DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE (GRAS) NOTICE

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000853	04-05-2019
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
Lori Gregg	Sr. Regulatory Affairs Manager

Company *(if applicable)*: Novozymes NA

Mailing Address *(number and street)*: 77 Perry 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
919-494-3000		lobg@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

Lysozyme produced by Trichoderma reesei

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 a lysozyme (EC 3.2.1.17). Lysozyme catalyzes the hydrolysis of peptidoglycans in the cell wall of gram-positive bacteria. The specific point of cleavage is 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues.

The lysozyme is not added to final foodstuffs but is used as a processing aid during the manufacture of ingredients produced by microbial fermentation such as xanthan gum, gellan gum and yeast extracts. The enzyme is used during processing to aid in the removal of cellular debris.
The maximum suggested use level in food ingredient processing is 300,000 LSU-F per kg food ingredient.

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	lysozyme	EC	3.2.1.17		
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.

Lysozyme, kDa:23; Liquid formula: water, sorbitol, sodium benzoate, potassium sorbate, enzyme concentrate.
Lysozyme is produced by a genetically modified Trichoderma reesei.

3. Synonyms

Provide as available or relevant:

1	Peptidoglycan N-acetylmuramoylhydrolase
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, Inc.
(name of notifier)

has concluded that the intended use*(s)* of Lysozyme produced by Trichoderma reesei
(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, Inc. *(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, Inc. *(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 Perry Chapel Church Rd., Franklinton, NC 27525
(address of notifier or other location)

Novozymes North America, Inc. *(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: 2019.04.05 13:11:23 -04'00'	Janet Oesterling, Regulatory Affairs	04/05/2019

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_Lysozyme_190405.pdf	Submission
	DecisionTree_Lysozyme_2019-04-05.pdf	Administrative
	Part 1_Lysozyme_2019-04-05.pdf	Submission
	Pariza and Johnson_Evaluating Safety Microbial Enzyme_2001-04-12.pdf	Administrative
	Sewalt etal_GRAS Process for Industrial Microbial enzymes.pdf	Administrative
	SummaryofToxicityData_lysozyme_2019-03-20.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 GRASnotice:

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 thenotifier:

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

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

The appropriately descriptive term for this notified substance is: Lysozyme enzyme produced by a genetically modified strain of *Trichoderma reesei.*

§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 lysozyme enzyme will be used as a processing aid during the manufacture of ingredients produced by microbial fermentation such as xanthan gum, gellan gum and yeast extracts. 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.

04/05/19

Lori Gregg Date
Sr. Regulatory Affairs Manager

Lysozyme produced by *Trichoderma reesei*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA

April 2019

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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 lysozyme enzyme preparation produced by submerged fermentation of a genetically modified *Trichoderma reesei* microorganism carrying the gene coding for lysozyme from *Acremonium alcalophilum.*

Classification	Lysozyme
IUBMB nomenclature:	peptidoglycan *N*-acetylmuramoylhydrolase
EC No.:	3.2.1.17
CAS No.:	9001-63-2
Specificity:	Hydrolysis of (1→4)-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins
Molecular weight:	23 kDa
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 *Trichoderma reesei (T. reesei)* production strain, designated AGJG-20A2, was derived from recipient strain RUTC30 (ATCC 56765), a natural isolate of *T. reesei* strain (1). RUTC30 is derived from the well-known wild type strain QM6a. QM6a is the parent of practically all *T. reesei* industrial production strains (2). *T. reesei* is classified as a Biosafety Level 1 microorganism by the American Type Culture Collection (ATCC) based on risk assessment from U.S. department of Public Health guidelines (3).

T. reesei complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (4). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (5) and later Pariza and Johnson (6) and several expert groups (4) (7) (8) (9) (10) (11).

The expression plasmid, used in the strain construction contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the sequence encoding a lysozyme from *Acremonium alcalophilum*.

2.2(b) Recipient Strain

The recipient strain used in the construction of the lysozyme production strain was modified by several rounds of classical mutagenesis of RUTC30 followed by screening for increased level of enzyme production. Furthermore, the strain was prepared for targeted homologous recombination in several loci.

2.2(c) Lysozyme Expression Plasmid

The expression plasmid used to introduce the lysozyme gene in the recipient strain is based on the replication origin of *E. coli*. However, no fragments of the vector backbone are introduced into the production strain. The plasmid contains the expression cassette consisting of a fragment of the *T. reesei cbh1* (cellubiohydrolase 1) promotor, the gene encoding the lysozyme, the transcriptional terminator of *cbh1* and finally a selective marker, *amdS*. The expression cassette and the *amdS* gene encoding an acetamidase are flanked by DNA regions used for targeted integration. Only the expression cassette is present in the final production strain. This has been confirmed by PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The *Trichoderma reesei* production strain, AGJG-20A2, was constructed from the recipient strain through the following steps:

1) The expression cassette from the plasmid was integrated into three specific loci in the recipient strain by targeted homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the lysozyme gene from the promoter.

2) The selection of transformants was achieved by growing on a minimal medium and subsequent screening for expression of the lysozyme.

The resulting lysozyme production strain containing one copy of the lysozyme gene in each of the three target loci was named AGJG-20A2.

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.

2.1(e) Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern blot hybridization. Analysis of samples from end of production using a lysozyme gene specific probe showed an identical band pattern compared to the reference production strain (AGJG-20A2), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the

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

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 IFBC (12) is satisfactorily addressed.

2.3 METHOD OF MANUFACTURE

This section of Part 2 describes the manufacturing process for the enzyme which follows standard industry practices (13) (14) (15). The quality management system used in the manufacturing process for the lysozyme complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (14) and 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 (16). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (17).

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 (13) (14) (15). 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 A/S, 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 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 lysozyme enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *T. reesei* 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, *T. reesei,* 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 standardized with sorbitol 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

The lysozyme enzyme preparation is sold in a liquid form. Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. Also, the enzyme preparation, that is the subject of this notification, 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*)	30%
Water	40 - 60%
Sorbitol	20 - 35%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The lysozyme enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (16). 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 (17).

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	Specification	1	2	3
Lysozyme Activity	LSU(F)/g	60300	58200	63300
Total viable count	Upper limit 50,000 CFU/ml	100	<100	<100
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

The active enzyme is a lysozyme (EC 3.2.1.17). Lysozyme catalyzes the hydrolysis of peptidoglycans in the cell wall of gram-positive bacteria. The specific point of cleavage is 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues.

The lysozyme is not added to final foodstuffs but is used as a processing aid during the manufacture of ingredients produced by microbial fermentation such as xanthan gum, gellan gum and yeast extracts. The enzyme is used during processing to aid in the removal of cellular debris.

Because this enzyme has an affinity for unviable cellular material, the benefit of using the enzyme is easier separation of the food ingredient (ie xanthan gum, gellan gum, yeast extract) from the fermentation organisms and improved clarity in the final foods produced with these food ingredients.

2.5(b) Use Levels

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

The dosage applied in practice by a food manufacturer depends on their particular process and the initial recommendation by the enzyme manufacturer. The dose is optimized to fit the process conditions. The maximum suggested use level in food ingredient processing is 300,000 LSU-F per kg food ingredient.

2.5(c) Enzymes Residues in the Final Food

The enzyme is typically added at the conclusion of fermentation prior to separation of the food ingredient from the fermentation biomass. The lysozyme catalyzes the

hydrolysis of the peptidoglycan cellular debris, thus facilitating separation of the food ingredient from the fermentation biomass.

The enzyme used during processing does not exert any enzymatic activity in the final food. This is due to a combination of various factors, depending on the process conditions used by the individual food producer. These factors include denaturation of the enzyme during heat processing, depletion of the substrate, physical removal of the enzyme, etc. In most cases, a heat treatment step is part of the manufacturing process for production of fermentation ingredients and this process will be sufficient to inactivate or denature the enzyme protein.

Consequently, the presence of residues of food enzymes in the final food does not lead to any effect in or on the final food. The enzyme action has taken place during the food manufacturing process and is over before the food product is available for delivery to consumers.

PART 3 - DIETARY EXPOSURE

In order to provide a “worst case” scenario for the calculation of the possible daily human exposure, an assumption was made that all the enzyme product is retained in the final food ingredient. The enzyme is used as a processing aid at very low dosages in the production of an ingredient that is then used in a final food. 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 xanthan gum, gellan gum, yeast extracts and similar food ingredients and that all processed foods and beverages contain this type of food ingredients. It is also assumed that the enzyme is always used at the maximum dose recommended.

The safety margin calculation derived from this method is highly conservative and exposure to the enzyme is negligible.

3(b) Food Consumption Data

The exposure assessment is based on the Budget Method (18) (19) which represents 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) According to the Budget Method, the maximum intake of liquids (other than milk) is 100 ml/kg body weight day. Assuming that 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.
c) It is assumed that all processed food is produced using food ingredients that have been processed with the enzyme. It is assumed that processed foods contain up to 5% xanthan gum, gellan gum, yeast extracts and similar types of ingredients.
d) The calculation is made assuming that all enzyme TOS remains in the final product.

The maximum recommended dosage is: 300,000 LSU(F)/kg food ingredient

This equates to: 544 mg TOS per kg food ingredient

Using the Budget Method assumptions for solid food:

544 mg TOS per kg food ingredient ÷ 1000 x 0.625 g = 0. 34 mg TOS per kg bw/day

Using the Budget Method assumptions for Liquids:

544 mg TOS per kg food ingredient ÷ 1000 x 1.25 g = 0.68 mg TOS per kg bw/day

This results in a Total Maximum Daily Intake (TMDI) of TOS:

0.34 + 0.68 = 1.02 mg TOS/kg body weight/day

Theoretical Maximum Daily Intake (TMDI)

The margin of safety is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The safety margin calculation derived from this method is highly exaggerated.

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on the lysozyme tox batch PPL41125 was the highest dosage possible, 1.132 g TOS/kg bw/day. See Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1132
*TMDI (mg TOS/kg bw/day)	1.02
Safety margin	1110

*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 general recognition of safety of the lysozyme enzyme preparation. The evaluation follows the generally recognized methodology and the decision tree by Pariza and Johnson (2001) (Appendix 1) and includes published information that provides the common knowledge element of the GRAS conclusion. Our safety evaluation in Part 6 follows the approach described in the Enzyme Technical Association publication (Sewalt et al 2016, Appendix 2) which 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 *T. reesei* production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (5) (6) (20). 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 (7). Pariza and Foster (5) 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”.

T. reesei has a long history of safe use in industrial scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. The original isolate, QM6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. A GRAS petition for Novozymes’ cellulase from *T. reesei* including a published safety article (21) was accepted for filing by FDA in 1979. A final rule was published by FDA in 1999 which resulted in the GRAS regulation 21 CFR 184.1250.

T. reesei is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC) and major culture collections worldwide (22). It is classified as a Biosafety Level 1 (BSL 1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines. BSL 1 microorganisms are not known to cause diseases in healthy adult humans.

Cellulases, hemicellulases, beta-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries (2). *T. reesei* strains are non-pathogenic for

healthy humans and animals (2). The safety of *T. reesei* has been discussed in several review papers (2) (23) (24). *T. reesei* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production.

All fungal species produce secondary metabolites to allow them to survive in nature. It is recognized that *T. reesei* is capable of producing a peptaibol compound (paracelsin) (23). However, the bulk of the literature investigating the capability of *T. reesei* to produce peptaibol is based on fermentation conditions designed either to mimic natural (and poor) growth conditions or attempt to optimize the conditions for secondary metabolite production. These methods are not representative of the conditions used in controlled industrial fermentation practices (25) (26) (23).

In 2012, the US EPA published a risk assessment (27) to support tiered exemption status for *T. reesei* QM6a and it's derivates. The EPA acknowledged in this assessment that under normal submerged fermentation conditions paracelsin is not produced. Novozymes has confirmed, by testing, that paracelsin is not produced by this production strain.

Enzyme preparations from *T. reesei* have been approved for use in food in Canada (Food and Drugs Act Division 16, Table V), France (Arrêté du 19 Octobre 2006), Denmark, Australia/New Zealand (Standard 1.3.3 processing aids), China, and Japan. More than 20 enzymes produced by *T. reesei* have been notified to FDA/CFSAN as GRAS for their intended uses (28). In addition, cellulase enzyme preparation from *T. reesei* is the subject of the regulation in 21 CFR §184.1250.

An evaluation of the genetically modified *T. reesei* production organism embodying the concepts initially outlined by Pariza and Foster, 1983 (5) and further developed by IFBC in 1990 (7), the EU SCF in 1991 (8), the OECD in 1993 (4), ILSI Europe Novel Food Task Force in 1996 (11), FAO/WHO in 1996 (10), JECFA in 1998 (17) and Pariza and Johnson in 2001 (6), demonstrates 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.

Novozymes' used the decision tree (Appendix 3) in Pariza and Johnson 2001 (6) as a basis for our safety assessment. The production strain is genetically modified as discussed in Part 2. The expressed enzyme product, lysozyme, has a history of safe use in food as discussed in Part 6 (c). The enzyme preparation is free of DNA encoding transferable antibiotic resistance DNA genes. 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.

Based on the information presented here it is concluded that the *T. reesei* production strain is considered a safe strain for the production of lysozyme enzyme (2).

6(b) Safety of the Donor Organism

The donor organism of the lysozyme gene is *Acremonium alcalophilum*. As indicated in Section 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the lysozyme 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 Lysozyme Enzyme

As indicated in Part 2, the subject of this GRAS notification is lysozyme, EC 3.2.1.17. Enzymes have a long history of use in food (6) (5) and animal feed (29). Enzyme proteins do not generally raise safety concerns (6) (5) . Pariza and Foster (5) note that very few toxic agents have enzymatic properties. The safety of the lysozyme was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 3).

Lysozyme is naturally occurring in most organisms including microorganisms, plants, animals and humans. It is found in human tears and human milk (30). It is present in foods that are part of the human diet such as turnip (31), cauliflower (32), and wheat germ (33). Lysozyme is found in high concentrations in chicken egg white (34) (35) Lysozyme, mainly from chicken egg white, is sold commercially by major dairy ingredient suppliers for use in cheese production (36). Lysozyme is also used in winemaking (37).

FDA did not raise any questions regarding the conclusion that egg white lysozyme is GRAS under the intended conditions of use described in GRAS notice 64 provided that the ingredient is labeled as egg white lysozyme to identify the source of the protein. Hen egg white lysozyme (part of the GH22 family of glycosyl hydrolases) is a known food allergen. However, this microbially derived lysozyme is a GH25 glycosyl hydrolase which did not show sequence homology to known allergens including hen egg white lysozyme (allergen Gal d 4 or UNIPROT:P00698). Allergenicity was evaluated as described in Section 6 (d) below.

A literature search was performed in March 2019 on lysozyme utilizing Scopus and the keywords “lysozyme”, “food”, “safety” and “toxicity”. Novozymes reviewed the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety for the lysozyme enzyme.

Based on the toxicological data provided in this notice and the fact that the enzyme and production strain have a history of safe use as indicated above, it is our

conclusion that this lysozyme is safe for its intended use as a processing aid in the manufacture of food ingredients.

6(d) Allergenic/Toxigenic Potential of the Lysozyme 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 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) (38).

Hen egg white lysozyme (part of the GH22 family of glycosyl hydrolases) is a known food allergen as it is one of the allergenic egg white proteins. However, this microbially derived lysozyme (subject of this notice) is a GH25 glycosyl hydrolase which did not show sequence homology to known allergens including hen egg white lysozyme (allergen Gal d 4 or UNIPROT:P00698).

In order to evaluate the possibility that the lysozyme 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 (39) and modified by Codex Alimentarius Commission, 2009 (40) the lysozyme 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.

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 and showed

no matches. Alignment of the lysozyme to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No homology was found between the lysozyme and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

A sequence homology of lysozyme produced by strain AGJG-20A2 to known toxins was assessed based on the information present in the UNIPROT database. This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 20% indicating that the homology to any toxin sequence in this database is random and very low.

On the basis of the available evidence it is concluded that oral intake of lysozyme produced by *T. reesei* is not anticipated to pose any food allergenic or toxigenic concerns.

6(e) Safety of the Manufacturing Process

The enzyme manufacturing process is based on generally available and accepted methods used for production of microbial enzymes (15) (13) (14).The lysozyme enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex* (16). As described in Section 2, the enzyme preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation.

6(f) Safety Studies

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

The following studies were performed on test batch PPL41125 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Human Lymphocyte Chromosome Aberration Assay
- 13 week oral toxicity study in rats

These tests are summarized in Appendix 4. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that the test preparation, represented by batch PPL41125 exhibits no toxicological effects under the experimental conditions described.

6 (f) (a) Description of test material

The toxicological testing of the lysozyme was conducted on a batch of lysozyme enzyme concentrate (batch PPL41125) which was produced according to the description given in section 2.3. The test batch is a lysozyme enzyme concentrate without addition of additives or other standardization or stabilization ingredients.

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 *T. reesei* and the limited and well-defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification, lysozyme 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

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3. Pariza and Johnson Decision Tree Analysis

4. Summary of Toxicity Data. Lysozyme, batch PPL41125, 20 March 2019, File 2019-05308-01

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Twenty-two pages have been removed in accordance with copyright laws. The removed references are:

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

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.

Appendix 3- This lysozyme enzyme preparation produced by *Trichoderma reesei* 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.

Department of Toxicology & Immunology

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SUMMARY OF TOXICITY DATA

Lysozyme, batch PPL41125

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1. ABSTRACT

The below series of toxicological tests were undertaken to evaluate the toxicological profile of Lysozyme, PPL41125. Lysozymes (EC 3.2.1.17) belong to the family of glycosyl hydrolytic enzymes. Lysozyme has been proposed for use as a processing aid in the manufacture of food ingredients from fermentation. It is added during the production process to degrade cellular debris and aid in the separation of the fermented food ingredient from the production organism.

All studies were carried out in accordance with OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Envigo, United Kingdom; Covance, United Kingdom; and Novozymes, Denmark, during the period of May 2016 to February 2017.

The main conclusions of the safety studies can be summarized as below:

- Lysozyme, batch PPL41125 showed no mutagenic activity in the Ames test (in the absence or presence of S-9), indicating no genotoxic potential.

- Lysozyme, batch PPL41125 did not induce chromosome aberrations in cultured human peripheral blood lymphocytes following treatment in the absence and presence of rat liver metabolic activation system (S-9) under the conditions tested.

- The oral administration of Lysozyme, batch PPL41125 to Han Wistar rats at doses up to 100% of the Lysozyme, batch PPL41125 for 13 weeks was well tolerated and did not cause any adverse change. The no-observed-adverse-effect level (NOAEL) was considered to be 100% of the Lysozyme, batch PPL41125 (equivalent to 1.132 g TOS/kg body weight/day or 384616 LSU(F)DV/kg body weight/day).

Based on the present toxicity data it can be concluded that Lysozyme, batch PPL41125 exhibits no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Lysozymes (EC 3.2.1.17) are glycoside hydrolases that cleave the β-1,4 glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan. The test item used for the toxicological studies was PPL41125, a light brownish and transparent liquid produced by *Trichoderma reesei*.

2.1 Characterization

The characterization of the test item is presented in Table 1.

Table 1. Characterization data of Lysozyme, batch no. PPL41125

Batch number	PPL41125
Physical description	Light brownish, transparent at room temp.
Main enzyme activity	36700 LSU(F)/g
N-Total	1.02% w/w
Water (Karl Fischer)	87.9% w/w
Dry matter	12.1% w/w
Ash (600ºC)	1.3% w/w
Total Organic Solids (TOS)#	10.8% w/w
Specific gravity	1.048 g/mL
pH	4.2
Total viable counts/g	200

#: TOS = 100 – (water + dry matter)

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

The test item Lysozyme, batch PPL41125, was tested for potential mutagenic activity using the Bacterial Reverse Mutation Assay in accordance to OECD guideline No. 471, adopted July 21st, 1997.

The experiments were carried out using histidine-requiring auxotroph strains *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and the tryptophan-requiring auxotroph strain of *Escherichia coli* (WP2uvrApKM101). Crude enzyme preparations like the present test substance, contain the free amino acids histidine and tryptophan in an amount that often exceeds the critical concentration for incorporation in the direct standard assay (plate incorporation method). To overcome this problem, all strains were exposed to the test substance in liquid culture (treat and plate assay). After incubation, the test substance was removed by centrifugation prior to plating.

The study was carried out in the absence and presence of metabolic activation system (S-9 mix). Two complete and independent experiments were performed. The bacterial strains were exposed to all six concentrations of Lysozyme (156, 313, 625, 1250, 2500, and 5000 µg TOS/mL), solvent control (sterile deionized water), and appropriate positive control substances. 5000 µg TOS/mL is the recommended maximum concentration according to current regulatory guidelines.
The test substance did not negatively affect the viability of any of the bacterial strains. The mean values of revertant colonies of the solvent control plates were within the general historical control range, and the reference mutagens showed the expected increase in the number of revertant colonies. Treatment of the five bacterial tester strains of *Salmonella typhimurium* and *Escherichia coli* with the test substance did not result in any increases in the number of revertant colonies that meet the criteria for a positive or equivocal response.
In conclusion, the test item Lysozyme, batch PPL41125, had no mutagenic activity in the examined bacterial strains under the test conditions of this study.

3.2 **In vitro** *human lymphocyte chromosome aberration assay*

Lysozyme was tested in an *in vitro* chromosome aberration assay conducted at Covance Laboratories Ltd (Harrogate, UK) to assess for clastogenic effects. The study was conducted in accordance with OECD Guideline 473 (2014).
Lysozyme, batch PPL41125 was tested in an *in vitro* chromosome aberration assay using duplicate human lymphocyte cultures prepared from the pooled blood of three male donors in a single experiment. 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 Chromosome Aberration Experiment were determined following a preliminary cytotoxicity Range-Finder Experiment. Treatments were conducted 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for chromosome analysis were selected by evaluating the effect of Lysozyme, batch PPL41125 on mitotic index. Chromosome aberrations were analyzed at three concentrations: 3000, 4000, and 5000 µg TOS/mL for 3+17 hour -S-9; 1000, 3000, and 5000 µg TOS/mL for 3+17 hour +S-9; 500, 2000, and 4000 µg TOS/mL for 20+0 hour -S-9.

Appropriate negative (vehicle) control cultures were included in the test system in the Chromosome Aberration Experiment under each treatment condition. The proportion of cells with structural

aberrations in these cultures fell within current historical vehicle control (normal) ranges. Mitomycin C (MMC) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of rat liver S-9 respectively. Cells receiving these were sampled 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural aberrations.

All acceptance criteria were considered met and the study was therefore accepted as valid. Treatment of cells with Lysozyme, batch PPL41125 in the absence and presence of S-9 resulted in frequencies of cells with structural chromosome aberrations which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analyzed. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within the 95th percentile of the current observed historical vehicle control (normal) ranges. No increases in the frequency of cells with numerical aberrations, which exceeded the normal range, were observed in cultures treated with Lysozyme, batch PPL41125 in the absence and presence of S-9.

It is concluded that Lysozyme, batch PPL41125, did not induce chromosome aberrations in cultured human peripheral blood lymphocytes following treatment in the absence and presence of rat liver metabolic activation system (S-9) under the conditions tested.

4. IN VIVO TOXICOLOGY

4.1 90-Day Oral Gavage Toxicity Study in Rats

Lysozyme, batch PPL41125 was tested in an *in vivo* study in accordance to OECD guideline No. 408, revised 1998, at Envigo (UK).

The purpose of this study was to assess the systemic toxic potential of Lysozyme, batch PPL41125, 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 Lysozyme, batch PPL41125 (equivalent to 0.113, 0.374 or 1.132 g TOS/kg body weight/day, or 38462, 126923 or 384616 LSU(F)DV/kg body weight/day). A control group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg body weight) as the treated groups.

The animals were grouped housed five of one sex per cage. Wood shavings were used as bedding and Aspen chew blocks and plastic shelters were provided as environmental enrichment. It was confirmed by analysis of samples that the test formulations were within a satisfactory range of the expected concentrations of the formulations prepared for administration in Week 1, 6 and 13 for the low, intermediate and high dose groups, confirming acceptable formulation.

During the study, clinical condition, detailed physical 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 behavior, sensory reactivity responses, grip strength and motor activity were not affected by treatment and there were no deaths. There was no effect of treatment on bodyweight gain or on food and water consumption. The hematology and blood chemistry investigations performed in Week 13 did not identify any toxicologically significant differences from controls. Organ weights were unaffected and there were no treatment-related macroscopic or microscopic findings.

It is concluded that the oral administration of Lysozyme, batch PPL41125 to Han Wistar rats at doses up to 100% of the Lysozyme, batch PPL41125 for 13 weeks was well tolerated and did not cause any adverse change. The no-observed-adverse-effect level (NOAEL) was considered to be 100% of the Lysozyme, batch PPL41125 (equivalent to 1.132 g TOS/kg body weight/day or 384616 LSU(F)DV/kg body weight/day).

5. REFERENCES

1. Lysozyme, batch PPL41125, Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli*. Novozymes A/S, Denmark, 25 November 2016. Study number 20168041.

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