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(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
000774	
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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 North America

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
919-494-8784		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

L-glutaminase from Bacillus licheniformis produced by a genetically modified strain of Bacillus licheniformis

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).*

L-glutaminase is considered a key enzyme for controlling the taste of fermented foods, such as soy sauce. L-glutaminase is used in various foods and ingredients such as; casein, whey protein, soy and wheat protein. It can also be used during the production of processed foods such as; breads, noodles, tofu, fish, cheese and seasonings. The level of use will not be higher than necessary to achieve an intended effect and in accordance with requirements for normal production following cGMP. The dosage applied in practice by a food manufacturer depends on the specific process. The maximum recommended use level is 1000 EGLU (A) per kilo of protein dry solids. The general population is the target population for consumption. There is no specific subpopulation.

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	L-glutaminase	EC	3.2.1.2		
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.

Classification: L-glutaminase
Systematic name: L-glutamine
EC No.: 3.5.1.2
CAS No.: 9001-47-2
Specificity: carboxylic acid amide hydrolysis
Molecular Weight: 64 kDa.
Typical composition of the enzyme preparation: enzyme solids, glycerol, water, sodium benzoate and potassium sorbate.

3. Synonyms

Provide as available or relevant:

1	L-glutaminase amindohydrolase
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 L-glutaminase from Bacillus licheniformis produced by a genetically modified strain of Bacillus *(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: 2018.03.13 11:10:12 -04'00'	Janet Oesterling, Regulatory Affairs	03/13/2018

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)
	GRASNotification_L-Glutaminase_2018-03-13.pdf	Submission
	DecisionTree_L-Glutaminase_2018-03-13.pdf	Administrative
	Part 1_L-Glutaminase_2018-03-13.pdf	Submission
	Sewalt etal_GRAS Process for Industrial Microbial enzymes.pdf	Administrative
	SummaryofToxicityData_L-Glutaminase_2018-03-12.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: Glutaminase enzyme from *Bacillus licheniformis* produced by *Bacillus licheniformis.*

§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 glutaminase enzyme will be used as a processing aid in various food and ingredients such as; casein, whey protein, dried egg whites, soy and wheat protein. It can also be used during the production of processed foods such as; breads, noodles, tofu, fish, cheese and seasonings. 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)

03/12/2018

Janet Oesterling — Date
Regulatory Affairs Specialist III

L-Glutaminase from *Bacillus licheniformis* Produced by a Genetically Modified Strain of *Bacillus licheniformis*

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

March 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 an L- glutaminase enzyme preparation produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism carrying the gene coding for L- glutaminase from *Bacillus licheniformis.*

Key enzyme and protein chemical characteristics of the L-glutaminase are given below:

Classification:	L-glutaminase
Systematic name:	L-glutamine amidohydrolase
EC No.:	3.5.1.2
CAS No.:	9001-47-2
Specificity:	carboxylic acid amide hydrolysis
Molecular Weight:	64 kDa.
Amino acid sequence:	The amino acid sequences have been verified.

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Bacillus licheniformis* production strain, designated SJ13263, was derived via the recipient strain, PP1897-3, from a natural isolate of *Bacillus licheniformis* strain DSM 9552.

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) (6) (1) (7) (8) (9).

The glutaminase expression plasmids pSJ13162 and pSJ13208, used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced L-glutaminase (*ggt*)is from *Bacillus licheniformis*.

2.2(b) Recipient Strain

The recipient strain PP1897-3 used in the construction of the L-glutaminase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding several proteases. Also, deletion of a gene essential for sporulation was performed, eliminating the ability to sporulate, together with the deletion of additional

genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity, safety and stability.

2.2(c) L-Glutaminase Expression Plasmid

The expression plasmid, pSJ13162 and pSJ13208, used to transform the *Bacillus licheniformis* recipient strain PP1897-3 is based on the well-known *Bacillus* vectors pE194 (10) and pUB110 (11) from *Staphylococcus aureus*. No elements of these vectors are left in the production strain. No elements of these vectors are left in the production strain. The plasmids contain the expression cassette consisting of a B. licheniformis promotor, the *ggt* sequence encoding L-glutaminase and a transcriptional terminator.

Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Bacillus licheniformis* SJ13263, was constructed from the recipient strain PP1897-3 through the following steps:

1) Plasmid pSJ13162 was integrated into a specific locus in strain PP1897-3 by targeted homologous recombination. Targeted integration of the expression cassettes at this locus allows the expression of the L-glutaminase gene *ggt* from the promoter.

2) Plasmid pSJ13208 was integrated into another specific locus in the strain PP1897-3 by targeted homologous recombination. Targeted integration of the expression cassettes at this locus allows the additional expression of the L-glutaminase gene *ggt* from the promoter.

The resulting L-glutaminase strain containing two copies of the *ggt* gene was named SJ13263.

Sequence confirmation of the inserted expression cassettes and the flanking regions at each of the integration loci was performed in the production strain.

2.2(e) Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern hybridization. Analysis of samples from end of production using a *ggt* gene specific probe showed an identical band pattern compared to the reference production strain (SJ13263), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Bacillus licheniformis*

chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

2.2(f) Antibiotic Resistance Gene

As a result of the genetic modifications, no functional antibiotic resistance genes were left in the strain. 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 IFBC (4) is satisfactorily addressed.

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the L-glutaminase enzyme preparation which follows standard industry practices (12) (13) (14). The quality management system used in the manufacturing process for the enzyme preparation 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. These methods are based on generally available and accepted methods used for production of microbial enzymes.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (15). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (16).

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 (12) (13) (14). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC (15). 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 sampled by the Quality Control Department and subjected to the appropriate analyses 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 onantifoams

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 L-glutaminase enzyme preparation is produced by pure culture, submerged, fed-batch fermentation of a genetically modified strain of *Bacillus licheniformis* as described in Part 2. 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, *Bacillus licheniformis,* described in Part 2. 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

The enzyme is recovered from the culture broth by the following series of operations:

1) Pretreatment - pH adjustment and flocculation (if required)

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

The enzyme concentrate is stabilized with glycerol. The product is formulated by addition of water and preserved with potassium sorbate and sodium benzoate. 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

Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the L-glutaminase enzyme preparation. Also, the enzyme preparation does not contain any major food allergens from the fermentation media.

Table 1. Typical compositions of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	11%
Glycerol	>50%
Water	40 - 50%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The L-glutaminase enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (15). 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 (16).

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

Table 2. Analytical data for three food enzyme batches

Parameter	Specifications	PPG47310	PPG46289	PPG46449
L-glutaminase activity	EGLU(A)/g	605	694	638
Total viable count	Upper limit 50,000	<100	<100	<100
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 4	< 4	< 4
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is L-glutaminase (EC 3.5.1.2). The enzyme L-glutaminase belongs to the class; hydrolytic enzymes. L-glutaminase catalyzes the hydrolysis of the γ-amido bond of L-glutamine to L-glutamate and ammonia (17).

L-glutaminase plays a significant role in the metabolism of cellular nitrogen. It is an effective agent in analytical and bio-sensing industrial applications (18). L-glutaminase also has a long history of use in the food industry due to this role as a flavour-enhancer (19).

2.5(b) Use Levels

L-glutaminase is considered a key enzyme for controlling the taste of fermented foods, such as soy sauce. L-glutaminase is used in various foods and ingredients such as; casein, whey protein, soy and wheat protein. It can also be used during the production of processed foods such as; breads, noodles, tofu, fish, cheese and seasonings.

The level of use will not be higher than necessary to achieve an intended effect and in accordance with requirements for normal production following cGMP.

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

The maximum recommended use level is 1000 EGLU (A) per kilo of protein dry solids.

2.5(c) Enzymes Residues in the Final Food

Enzymes do not exert enzymatic activity in the final food due to a variety of factors specific to the application and the process conditions used by the individual food producer. These factors include denaturation of the enzymes during processing, depletion of the substrate, lack of water activity, adverse pH, filtration, carbon treatment, ion exchange, evaporation and drying etc. Given these conditions, the glutaminase enzyme will not be functional in the final food product.

PART 3 - DIETARY EXPOSURE

To provide a “worst case” scenario for the calculation of possible daily human exposure, an assumption was made that all the enzyme product is retained in the final food product.The general population is the target population for consumption. There is no specific subpopulation.

3(a) Assumptions in Dietary Exposure

The assumptions are highly exaggerated since the enzyme protein and the other substances are diluted or removed in certain processing steps.

Furthermore, all processed foods and beverages produced with the enzyme are not always produced with the maximum recommended dosage. Therefore, the safety margin calculation derived from this method is highly conservative.

The exposure assessment represents a “maximum worst case” situation of human consumption. Overall, the human exposure to the L-glutaminase will be negligible because the enzyme preparation is used as a processing aid and in very low dosages therefore the safety margin calculation derived from this method is highly conservative.

3(b) Food Consumption Data

Budget Method

Solid Food: The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (bw) per day. Fifty kcal corresponds to 25 g food. Therefore, adults ingest 25 g food per kg body weight per day.

Assuming 50% of the food is processed food, the daily consumption of processed food will be 12.5 g processed foods per kg body weight.

It is further assumed that, on average, all processed food contains 10% protein hydrolysates = 1.25 g protein hydrolysates per kg body weight per day.

The L-glutaminase has an average activity of 646 EGLU(A)/g and approximately 11% TOS (Total Organic Solids) content.

The highest dose given for solid foods is: 1000 EGLU(A)/kg of protein dry solids, which corresponds to 0.17 mg TOS per g of dry protein solids.

Based on this 1.25 g protein dry solids will maximally contain:

0.17 mg TOS per g protein dry solids x 1.25 g protein dry solids/kg bw/day = 0.2125 mg TOS/kg bw/day

The Total Maximum Daily Intake (TMDI) of the food enzyme by consumers based on starch contribution is:

0.2125 mg/TOS/kg bw/day.

The safety margin calculation derived from this method is highly exaggerated.

Theoretical Maximum Daily Intake (TMDI)

The safety margin is calculated as the dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13-week oral toxicity study in rats conducted on L-glutaminase, PPG45609 was the highest dosage possible, 702 mg TOS/kg bw/day. See the *Summary of Toxicology Data* included in this submission and Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	702
*TMDI (mg TOS/kg bw/day)	0.2125
Safety margin	3303

*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 L-glutaminase enzyme preparation. 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.

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (4) (6) (1) (7) (8) (9). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Part 2.

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 L-glutaminase, *Bacillis licheniformis,* is discussed in Part 2 and in this Part.

The production strain is genetically modified by rDNA techniques, as discussed in Part 2. The enzyme preparation is free of DNA that may encode transferable, antibiotic resistance DNA and the introduced DNA is well characterized and safe for the construction of microorganisms 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.

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 (20). Pariza and Foster 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” (2).

Bacillis licheniformis has a long history of safe industrial use in the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (20). *Bacillis licheniformis* is not a human pathogen and it is nottoxigenic (21).

An evaluation of the genetically modified *Bacillus licheniformis* production organism embodies the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (20), the EU SCF in 1991 (6), the OECD in 1992 (1), ILSI

Europe Novel Food Task Force in 1996 (9), FAO/WHO in 1996 (8), JECFA in 1998 (16) and Pariza and Johnson in 2001, demonstrating the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient 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 it are given in Part 2. Also, Novozymes' has repeatedly used the decision tree procedures outlined by Pariza and Johnson and is the basis for our safety assessment. See Appendix 1.

In addition, (GRAS) Notices have been submitted to the US FDA for several food enzymes from genetically modified *Bacillus licheniformis* strains, including; phospholipase (GRN 728 and 689), pullulanase (GRN 645), acetolactate decarboxylase (GRN 587), lactase (GRN 572) and protease (GRN 564) (22). Based on the information provided in these GRAS Notices, the agency did not question the conclusion that food enzyme preparations from *Bacillis licheniformis* are GRAS under the intended conditions of use.

In addition, *Bacillis licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

Based on the information presented here it is concluded that the *Bacillus licheniformis* production strain is considered a safe strain for the production of L-glutaminase enzyme.

6(b) Safety of the Donor Organism

The donor organism of the L-glutaminase is *Bacillus licheniformis*. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the L-glutaminase 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 Glutaminase Enzyme

The subject of this GRAS notification is an L-glutaminase, EC 3.5.1.2. Enzymes, including glutaminase, have a long history of use in food.

A wide variety of enzymes are used in food processing. And, according to Pariza and Johnson, enzyme proteins do not generally raise safety concerns (3) (2).

Research on L-glutaminase started in 1956 when Alexander B. Gutman and Tsai-Fan discovered the importance of L-glutaminase as a therapeutic enzyme (23). In 1974,

Shusaku Yamamoto and Hitoshi Hirooka observed the role L-glutaminase plays in improving the taste of food (24).

L-glutaminase is regarded as a key enzyme in fermented foods such as soy sauce, and has been used in food processing in Japan for many years (17).

Glutaminase enzymes sourced from *Bacillus amyloliquefaciens* have a long history of use in Japan as they were first reported in the publicly available literature in 1988 (25). Specifically, glutaminase has been used in the production of soy sauces since 1991, the production of miso since 1992 and the production of hydrolyzed vegetable protein since 2003 (26).

L-glutaminase is currently on the 'List of Existing Food Additives' published by the Ministry of Health and Welfare of Japan (27). In July 2009 AFSSA (French Food Safety Agency) approved the use of glutaminase from *bacillus amyloliquefaciens* for the production of protein hydrolysates and yeast extracts, stating that there were no health risks associated with glutaminase (28). In May 2016, glutaminase from *Bacillus amyloliquefaciens* was approved for use as a processing aid by FSANZ (*Food Standards Australia New Zealand).* FSANZ determined that the use of glutaminase from *B. amyloliquefaciens* as a processing aid did not pose public health or safety risks (26).

Novozymes completed an extensive literature search using Medline, ToxCenter, SciSearch, Chemlist, Scopus and a Google Scholar. Key words such as "glutaminase", "toxicity", "human consumption", "food" and others, was used for the search. The literature search produced no health or safety issues associated with the use of the glutaminase enzyme from *Bacillus licheniformis* for the intended uses listed in 2.5(b) Use Levels.

Based on the publicly available, scientific data from the literature and additional supporting data generated by Novozymes it has been concluded that glutaminase enzyme produced by *Bacillus licheniformis* is safe and suitable for the intended use.

6(d) Allergenic/Toxigenic Potential of the L-glutaminase Enzyme

The ingestion of a food enzyme protein is not considered a concern as a 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. Based on previous experience, food enzymes are not homologues to known

allergens, which make it very unlikely that an enzyme would be afood allergen.

3) Enzymes in foods are added in concentrations in the low range of parts per million. 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) (29).

In order to further evaluate the possibility that the L-glutaminase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (30) and modified by Codex Alimentarius Commission, 2009 (31) the glutaminase 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 80 amino acid stretches within the sequence that have greater than 35% identity to the expressed protein showed no homology to food allergens. Full alignment of the L-glutaminase with greater than 35% identity over the full length of the alignment was also analyzed. No homology to food allergens was found between the L-glutaminase and any of the allergens from the databases referenced above. A search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

Also, a search for homology of the L-glutaminase sequence to known toxins was assessed based on the information present in the UNIPROT database (2018-01-25). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 17% indicating that the homology to any toxin sequence in this database is low and random.

Consequently, oral intake of the L-glutaminase is not anticipated to pose any food allergenic or toxin concerns.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the L-glutaminase, which follows standard industry practices (14) (13) (12).

The quality management system used in the manufacturing process for the glutaminase 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, 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 (15). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (16).

6(f) Safety Studies

This section describes the studies and analysis performed to evaluate the safety of the use of the glutaminase.

The following studies were performed on test batch PPG45609 with favourable results:

Bacterial Reverse Mutation Assay (Ames test)

In vitro Micronucleus Test in Cultured Human Lymphocytes

13-week oral toxicity study

These tests are described in Appendix 2. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that L-glutaminase, represented by batch PPG45609, exhibits no toxicological effects under the experimental conditions described.

6(g) Results and Conclusion

Novozymes has reviewed the available data and information. We are not aware of any data and/or information that is, or appears to be, inconsistent with our conclusion of GRAS. Based on this critical review and evaluation, a history of safe use of *Bacillus licheniformis* and the limited and well-defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; glutaminase enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices. Thus, it 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, Glutaminase PPG45609. March 12, 2018. LUNA No. 2017-13680-04.

3. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology, Vol. 12, No. 5. October 2016.

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Bonnette, Richard

Subject: FW: GRAS submission for L-glutaminase - USDA uses
Attachments: MODIFIED - Part 1_L-Glutaminase_2018-04-10.pdf

From: JAO (Janet Oesterling) [mailto:JAO@novozymes.com]
Sent: Tuesday, April 10, 2018 4:54 PM
To: Bonnette, Richard <Richard.Bonnette@fda.hhs.gov>
Subject: RE: GRAS submission for L-glutaminase - USDA uses

Hello Mr. Bonnette,

Thank you for your email below. Please disregard the inclusion of the USDA regulated product (dried egg whites) as an intended use, which is listed in Part 1 of the notification. I have attached a modified Part 1 for your review.

Many thanks for your kindness in bringing this to my attention.

Best regards,

Janet Oesterling
Regulatory Affairs Specialist III

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Appendix 3

This glutaminase enzyme preparation from *Bacillus licheniformis* produced by *Bacillus licheniformis* was evaluated according to the decision tree published in Pariza and Johnson, 2001 (1). The result of the evaluation is presented below.

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

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.

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Toxicology & Immunology

Date: March 12, 2018
File: 2017-13680-04
Ref.: BTR

SUMMARY OF TOXICITY DATA

Glutaminase, batch PPG45609, from *Bacillus licheniformis*

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CONTENT

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Glutaminase, batch PPG45609.

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 Envigo (UK) and Covance (UK) during the period May 2017 to February 2018.

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

- Glutaminase, batch PPG45609, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Glutaminase, batch PPG45609, did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9).
- In a 13 week oral toxicity study in rats Glutaminase, batch PPG45609 was well tolerated and did not cause any toxicologically significant changes at any dose level tested.

2. TEST SUBSTANCE

The testsubstance is a glutaminase (E.C. 3.5.1.2).

2.1 Characterization

The toxbatch Glutaminase, batch PPG45609, 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 Glutaminase, batch PPG45609

Batch number	PPG45609
Activity	264 EGLU(A)/g
N-Total (% w/w)	0.88
Water (KF) (% w/w)	90.4
Dry matter (% w/w)	9.6
Ash (% w/w)	2.9
Total Organic Solids (TOS[1]) (% w/w)	6.7
Specific gravity (g/mL)	1.048

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

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Glutaminase, batch PPG45609 was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmonella typhimurium, and one tryptophan-requiring strain (WP2 uvrA pKM101) of Escherichia coli, both in the absence

and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments. A 'treat and plate' procedure was used for all treatments in this study as Glutaminase, batch PPG45609 may contain free amino acids i.e. histidine and tryptophan (which may cause artefacts through growth stimulation in a standard plate-incorporation test).

All Glutaminase, batch PPG45609 treatments in this study were performed using formulations prepared in water for irrigation (purified water), and all concentrations stated in this report include a correction to account for Total Organic Solids (TOS) content of 6.7% w/w, using a correction factor of 14.93.

Mutation Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using final concentrations of Glutaminase, batch PPG45609 at 5, 16, 50, 160, 500, 1600 and 5000 µg TOS/mL. Following these treatments, evidence of toxicity was observed on the mutation plates treated at 1600 µg TOS/mL and above in all the Salmonella strains in the absence of S-9, and also at 500 µg TOS/mL in strain TA1537 in the absence of S-9, and at 5000 µg TOS/mL in strain TA1535 in the presence of S-9.

Mutation Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S-9. The maximum test concentration of 5000 µg TOS/mL was retained for all strains. Narrowed concentration intervals were employed covering the range 160-5000 µg TOS/mL, in order to examine more closely those concentrations of Glutaminase, batch PPG45609 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, evidence of toxicity was only observed on the mutation plates treated at 5000 µg TOS/mL in strain TA1537 in the absence of S-9.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were all acceptable for vehicle control treatments, and were elevated by positive control treatments.

Following Glutaminase, batch PPG45609 treatments of all the test strains in the absence and presence of S-9, no concentration-related increases in revertant numbers were observed, and none that were ≥2-fold (in strains TA98, TA100 and WP2 uvrA pKM101) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of any Glutaminase, batch PPG45609 mutagenic activity in this assay system.

It was concluded that Glutaminase, batch PPG45609 did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmonella typhimurium, and one tryptophan-requiring strain (WP2 uvrA pKM101) of Escherichia coli when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg TOS/mL (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S-9) using a modified Treat and Plate methodology.

3.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

Glutaminase, batch PPG45609 was tested in an in vitro micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in two experiments. 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 purified water and the highest concentrations tested in the Micronucleus Experiments (limited by toxicity), were determined following a preliminary cytotoxicity Range-Finder Experiment.

Treatments were conducted (as detailed in the following summary table) 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of Glutaminase, batch PPG45609 on the replication index (RI). Micronuclei were analysed at four to six concentrations.

In order to further investigate a weak but statistically significant increases in MNBN cells observed following the initial 24+24 hour -S-9 treatment, a second experiment (Micronucleus Experiment 2) was conducted to look for reproducibility of effect and to aid interpretation of biological relevance. This was performed using a separate frozen aliquot of the same batch of test article.

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in these cultures fell within the 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 Experiments 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.

In Experiment 1 treatment of cells with Glutaminase, batch PPG45609 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 the majority of concentrations analysed (all treatments). Exceptions to this were observed for two intermediate concentrations analysed following 24+24 hour -S-9 treatment (100 and 150 µg TOS/mL, inducing 46% and 59% cytotoxicity respectively) where statistically significant increases were observed. However, in both instances, these statistical increases were small with just single cultures at each concentration exhibiting MNBN cell values that marginally exceeded the 95th percentile of the normal range. The MNBN cell values of the replicate cultures and for higher and lower concentrations analysed (and all other treatment concentrations) fell within normal ranges. As such, these statistical increases were considered of questionable biological relevance.

In Experiment 2 treatment of cells with Glutaminase, batch PPG45609 for 24+24 hours in the absence of S-9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all six concentrations analysed. The MNBN cell values of all Glutaminase, batch PPG45609 treated cultures (all concentrations) fell within normal values.

Overall the data from Experiment 1 and 2 indicated a negative response. The weak statistical increases in MNBN cell frequency observed following 24+24 hour -S-9 treatment

in Experiment 1 were not reproduced in Experiment 2 where a similar (but extended) concentration range was investigated.

It was concluded that Glutaminase, batch PPG45609 did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations were tested up to recommended limits of cytotoxicity (in accordance with current regulatory guidelines for the in vitro micronucleus assay).

4. GENERAL TOXICITY

4.1 Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks

The purpose of this study was to assess the systemic toxic potential of Glutaminase, batch PPG45609 when administered orally by gavage to Han Wistar rats for 13 weeks. Three groups, each comprising 10 males and 10 females, received doses of 10, 33 or 100% of the Glutaminase, batch PPG45609 (equivalent to 70.22, 231.71 or 702.16 mg TOS/kg body weight/day, or 276.7, 913.0 or 2766.7 EGLU(A)/kg body weight/day). A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume dose.

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

The general appearance and behaviour of the animals and sensory activity, grip strength and motor activity were unaffected by treatment and there were no deaths. There was no effect of treatment on body weight gain or food and water consumption. There were no treatment related haematology or blood chemistry findings.

Organ weights were unaffected and there were no treatment-related macroscopic and microscopic findings.

It is concluded that oral administration of Glutaminase, batch PPG45609 to Han Wistar rats at doses up to 100% of the test batch (equivalent to 702.16 mg TOS/kg body weight/day, or an enzyme activity of 2766.7 EGLU(A)/kg body weight/day) for 13 weeks was well tolerated, with no evidence of any adverse finding at any of the administered doses. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 702.16 mg TOS/kg body weight/day or 2766.7 EGLU(A)/kg body weight/day.

5. REFERENCES

5.1 Study reports

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Covance: Study No.: 8366019; Novozymes Reference No.: 20176012: Glutaminase, Batch PPG45609: *In vitro* Human Lymphocyte Micronucleus Assay. (September 2017). LUNA file: 2017-13498.

Envigo: Study No.: WK31KY; Novozymes Reference No.: 20176010: Glutaminase, Batch PPG45609: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. (February 2018). LUNA file: 2018-02622.