GRAS notices outlined in Table 4 below all with the FDA concurring "No Questions".

Table 4. Safe Strain Lineage from *Aspergillus oryzae* strains

Enzyme	IUBMB no	Predecessor strain[1]	Donor strain	Safety studies[2]
Triacylglycerol lipase (GRN No. 43)	3.1.1.3	*Aspergillus oryzae* (A1560)	*Humicola (now Thermomyces) lanuginosa*	Yes
Mucorpepsin (GRN No. 34)	3.4.23.23	*Aspergillus oryzae* (A1560)	*Rhizomucor miehei*	Yes
Triacylglycerol lipase (GRASP 7G0323)	3.1.1.3	*Aspergillus oryzae* (A1560)	*Rhizomucor miehei*	Yes
Triacylglycerol lipase (GRN No. 75)	3.1.1.3	*Aspergillus oryzae* (JaL 228)	*Fusarium oxysporum*	Yes
6-phytase	3.1.1.26	*Aspergillus oryzae* (JaL 228)	*Peniophora lycii*	Yes
Xylanase	3.2.1.8	*Aspergillus oryzae* (BECh2)	*Thermomyces lanuginosus*	Yes
Glucose oxidase (GRN No. 106)	1.1.3.4	*Aspergillus oryzae (*BECh2)	*Aspergillus niger*	Yes
Triacylglycerol lipase (GRN No. 103)	3.1.1.3	*Aspergillus oryzae* (BECh2)	*Thermomyces lanuginosus/ Fusarium oxysporum*	Yes
Phospholipase (GRN No. 142)	3.1.1.32	*Aspergillus oryzae* (BECh2)	*Fusarium venenatum*	Yes
Xylanase	3.2.1.8	*Aspergillus oryzae* (BECh2)	*Aspergillus oryzae*	Yes

Asparaginase (GRN No. 201	3.5.1.1	*Aspergillus oryzae* (BECh2)	*Aspergillus oryzae*	Yes
Triacylglycerol lipase	3.1.1.3	*Aspergillus oryzae* (JaL731)	*Thermomyces lanuginosus*	Yes
Asparaginase	3.5.1.1	*Aspergillus oryzae* (JaL731)	*Aspergillus oryzae*	Yes

Table 4. Novozymes products derived from *A. oryzae* strains. [1]The predecessor strain shows strains in the GM construction pathway that are in common with the recipient strain lineage. [2]At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13-week sub chronic oral toxicity study in rats

Novozymes has both published and unpublished toxicology studies that support the conclusion that the lineage to which these safety studies belong, is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Based on the evidence above, Novozymes believes that the elements needed to establish a safe strain lineage, as defined in Pariza and Johnson, have been met (3).

6(b) Safety of the Donor Organism

The donor organism of the phospholipase is *Valsaria rubricosa* . As indicated in Part 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the phospholipase coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

6(c) Safety of the Phospholipase Enzyme

A wide variety of enzymes are used in food processing (3). Phospholipases are classified as hydrolases which are a group of enzymes that hydrolyze various bonds. Phospholipases, like other hydrolases used in food processing, breakdown substrates into smaller units that are readily metabolized by the human body. As noted above in Part 4, phospholipases hydrolyze the fatty acyl ester bond at the sn-1 position of the glycerol moiety resulting in the formation of 2-acyl-1-lysophospholipids and free fatty acids.

Phospholipases are found in animal and plant tissue and in many cells and tissues of various organisms, including animal pancreas and small intestines. They are also part of the digestive enzymes present in pancreatic juice of mammals including humans. Animal derived phospholipase have been safely used to manufacture enzyme modified lecithin (26) (27) (28) (29) (30) (31).

FDA's acceptance of phospholipases as common to man is because phospholipase activity is found in most cells and tissues, including animal tissues that are consumed by man. It is concluded that phospholipases would be digested like any other protein in food (31).

Novozymes has several commercial lipases that exhibit this phospholipase activity under certain conditions. Two current enzyme preparations are; Phospholipase A1 produced by a genetically modified strain of *Aspergillus niger* carrying the gene coding for phospholipase from *Talaromyces leycettanus* (GRN 651) and Phospholipase A1 produced by *Aspergillus oryzae* expressing the gene encoding a phospholipase from *Fusarium venenatum* (GRN 142).

A literature search was performed in July 2018 on phospholipase A1 utilizing the database *Web of Science* and *Google Scholar* and the keywords “phospholipase A1”, “toxicity”, “human consumption” and “safety”. Novozymes reviewed the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety for the phospholipase A1 enzyme.

Based on the toxicological data provided in these Notices and the fact that the production strain has a history of safe use as indicated above, it is our conclusion that the phospholipase is safe for its intended use as a processing aid in bakery type applications.

6(d) Allergenic/Toxigenic Potential of the Phospholipase Enzyme

The ingestion of a food enzyme protein is not considered a concern for food allergy. This is based on the following considerations:

1) Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

2) The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

3) Enzymes in foods are added in concentrations in the low range of part per millions. The enzyme is typically removed or denatured during food processing, and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (32).

To further evaluate the possibility that the phospholipase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by

FAO/WHO, 2001 (33) and modified by Codex Alimentarius Commission, 2009 (14) the phospholipase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a gap penalty was done. Also, an alignment of the phospholipase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed.

The analyses identified one fungal (Sch c 1) allergen, having an identity with phospholipase above the threshold of 35% across an 80-amino acid window.

Sch c 1 is not registered as a food allergen (**http://www.allergen.org**). Further, an additional screen of the current literature did not find any evidence that Sch c 1 can trigger oral sensitization.

In addition, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food, performed an in-depth analysis of the allergenicity of enzyme products. In this paper, Dauvrin and colleagues conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization. There is compelling evidence that persons affected by occupational asthma can ingest the respiratory allergen without acquiring clinical symptoms of food allergy, suggesting that inhalation is not likely to result in food allergy. Only one single case has been reported in the literature and this case was not verified as a bona fide oral sensitization to enzymes in food (34).

This is backed up by the study conducted by Bindslev-Jensen et al (32) using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with a diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes could be found. Further, there were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens causing clinical symptoms in the patients included in the study. Also, a search for 100% identity over 8 contiguous amino acids was completed. No homology was found.

Additionally, a search for homology of the phospholipase sequence to known toxins was assessed based on the information present in the UNIPROT database (04-25-2018). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 18%, indicating that the homology to any toxin sequence in this database is low and random.

Based on the available evidence and supporting scientific literature, it is concluded that oral intake of phospholipase produced by the *Aspergillus oryzae* strain is not anticipated to pose any food allergenic concern.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the phospholipase, which follows standard industry practices (11) (9) (10). The quality management system used in the manufacturing process for the phospholipase complies with the requirements of ISO 9001. It is manufactured in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

6(f) Safety Studies

Novozymes considers the phospholipase enzyme preparation, produced by the *Aspergillus oryzae* production organism, to be safe. This was determined through scientific procedure and is based on a review of the toxicological studies conducted on strains within Novozymes' *Aspergillus oryzae* safe strain lineage.

The toxicological studies include genotoxicity, cytotoxicity and general toxicity activities. Table 4, shown above, list the strains within the lineage where safety studies have been performed

Due to the consistent past findings of the toxicology studies supporting the safety of the enzymes produced by *Aspergillus oryzae* strains within the safe strain lineage, it is reasonable to expect and conclude that enzymes produced by *Aspergillus oryzae* strains within the safe strain lineage will show similar toxicological profiles.

The results from these studies indicate that the test enzyme concentrates did not exhibit any toxic or mutagenic effects under the conditions of the test. Concluding, that strains within the Novozymes' *Aspergillus oryzae* safe strain lineage (see Table 4) can be safely used as production organisms for food enzymes.

6.1(f) Description of the Test Material

As mentioned, Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate the enzymes derived from *Aspergillus oryzae* production strains (3). As described above, Novozymes has concluded, through scientific procedure, that strains within the safe strain lineage of *Aspergillus oryzae* pose no safety concerns. Repeated toxicology studies conducted on the enzymes produced

by the *Aspergillus oryzae* production strains support the safety of these enzymes produced by this strain.

Based on strain lineage, the phospholipase production strain is closely related to the *Aspergillus oryzae* strain producing asparaginase. The toxicology studies conducted on the asparaginase can be extrapolated to phospholipase from *Aspergillus oryzae*, the subject of this notification. This approach is in line with the Safe Strain Lineage concept as outlined by Pariza and Johnson (3).

All toxicology studies produced for the asparaginase from *Aspergillus oryzae* were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Novozymes A/S (Denmark), Huntingdon Life Sciences (UK) and Covance Laboratories Ltd. (UK) during the period November 2013 to July 2014.

A Summary of the toxicology studies for the asparaginase enzyme produced by *Aspergillus oryzae* is included as Appendix 2.

Based on the presented toxicity data, the history of safe use and the safe strain lineage of the production strain, it can be concluded that the test preparation exhibits no toxicological effects under the experimental conditions described in the Summary.

6(g) Results and Conclusion

Results of the toxicity and mutagenicity tests described in Appendix 2 showed no toxicity or mutagenicity of the asparaginase produced by *Aspergillus oryzae*, batch PPV35776.

A critical review and evaluation of the phospholipase enzyme preparation (subject of this notification) and the asparaginase enzyme preparation (subject of the test material) was done following the concepts of the Pariza papers (2) (3) and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (20).

Based on the available scientific data provided in this notification, the known history of safe use of the production organism and the limited and well-defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; phospholipase A1 enzyme preparation, is generally recognized, among qualified experts, to be safe under the conditions of its intended use.

PART 7 – SUPPORTING DATA AND INFORMATION

All information indicated in the List of Appendices and References is generally available

APPENDICES

1. Pariza and Johnson Decision Tree Analysis

2. Summary of Toxicity Data, Asparaginase from *Aspergillus oryzae*, batch PPV35776. August 2014, 2002-03563-01.

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Appendix 3- This phospholipase A1 enzyme preparation produced by *Aspergillus oryzae* was evaluated according to the decision tree published in Pariza and Johnson, 2001 [(1)].

The result of the evaluation is presented below.

Decision Tree

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
      YES, *go to 3c.*

   c. Is the test article free of transferable antibiotic resistance gene DNA?
      YES, go to 3e.

   e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?
      YES*, go to 4*.

4. Is the introduced DNA randomly integrated into the chromosome?
   NO*, go to 6*.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   YES. ***If yes the test article is ACCEPTED.***

LIST OF REFERENCES

1. Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.

Toxicology & Product Safety

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SUMMARY OF TOXICITY DATA

Asparaginase, batch PPV35776 from *Aspergillus oryzae*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Asparaginase, batch PPV35776.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Novozymes A/S (Denmark), Huntingdon Life Sciences (UK) and Covance Laboratories Ltd. (UK) during the period November 2013 to July 2014.

The main conclusions of the studies can be summarized as follows:

- Asparaginase, batch PPV35776 did not induce gene mutations in the Ames test, neither in the presence or absence of S-9 mix.
- Asparaginase, batch PPV35776 did not cause an increase in the induction of micronuclei in cultured human lymphocytes in this *in vitro* micronucleus test using human lymphocytes, either in the presence or in the absence of S-9 mix.
- Daily oral administration (by gavage) of Asparaginase, batch PPV35776 to rats at dosages of up to 10.0 mL/kg bw/day for thirteen weeks resulted in no treatment-related effects. Consequently, the No Observed Adverse Effect Level (NOAEL) was considered to be 10 mL/kg bw/day (equivalent to 1.182 g TOS/kg bw/day or 140164 ASNU(F)/kg bw/day).

Based on the present toxicity data it can be concluded that Asparaginase, represented by batch PPV35776, exhibits no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Asparaginase is a liquid enzyme concentrate containing an asparaginase (E.C. number 3.5.1.1), which is used for conversion of asparagine to aspartic acid.

2.1 Production organism

The production organism is a genetically modified strain of the fungus *Aspergillus oryzae*. *A. oryzae* strains have a long history of safe use for the production of food enzymes. The recipient *A. oryzae* strain was developed from the parental strain through a series of modification steps including classical mutagenesis and genetic modification. The classical mutagenesis steps included γ-irradiation mutagenesis, resulting in the deletion of gene clusters required for the synthesis of the mycotoxins cyclopiazonic acid and aflatoxin, and UV irradiation mutagenesis, resulting in a drastically reduced potential to produce kojic acid.

Asparaginase, batch PPV35776, has been analysed for the presence of kojic acid and ß-nitropropionic acid. None of these mycotoxins were detected in significant amounts. The present production strain does not contain any resistance markers and the test article does not contain the production strain.

2.2 Characterization

The toxbatch Asparaginase, batch PPV35776 was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Asparaginase, batch PPV35776

Batch number	PPV35776
Activity	13400 ASNU(F)/g
Water (KF) (% w/w)	88.2
Dry matter (% w/w)	11.8
Ash (% w/w)	0.5
Total Organic Solids (TOS[1]) (% w/w)	11.3
Specific gravity (g/mL)	1.046

[1] % TOS is calculated as 100% - % water - % ash - % diluents.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Asparaginase, batch PPV35776 was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, and TA98 and *Escherichia coli* WP2uvrA. The study was carried out according to the OECD test guideline 471 (adopted in 1997) and in compliance with GLP.

Crude enzyme preparations, like the present batch of Asparaginase, contain the free amino acids histidine and tryptophan, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay. To overcome this problem all strains were exposed to Asparaginase in liquid culture known as “treat and plate assay”.

Two independent experiments were performed, with and without the inclusion of metabolic activation (S-9 mix). In each experiment cultures of bacteria were exposed to six doses of the test substance (5000, 2500, 1250, 625, 313, and 156 µg dry matter/mL) in a phosphate buffered nutrient broth for 3 hours. After incubation, the test substance was removed by centrifugation prior to plating.

No toxicity of the test substance to the bacteria was observed. No treatments of any of the bacterial strains with the test substance resulted in dose related and reproducible increases in revertant numbers that exceeded a doubling in the mean number of revertants per plate compared to the appropriate solvent control either in the presence or absence of S-9 mix.

The results obtained with the diagnostic mutagens and the solvent control demonstrated the sensitivity of the tests and the efficacy of the S-9 mix metabolic activation system.

It was concluded that Asparaginase, batch PPV35776 did not induce gene mutations in bacteria either in the presence or absence of metabolic activation when tested under the conditions employed in this study.

3.2 *In vitro* Micronucleus assay

In order to assess the clastogenic and the aneugenic activity of Asparaginase, batch PPV35776 its effects on the frequency of micronuclei was investigated in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted according to GLP, in compliance with the OECD Guideline for the testing of chemicals; Guideline 487: *In vitro* micronucleus test (Adopted in 2010).

Heparinized whole blood cultures, pooled from two male donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test article was formulated in water for irrigation (purified water) and the highest concentration tested in the Micronucleus Experiment, 5000 µg test substance (weighed out as received)/mL, was determined following a preliminary cytotoxicity Range-Finder Experiment.

Treatments were conducted for 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of Asparaginase, batch PPV35776 on the replication index (RI).

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in the vehicle cultures fell within current 95th percentile of the observed historical vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9.

Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells receiving these were sampled in the Micronucleus Experiment at 24 hours (CPA, MMC) or 48 hours (VIN) after the start of treatment. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei. All acceptance criteria were considered met and the study was therefore accepted as valid.

Treatment of cells with Asparaginase, batch PPV35776 in the absence and presence of S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all treated cultures fell within the 95th percentile of the observed historical vehicle control (normal) ranges.

It was concluded that Asparaginase, batch PPV35776 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of an aroclor induced rat liver metabolic activation system (S-9).

4. GENERAL TOXICITY

4.1 13 Week Oral Toxicity Study in Rats

The objective of this study was to assess the systemic toxic potential of Asparaginase, batch PPV35776, when administered orally by gavage to Sprague Dawley (Crl:CD(SD)) rats for 13 weeks. Three groups, each comprising 10 males and 10 females, received doses of 10, 33 or 100 % of Asparaginase, batch PPV35776 (equivalent to 0.118, 0.390 or 1.182 g TOS/kg bw/day corresponding to 14016, 46254 or 140164 ASNU(F)/kg bw/day). A control group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg bw).

During the study, clinical condition, detailed physical, arena observations, sensory reactivity, grip strength, motor activity, body weight, food consumption, water consumption (by visual assessment), ophthalmoscopy, haematology (peripheral blood), blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

The general appearance and behaviour of the animals during the detailed physical examination and the arena observations were not affected by treatment and no animals died during the treatment period.

At the motor activity assessment, males receiving 100% of the test compound had slightly increased low and high beam scores (cage floor and rearing activity, respectively) compared with concurrent controls but statistical significance was restricted to only a small number of the 6 minute interval scores during the 1 hour recording period for low beam scores where none of the group mean values was above the historical control data range. This finding was attributed to low scores in the concurrent control group where a number of the group mean values, particularly during the latter half of the recording period, and the overall total scores, were below the historical control data range. Furthermore, there was no similar finding in the females. Consequently, this finding was considered incidental and not related to treatment with Asparaginase, batch PPV35776.

The haematological investigation revealed a small (<2.5 seconds) prolongation of prothrombin time in males receiving 33% of the Asparaginase batch and in both sexes receiving 100% of the Asparaginase batch, but the difference from control was small and histopathological examination of a range of tissues did not reveal any test article related effects associated with abnormal clotting function. Consequently, this was considered a non-adverse finding in this study. There was also a slight prolongation of activated partial thromboplastin time at all doses in males, but with no dose-response, and in females receiving 100% of the Asparaginase batch, but as all individual values were well within the background range these differences from controls were considered to be non-adverse findings in this study.

The biochemical examination of the blood plasma did not identify any differences from controls that were attributable to treatment. All inter-group differences from controls, including those which attained statistical significance, were generally confined to one sex only and/or lacked dose relationship and were therefore attributed to normal biological variation. Such findings included the slightly low urea concentration, alkaline phosphatase and aspartate amino transferase activities in females receiving 33 or 100%

and high glucose concentrations in females receiving 100%. No similar findings were evident in males and only a few females had values that were outside the historical background range. There was also a small increase of albumin concentration in females receiving 33 or 100% but there was no dose response and the difference from controls for the high dose females was minimal.

There were no treatment-related ophthalmoscopic findings and no macroscopic or microscopic findings that were attributable to treatment.

It is concluded that oral administration of Asparaginase, batch PPV35776, to Sprague-Dawley rats at doses up to 100% (equivalent to 1.182 g TOS/kg bw/day corresponding to 140164 ASNU(F)/kg bw/day) for 13 weeks was well-tolerated and did not cause any adverse change. The no-observed-adverse-effect level (NOAEL) was thus considered to be high dose (100%) of the Asparaginase batch (equivalent to 1.182 g TOS/kg bw/day corresponding to 140164 ASNU(F)/bw kg/day).

5. REFERENCES

5.1 Study reports

Novozymes A/S: Study No.: 20138059. Asparaginase, batch PPV35776: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. (1st Amended Report, March 2014). LUNA file: 2014-02370.

Covance Laboratories: Study No.: 8294711. Novozymes Reference No.: 20136084: Asparaginase, PPV35776: Induction of micronuclei in cultured human peripheral blood lymphocytes. (March 2014). LUNA file: 2014-04056.

Huntingdon Life Sciences: Study No.: LKG0092. Novozymes Reference No.: 20136078: Asparaginase, batch PPV35776: Toxicity Study by Oral Gavage Administration to Sprague-Dawley Rats for 13 Weeks. LUNA file: 2014-10500.

Viebrock, Lauren

From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Wednesday, April 03, 2019 2:32 PM
To: Viebrock, Lauren
Subject: GRN 000811
Attachments: GRN 811 - Response to FDA Questions.pdf

Hi Lauren,

Hope this email finds you well. Attached is our response to your questions on GRN 811. Please do not hesitate to contact me if you need anything else.

Best regards,

Janet Oesterling
Regulatory Affairs Specialist III

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Mobile: +1 2529151444
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From: Viebrock, Lauren [mailto:Lauren.Viebrock@fda.hhs.gov]
Sent: Thursday, March 28, 2019 4:23 PM
To: JAO (Janet Oesterling) <JAO@novozymes.com>
Subject: RE: GRN 000811

Dear Ms. Oesterling,

During our review of GRAS Notice No. 000811, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
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U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock]@fda.hhs.gov

From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Thursday, March 14, 2019 9:30 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: GRN 000811

Hello Lauren,

Hope this email finds you well. I am wondering about the status of GRN 000811, phospholipase A1 from A. oryzae? The Acknowledgement letter was received on September 25, 2018 and I wanted to check in with you on the progress of the review.

Many thanks for the update,
Janet

Janet Oesterling
Regulatory Affairs Specialist III

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April 3, 2019

Phospholipase A1 from Aspergillus oryzae – GRN 811

1. Please provide the number of amino acids in the primary protein sequence and the theoretical molecular weight of the enzyme in kilodaltons of the phospholipase A1 enzyme.
ANSWER: The primary sequence of the protein is 308 amino acids and the molecular weight is 31 kDA.

2. Please state whether the gene encoding phospholipase A1 is synthetic or modified compared to wild-type sequence. Please comment on whether any difference in the enzyme sequences would bear on safety.
ANSWER: The gene encoding the phospholipase A1 is the wild-type. There is no safety concern.

3. Please provide the strain name for the *A. oryzae* strain used as recipient strain. Please state whether the recipient or member of recipient lineage has been deposited in a culture collection.
ANSWER: The *Aspergillus oryzae* recipient strain, AT604, developed from the *A. oryzae* A1560 cell lineage. Strain A1560 was obtained from Institute for Fermentation Osaka (IFO)

4. Please confirm that the genus and species that is the source of the plasmid DNA encoding the nitrate reductase (*niaD*) is *A. oryzae*.
ANSWER: *Aspergillus oryzae* is confirmed.

5. Please provide the genus and species of the donor organism that is the source of the DNA encoding the orotidine 5-phosphate decarboxylase (*pyrG*).
ANSWER: *Aspergillus oryzae*

6. Please specify whether the specifications and batch analyses data given on p.9 of the GRAS notice correspond to the unformulated enzyme concentrate or formulated enzyme preparation.
ANSWER: Enzyme concentrate

7. The GRAS notice includes unpublished toxicological studies on a structurally and biochemically different enzyme (asparaginase) to support your GRAS conclusion. Please state clearly that your GRAS conclusion is based on published information that establish that the article of commerce, i.e. phospholipase A1 from *V. rubricosa*, is generally recognized as safe.
ANSWER: The summary of the studies and findings for the asparaginase produced by the production strain most closely related to the phospholipase A1 production strain, is included in GRN 811. The GRAS conclusion for the phospholipase A1 is established based on the studies for the asparaginase, which are published and available for review.

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8. Bioinformatic analyses of the phospholipase A1 protein sequence identified greater than 35% identity over 80 amino acid window with Sch c 1. Please confirm that Sch c 1 (glucoamylase) is associated with bronchopulmonary mycosis and that there are other amylases considered occupational allergens but safe for oral exposure.
ANSWER: The organism Schizophyllum commune may cause bronchopulmonary mycosis. Sch c 1 is recognized by the host immune system of patients with S. commune-induced mycosis as an antigen/allergen. The purified glucoamylase Sch c 1 is a candidate antigen for the serodiagnosis of this disease. Workers exposed to enzymes for use in the food industry may develop allergy to the enzymes via inhalation whereas sensitization to the enzymes by oral route in the consumers has not been described.

A large and very thorough study on allergic reactions to commercial food enzymes (including amylases) was conducted by Bindslev-Jensen el al. (2006). Here is shown that even hyper sensitive people can ingest enzymes. Bindslev-Jensen and colleagues found no allergenic effects of clinical relevance and concluded that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy.

References

Bindslev-Jensen, C., Skov, P., S., Roggen, E., L., Hvass, P., Brinch, D., S. Food and Chem. Toxicol. 44; 1909-1915. 2006.

Michael W. Pariza, Eric A. Jonson, Evaluating the Safety of Microbial enzyme Prepations Used in Food Processing: Update for a New Century. Reg. Tox & Pharma., 33, 173-186. 2001.

Best regards,

(b) (6)

Janet Oesterling
Regulatory Affairs Specialist
Novozymes North America, Inc.