Transglutaminase produced by *Bacillus licheniformis* carrying the gene coding for transglutaminase from *Streptomyces mobaraensis*

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

April 2023

PART 1- SIGNED STATEMENT AND CERTIFICATION

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 transglutaminase enzyme preparation 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 transglutaminase enzyme preparation is used as a processing aid during food manufacturing to catalyze the formation of an isopeptide bond between glutamine and lysine residues in proteins. This results in a protein gelling through an intra- and intermolecular crosslinking of proteins present in food matrices that contain milk, plant, or other proteins. The enzyme can be used in any food application where the protein that is present can be linked by the transglutaminase. Transglutaminase can be used in a wide range of applications such as processing of dairy foods (milk, fermented milk, yogurt, cheese) and plant-based dairy analogues including plant-based egg analogues, plant-based meat analogues, tofu, processed fish (non-Siluriformes only) and seafood and plant-based analogues. There are also uses in baking, cereal, and grain-based products. Finally, transglutaminase can be used in brewing and other cereal-based or plant-based beverages. 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.

April 3, 2023

Lori Gregg Date
Sr. Regulatory Affairs Manager

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 transglutaminase enzyme preparation produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism carrying the gene coding for transglutaminase from *Streptomyces mobaraensis*. The amino acid sequence has been determined by Novozymes.

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

Classification:	transglutaminase
Systematic name:	protein-glutamine:amine gamma-glutamyltransferase
EC No.:	2.3.2.13
CAS No.:	80146-85-6
Specificity:	formation of isopeptide bond between glutamine and lysine
Molecular weight:	38 kDa

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Bacillus licheniformis* production strain, designated JA4767, was derived via the recipient strain, SJ13672, 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 (1) (4) (5) (6) (7) (8) (9).

The transglutaminase expression plasmid pJA4762, used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced transglutaminase (*tgsSM-1*) is from *Streptomyces mobaraensis*.

2.2(b) Recipient Strain

The recipient strain SJ13672 used in the construction of the transglutaminase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding several proteases. Also, several genes were deleted including a gene essential for sporulation (eliminating the ability to sporulate), as well as additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these genes represents improvements in the product purity, safety, and stability.

2.2(c) Expression Plasmid

The expression plasmid, pJA4762, used to transform the *Bacillus licheniformis* recipient strain SJ13672 is based on the well-known *Bacillus* vector pE194 (10) and pUB110 (11) from *Staphylococcus aureus*. No elements of these vectors are left in the production strain. The introduced DNA consists of a fragment of a hybrid *Bacillus* promotor with promotor elements from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus thurigiensis*, the transglutaminase coding sequence and finally a transcriptional terminator.

The expression cassette is flanked by non-coding DNA sequences to enable targeted integration of the transforming DNA into the genome of the recipient strain. 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 PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Bacillus licheniformis* JA4767, was constructed from the recipient strain SJ13672 through the following steps:

1. Plasmid pJA4762 was integrated into specific loci in strain SJ13672 by targeted homologous recombination. Targeted integration of the expression cassettes at the loci allows the expression of the transglutaminase gene *tgsSM-1* from the promoter.

The resulting transglutaminase strain containing three copies of the *tgsSM-1* gene was named JA4767.

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 DNA is integrated into the *Bacillus licheniformis* chromosome. Thus, it is as such poorly mobilized for genetic transfer to other organisms and is considered mitotically

stable. The phenotypic and genetic stability of the *Bacillus licheniformis* is proven by its capacity to produce a constant level of the transglutaminase enzyme. This was assessed by measuring the enzyme activity in three independent batches of the food enzyme, as outlined in Table 2 below.

Furthermore, the protein spectrum for 3 batches showed identical expression profiles. Thus, stable enzyme production of the desired enzyme combined with the identical protein expression profile in 3 batches confirm the stability of the *Bacillus licheniformis* production strain.

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

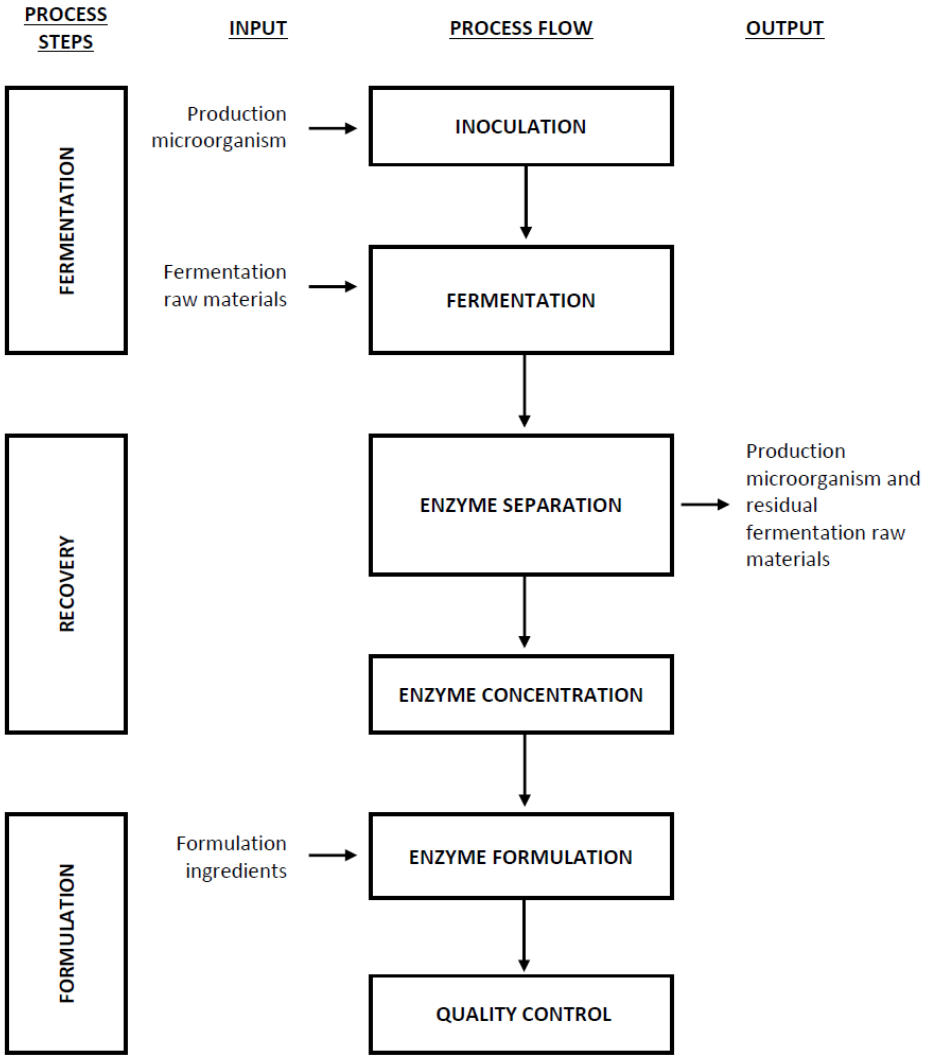
The absence of the production organism is an established specification for the commercial product.

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the transglutaminase 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.

A flow chart of the standard manufacturing process for fermentation-based enzymes is included below:[1]

[1] Figure taken from the Enzyme Technical Association white paper, “Standard Manufacturing Process for Fermentation-based Enzymes Used in Food.” Available at: https://www.enzymetechnicalassociation.org/wp-content/uploads/2021/08/ETA-Standard-Manufacturing-Process-for-Fermentation-based-Enzymes-Used-in-Food.pdf



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, food safe 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 to ensure suitability and acceptability of use in food enzyme production. As part of the overall Quality program, Novozymes Quality Department follows a raw material approval program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt. 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 in the product is not greater than 1%.

2.3(b) Fermentation Process

The transglutaminase enzyme preparation is produced by pure culture, submerged, fed-batch fermentation of the genetically modified strain of *Bacillus licheniformis* described in Part 2.

During fermentation, the transglutaminase enzyme that is produced by *Bacillus licheniformis* is secreted into the fermentation media.

All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. Physical and chemical control measures are applied during all steps of fermentation. Microbiological analyses are conducted to ensure absence of foreign microorganisms and to 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.

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.

2.3(f) Formulation Process

The formulation process consists of preservation and stabilization of the liquid enzyme concentrate. The enzyme concentrate is stabilized with glycerol and formulated by addition of water. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

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

2.4(a) Typical Composition

Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives, and inert raw materials used in the transglutaminase enzyme preparation. 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*)	3%
Glycerol	60%
Water	37%

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

2.4(b) Specifications

The transglutaminase enzyme preparation complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex* (15). These requirements include limits on impurities, microbial limits, use of food safe ingredients, and a requirement for production in accordance with good manufacturing practices. 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.[2]

Table 2. Analytical data for three food enzyme batches

Parameter	Specifications	PPN88445	PPN88446	PPN88485
Transglutaminase activity	TGHU(A)/g	963	917	1160
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
Production Organism	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is transglutaminase (EC 2.3.2.13, CAS 80146-85-6) which is the common name for this acyl-transfer catalyzing enzyme also known as protein-glutamine γ-glutamyltransferase. Transglutaminase catalyzes the formation of an ε-(γ-glutamyl) lysine bonds between the carboxyamide group of protein glutamine residues with the ε-amino group of protein lysine residues (17). This results in protein gelling through an intra- and intermolecular crosslinking of proteins present in food matrices that contain milk, plant, or other proteins.

Transglutaminase is used in manufacturing a wide range of food products where protein gelling can:

- Increase viscosity
- Reduce syneresis (whey separation) in dairy products
- Improve separation efficiency and increase yields

[2] These batches are registration batches designed to show the consistency of the commercial manufacturing process, and therefore the specification values may not align with commercial requirements. For example, the average activity level in commercial batches is 200 TGHU(A)/g.

- Reduce the need for addition of texturizing agents and/or starches
- Increase strength of the protein network resulting in unique textures and firmness (*i.e.*, tofu)
- Improve juiciness and reduce cooking loss in meat analogues

Transglutaminase can be used in a wide range of industries in the processing of dairy foods (milk, fermented milk, yogurt, cheese) and plant-based dairy analogues including plant-based egg analogues, plant-based meat analogues, tofu, processed fish (non-Siluriformes only) and seafood, and plant-based analogues. There are also uses in baking, cereal and grain-based products and pasta and noodles. Finally, transglutaminase can be used in brewing and other cereal-based or plant-based beverages. In plant-based analogues the sources of protein can be a wide range of ingredients such as: fruits, vegetables, grains (soy, oat, wheat, ancient grains, amaranth, quinoa, millet), legumes (pea, chickpea), seeds (nuts), fava beans, and yeast.

2.5(b) Use Level

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for production of the final food product following cGMP. The dosage applied in practice by a food manufacturer depends on the particular process. It is based on an initial recommendation by the enzyme manufacturer and optimized to fit the product and process conditions.

Typical use level in most applications is 2 to 8 TGHU (A) per gram protein. The maximum dose applied is typically 8 TGHU(A) per gram protein.

2.5(c) Enzymes Residues in the Final Food

In most cases, 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. Transglutaminase exerts its technical effect during the manufacturing of foods but is expected to have no continued technical or functional effect in the final food product. In many cases, the enzyme will be inactive due to a heat treatment during processing of the final food or due to the pH of the final food product.

PART 3 - DIETARY EXPOSURE

The transglutaminase can be used in a variety of applications.

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.

3.1 ESTIMATES OF HUMAN CONSUMPTION AND SAFETY MARGIN

The exposure assessment is based on the Budget Method (18) which represents a “maximum worst case” situation of human consumption. The assumptions are highly exaggerated since the enzyme protein and the other substances are often diluted or removed during the manufacture of the final food products. 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.

3.2 FOOD CONSUMPTION DATA ASSUMPTIONS IN THE BUDGET METHOD

The transglutaminase concentrate has an average activity of 200 TGHU(A)/g and approximately 3% TOS (Total Organic Solids) content. This is 30 mg TOS in 200 TGHU(A) per gram enzyme preparation. A dose rate of 8 TGHU(A) per gram protein has 1.2 mg TOS per gram protein or 1200 mg TOS/ kg protein.

Solid Food: The maximum energy intake for adults 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.

Liquids: The maximum intake of (non-milk) liquid 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. It is assumed that the densities of the beverages are ~ 1.

The following table provides the enzyme dose level and assumptions for several applications:

Application	Enzyme Dose (TGHU/g protein)*	Assumed % protein in food*	Budget method Calculation for liquid or solids	mg TOS/kg bw/day
Dairy beverages and plant-based analogues	8	3.4	25 ml processed beverages/kg bw/day X 3.4%=0.85 and (1200mg TOS/kg protein:1000 g) x 0.85 g protein/kg bw/day	1.0
Yogurt and plant-based analogues	5	4	12.5 g processed food/kg bw/day x 4% protein = 0.5 g protein/kg BW/day and (750 mg TOS/kg protein:1000 g) x 0.5 g protein/kg bw/day	0.375
Tofu	4	8	12.5 g processed food/kg BW/day x 8% protein = 1 g protein/kg BW/day and (600 mg TOS/kg protein:1000 g) x 1 g protein/kg bw/day	0.6
Baking	66.67 TGHU/kg flour	1kg flour produces average 1.6 kg bread	10 mg TOS/1.6 kg bread = 6.25 mg TOS/kg bread and (6.25 mg TOS/kg bread:1000 g) x 12.5 g/kg bw/day	0.08
Pasta	100 mg TOS/kg pasta flour	1kg pasta flour produces ~3.8 kg pasta	100 mg TOS/3.8 kg bread = 26.3 mg TOS/kg pasta and 26.3 mg TOS/kg bread:1000 g) x 12.5 g/kg bw/day	0.3
Processed fish (non-Siluriformes only) and seafood	5	20	12.5 g processed food/kg bw/day x 20% protein = 2.5 g protein/kg bw/day and 750 mg TOS/kg : 1000 g) x 2.5 g protein/kg bw/day	1.9
Plant-based meat and fish analogues	5	20	12.5 g processed food/kg bw/day x 20% protein = 2.5 g protein/kg bw/day and 750 mg TOS/kg:1000 g) x 2.5 g protein/kg bw/day	1.9
Brewing	5 mg TOS/kg beer	-	Assuming that 25% of the beverages is processed, the daily consumption will be 25 ml processed beverages/kg bw. (5 mg TOS/kg beer:1000 g) x 25 g/kg bw/day	0.125
Plant based egg substitute	5	12	12.5 g processed food/kg bw/day x 12% protein = 1.5 g protein/kg bw/day and 750 mg TOS/kg:1000 g) x 1.5 g protein/kg bw/day	1.1

*except where noted otherwise *i.e.*, baking, pasta, brewing

Theoretical Maximum Daily Intake (TMDI)

As the enzyme intake from fish (non-Siluriformes only), plant-based meat and plant-based fish analogues and dairy beverages represent the highest contribution from solid foods and liquids, respectively, the theoretical enzyme intake, the TMDI of the food enzyme is:

1.9 mg TOS/kg bw/day from solid foods (fish (non-Siluriformes only), plant-based meat and plant-based fish analogues) + 1.0 mg TOS/kg bw/day from liquids (dairy beverages) = 2.9 mg TOS/kg bw/day

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 transglutaminase, PPN81685 was the highest dosage possible, 372 mg TOS/kg bw/day. See the *Summary of Toxicity Data*, Appendix 1, included in this submission and Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	372
*TMDI (mg TOS/kg bw/day)	2.9
Safety margin	128

*based on the worst-case scenario

CONCLUSION

As demonstrated by the calculations above, the TMDI is 2.9 mg TOS/kg bw/day, which is far below the NOAEL level in the chronic toxicity studies, which was 372 mg TOS/kg bw/day. Given that the Budget Method uses a conservative estimate of exposure, it is likely that the actual daily intake is even lower. Therefore, the anticipated exposure to the transglutaminase is well below the safe limit demonstrated in toxicity studies.

PART 4 - SELF-LIMITING LEVELS OF USE

Enzyme preparations are generally used in quantum satis. While the average dosage of the enzyme depends on the type and quality of the raw materials used, the process conditions, and the nature of the final food product, excessive amounts of enzyme would be expected to adversely affect the organoleptic and/or functional properties of the food.

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 conclusion of the general recognition of safety for the transglutaminase enzyme preparation. The evaluation follows generally recognized methodology (1) (4) (6) (7) (8) (9) and the decision tree by Pariza and Johnson 2001 (3). Our safety evaluation in Part 6 follows the approach described by Sewalt *et al.,* 2016 (19) which includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme, and the manufacturing process. In addition to the factors outlined in the decision tree below, toxicology studies were also conducted to corroborate the safety of the enzyme.

6.1 DECISION TREE

This transglutaminase enzyme preparation produced by *Bacillus licheniformis* was evaluated according to the decision tree published in Pariza and Johnson, 2001 (3). The result of the evaluation is presented below in the 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.
3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
YES
If yes, go to 3c.

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

3e. 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
If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
NO
If 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
Test article is accepted

6.2 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). If the organism is non-toxigenic and non-pathogenic, the enzyme products that are derived from the organism are safe, assuming they are made under Good Manufacturing Practices, and would not introduce a safety risk into food products that incorporate these enzymes during manufacture. 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). *Bacillus licheniformis* is not a human pathogen and it is not toxigenic (20).

Bacillus licheniformis is widely distributed in nature. It is a Class 1 organism according to the NIH guidelines: Guidelines for Research Involving Recombinant DNA Molecules, Federal Register, Dec.19, 2001 (66 FR 57970). Risk Group 1 organisms are those not associated with disease in healthy adult humans. In addition, it has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority (EFSA) (21). EFSA reviews the available information for strains, evaluating the strains for safety with regard to humans, animals, and the environment. The qualifications reviewed for *Bacillus licheniformis* include the lack of antibiotic resistance genes in the strain, and the absence of toxigenic activity. *Bacillus licheniformis* was also included in a publication by staff at the US Food and Drug Administration (FDA), which characterized the strain as having a long history of safe use, and identified the strain as non-pathogenic and distinct from other *Bacillus* species considered pathogenic (22). Finally, the strain was evaluated by the Environmental Protection Agency, and was included on its list of recipient microorganisms for tiered exemption, as it found the use of the strain in industrial settings presents no unreasonable risk, and a low risk of adverse effects to human health or the environment (5).

Bacillus licheniformis has a long history of safe industrial use in the production of enzymes used in human food. It has been used by Novozymes for the production of food grade enzymes for more than 30 years. *Bacillus licheniformis* is used in the fermentation industry for the production of enzymes, many of which have been reviewed by the US Food and Drug Administration and are considered GRAS substance (GRNs 265, 277, 472, 572, 587, 645, 689, 728, 774, 975) (23).

The *Bacillus licheniformis* used as the production strain for this transglutaminase is genetically modified by rDNA techniques. 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.

The enzyme preparation is free of DNA that may encode transferable, antibiotic resistance DNA. The genetic modifications of this *Bacillus licheniformis* are well characterized and specific utilizing well-known plasmids for the vector constructs, and

the introduced genetic material is not known to encode or express any harmful or toxic substances.

In conclusion, the safety of the production organism has been reviewed by multiple governmental agencies and is widely used in the production of enzymes for food products. As noted by Pariza and Johnson and reaffirmed by Olempska-Beer, the critical component of an enzyme safety assessment is the pathogenic and toxigenic potential of the production organism (2) (3) (22). *Bacillus licheniformis* is non-pathogenic and non-toxic, and its use in the production of the transglutaminase enzyme does not present a safety concern.

6.3 SAFE STRAIN LINEAGE

The safety of this *Bacillus licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4). The concept of the safe strain lineage has been widely adopted in the enzyme industry and includes an assessment and characterization of the host organism, an assessment of the safety of introduced DNA, and an assessment of the procedures used to create the strain.

Novozymes has used *Bacillus licheniformis* as a production strain for a variety of enzymes for decades. Table 4 below outlines some of Novozymes enzyme preparations produced by *Bacillus licheniformis* production strains within the safe strain lineage and the safety studies conducted on those enzyme concentrates.

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Alpha-amylase (GRASP 0G0363)	3.2.1.1	*Bacillus licheniformis* Si3	*Bacillus stearothermophilus*	Yes
Alpha-amylase (GRN 22)	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Cyclodextrin glucanotransferase	2.4.1.19	*Bacillus licheniformis* SJ1707	*Thermoanaerobacter sp.*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1904	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus stearothermophilus*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus amyloliquefaciens*	Yes
Serine protease (GRN 564)	3.4.21.1	*Bacillus licheniformis* MDT223	*Nocardiopsis prasina*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Xylanase (GRN 472)	3.2.1.8	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
L-Glutaminase (GRN774)	3.5.1.2	*Bacillus licheniformis MDT223*	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis PP3579*	*Bacillus licheniformis*	Yes

Beta-amylase	3.2.1.1	*Bacillus licheniformis PP3579*	*Bacillus flexus*	Yes

Table 4. Novozymes products derived from *B. licheniformis* strains. [1]The predecessor strains show common strains in the GM construction pathway. [2]At least the following: in vitro test for gene mutations in bacteria (Ames); in vitro test for chromosomal aberration or in vitro micronucleus assay; 13-week sub chronic oral toxicity study in rats. The conclusions of these studies were in all cases favorable.

The *Bacillus licheniformis* production strain used in this notice is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (*i.e.,* 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay). All toxicological studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view that strains derived from the *Bacillus licheniformis* strain lineage can be used safely for the production of food enzymes.

The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed transglutaminase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized by Novozymes, and free of any sequences that would render the strain unsafe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (3) have been met.

The fact that no issues are observed in safety studies on different enzymes (e.g., amylases, protease etc.) produced by strains derived from a common predecessor to the strain used in this GRAS notice (*B. licheniformis* MDT233), strongly supports the safety of the *B. licheniformis* strain lineage, independent of which enzyme is produced.

Based on the information presented in Parts 6 (a) (b) and (c), it is concluded that the *Bacillus licheniformis* production strain is part of a safe strain lineage and is considered a safe strain for the production of the transglutaminase enzyme.

6.4 SAFETY OF THE DONOR ORGANISM

The donor organism of the transglutaminase enzyme is *Streptomyces mobaraensis*. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the transglutaminase 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.

Streptomyces mobaraensis, also known as *Streptoverticillum mobaraense*, has been included in multiple GRAS notices reviewed by the FDA, and provided with No

Questions letters.[3] Further, FDA reviewed and issued a No Questions letter for a transglutaminase enzyme produced in *Streptomyces mobaraensis* in 2022, in GRN 1021, Transglutaminase from *Streptomyces mobaraensis* M2020197.

6.5 SAFETY OF THE TRANSGLUTAMINASE ENZYME

As Pariza and Johnson noted, enzymes generally do not raise safety concerns, and few toxic agents have enzymatic properties (2) (3). The subject of this GRAS notification is transglutaminase, EC 2.3.2.13. Enzymes, including transglutaminase, have a long history of use in food. Transglutaminase from *Streptoverticillum mobaraense* (synonym for *Streptomyces mobaraensis*) is the subject of 5 GRAS notices submitted to FDA from 1998 to 2022. Further, transglutaminase is recognized in the Food Chemical Codex monograph on Enzyme Preparations (15).

As FDA authors noted, “Enzymes naturally present in the human diet have not been associated with toxicity and are considered intrinsically safe.” (22) Humans have consumed foods containing transglutaminase since ancient times in the form of raw fish, oysters, fruits, and vegetables. Transglutaminase occurs naturally in fish (such as pollack, mackerel, and sardines) that are used in the manufacture of traditional Japanese kamaboko, which has been consumed by humans since the 14th century. The intrinsic transglutaminase in surimi forms crosslinks between myosin which gives elasticity to the kamaboko (24) (25) (26). Additionally, transglutaminase is present in most animal tissues and body fluids and is involved in biological processes such as blood clotting and wound healing (17).

Novozymes completed an extensive literature search using Medline, ToxCenter, SciSearch, Chemlist, Scopus and a Google Scholar. Key words such as “transglutaminase”, “toxicity”, “human consumption”, “food” and others, were used for the search. The literature search produced no health or safety issues associated with the use of the transglutaminase enzyme from Bacillus licheniformis for the intended uses listed in Section 2.5.

A general recognition of safety assessment must include a summary of the positive literature, but also the potential negative information that exists about a substance. Novozymes is aware of a number of review articles (27) (28) (29) (30) (31) (32) (33) (34), all from the same research group lead by Aaron Lerner and Torsten Matthias, all of which speculate on a link between microbial transglutaminase (mTGase) use in food processing and celiac disease. Importantly, these articles are all review articles or theoretical in nature, and the authors have not presented data to support their hypothesis. In fact, the authors themselves state that these hypotheses need substantiation, and that further studies are needed, none of which have been conducted. These articles only present a hypothesis that mTGase may be a new environmental factor for the induction of celiac disease, based on an increased incidence of the disease which they purport aligns with increased food additive use, and in particular,

[3] See GRAS Notices 4, 29, 55, 95, 1021.

microbial transglutaminase. The authors seemingly ignore other potential confounding factors, such as increased detection methods, other dietary changes, and increased disease awareness. Furthermore, they do not consider the fact that most enzymes are inactivated in the final food product and are often subject to rapid degradation in the stomach after exposure to pepsin, as noted with transglutaminase (35). The authors base their hypotheses on an artificial scenario in which the mTGase remains in the stomach intact, defying critical biological processes that come with eating food, such as digestion and metabolism.

Despite the fact that these hypotheses are unsubstantiated and detached from the realities of actual use of the enzyme, in the interest of safety, Novozymes collaborated with a pediatric gastroenterologist with a longstanding interest in the immunology and epidemiology of celiac disease to evaluate the relevance of the concern regarding using TGase as a food processing aid. He is a medical doctor and research professor with more than 185 scientific papers related to celiac disease. A careful review of the current body of knowledge on TGase and the epidemiology of celiac disease gives no reason for toxicological concern. The assessment of Novozymes Toxicology Department and the gastroenterologist are presented in Appendix 2.

In summary, a review of the literature, and the results of the toxicology studies reveal that microbial transglutaminase has a history of safe use in food and the proposed use of the microbial transglutaminase from *Streptomyces mobaraensis* in food products does not present a safety concern.

6.6 ALLERGENIC/TOXIGENIC POTENTIAL OF THE TRANSGLUTAMINASE ENZYME

Enzymes have a long history of safe use in food. They are typically added in foods at very low concentrations, often in the 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. Transglutaminase has been specifically shown to be sensitive to pepsin found in the stomach, with complete cleavage of microbial transglutaminase from *S. mobaraense* within 1 min at a pH of 2.5 (35). In fact, a study of 19 commercial enzymes used in the food industry declared after extensive allergy testing in 400 allergic individuals that ingestion of food enzymes does not raise concerns of food allergy (36).

In order to further evaluate the possibility that the transglutaminase 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 (37) and modified by Codex Alimentarius Commission, 2009 (38) the transglutaminase was compared to allergens from the Food Allergy Research and Resource Program (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 transglutaminase with greater than 35% identity over the full length of the alignment was also analyzed. No homology to food allergens was found between the transglutaminase 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.

Additionally, Pedersen and colleagues has reported a thorough evaluation of the potential allergenicity of *S. mobaraense* transglutaminase (m-TG), using the FAO/WHO decision tree (35). In agreement with Novozymes' risk assessment, they reported a database search with no homology between transglutaminase and known allergens, down to a match of six contiguous amino acids, which meets the requirements of the FAO/WHO decision tree. However, they found a match of five contiguous amino acid to Gad c1 – a codfish allergen. The potential cross reactivity between m-TG and Gad c1 was investigated in radioallergosorbant test (RAST) using sera from 25 documented cod-allergic patients and an extract of raw codfish. No binding between patient IgE and m-TG was observed. Pedersen et al., concluded that no safety concerns with regard to the allergenic potential of m-TG (35).

Also, a search for homology of the transglutaminase sequence to known toxins was assessed based on the information present in the UNIPROT database (2021-02-15). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 18% indicating that the homology to any toxin sequence in this database is low and random.

Consequently, ingestion of food containing transglutaminase is not anticipated to pose any food allergenic or toxin concerns due to the presence of the enzyme.

6.7 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process for the transglutaminase is described in section 2 and follows standard industry practices (12) (13) (14). Only food-grade raw materials are used, and strict specifications are imposed on the product to ensure purity and safety.

The quality management system used in the manufacturing process for the transglutaminase 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).

6.8 SAFETY STUDIES

Studies Conducted with Other Bacillus licheniformis Products as Part of the Safe Strain Lineage

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate enzymes derived from Bacillus licheniformis production strains (3). As described in Part 6(c), Novozymes has concluded, that strains within the safe strain lineage of Bacillus licheniformis pose no safety concerns. Table 4 lists the strains within this lineage, with many having corresponding GRNs on file with the FDA, where toxicological safety studies have been performed.

The toxicological studies include genotoxicity, cytotoxicity, and general toxicity tests. These toxicology studies have produced consistent findings indicating that the test article (enzyme concentrate) did not exhibit any toxic or mutagenic effects under the conditions of the test, thus supporting the safety of the enzymes produced by Bacillus licheniformis strains that are within this lineage.

It is reasonable to expect and conclude that enzymes produced by Bacillus licheniformis strains within this safe strain lineage will show similar toxicological profiles and further supports our conclusion that Bacillus licheniformis strains are safe hosts for the expression of enzymes (20) (39).

Safety Studies with the Transglutaminase Enzyme Preparation

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

The results from those studies indicate that the enzyme concentrates did not exhibit any toxic or mutagenic effects under the conditions of the test.

Additionally, Novozymes conducted the following safety studies on the transglutaminase enzyme, subject of this notification, concentrate test batches PPN66927 and PPN81685:

- Bacterial Reverse Mutation Assay (Ames test), batch PPN66927
- *In vitro* Micronucleus Test in Cultured Human Lymphocytes, batch PPN66927
- 13-week oral toxicity study, batch PPN81685

All toxicology studies performed on the transglutaminase enzyme concentrate were carried out in accordance with current OECD guidelines and in compliance with the

OECD principles of Good Laboratory Practice (GLP). A summary of the toxicology studies is included below, and a summary report is included as an Appendix.

Mutagenicity:
Bacterial Reverse Mutation assay (Ames test)

In this *in vitro* assessment of the mutagenic potential of transglutaminase batch PPN66927, histidine-dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 uvrA (pKM101), were exposed to transglutaminase batch PPN66927, according to the treat-and-wash method. Two independent mutation tests were performed, with concentrations of up to 5000 µg TOS/mL transglutaminase tested.

No signs of toxicity towards the tester strains were observed in either mutation test following exposure to transglutaminase. No evidence of mutagenic activity was seen at any concentration of transglutaminase, Batch PPN66927 in either mutation test. It was concluded that transglutaminase, Batch PPN66927, showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

In vitro Micronucleus Test in Cultured Human Lymphocytes

This study was designed to assess the potential of transglutaminase batch PPN66927 to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes in vitro.

The study consisted of a preliminary toxicity test and a main micronucleus test. Human lymphocytes in whole blood culture, were exposed to Transglutaminase, batch PPN66927 for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 3000 µg TOS/mL, dosed at 10% v/v.

In both the absence and presence of S9 mix, following 3-hour treatment, and in the absence of S9 mix, following 20-hour treatment, transglutaminase batch PPN66927 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls. There was no evidence of any dose-concentration relationship and all the mean micronucleus frequencies for the vehicle and test item treated cultures were within the laboratory historical 95% confidence limits. These results all fulfilled the criteria for clearly negative results.

It was concluded that transglutaminase batch PPN66927 did not show evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this in vitro test system under the experimental conditions described.

13-Week Toxicity Study
The objective of this study was to assess the systemic toxic potential of transglutaminase batch PPN81685 (an enzyme used in the food industry) when

administered in the diet to Han Wistar rats for 13 weeks.

Three groups, each comprising ten male and ten female Han Wistar rats, received transglutaminase batch PPN81685 in the diet at concentrations of 16448, 32895 and 49343 ppm (equivalent to 1875, 3750 and 5625 ppm in terms of TOS). The overall mean achieved dosages were 122, 244 and 372 mg TOS/kg bwt/day in males and 138, 292 and 447 mg TOS/kg bwt/day in females (equivalent to 122, 2761 and 4209 TGHU(A)/kg bwt/day in males and 1562, 3304 and 5058 TGHU(A)/kg bwt/day in females, respectively). A similarly constituted control group received the basal diet containing water at the same concentration as the high group (49343 ppm, or ~4.9%).

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

Results

The general appearance and behavior of the animals and sensory activity, grip strength and motor activity were unaffected by treatment. There were no premature deaths associated with Transglutaminase, batch PPN81685 (one high dose male died during the study but the death was not attributable to treatment). There was no effect of treatment on body weight gain or food and water consumption. There were no treatment-related ophthalmoscopic findings. The haematological and blood chemistry investigations did not indicate any toxicologically significant findings. Estrous cycles at the end of the treatment period were unaffected. There was also no effect of treatment on testicular pathology. Serum triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations were unaffected by treatment. Organ weights were unaffected and there were no treatment-related macroscopic or microscopic findings.

It was concluded that the dietary administration of transglutaminase batch PPN81685 to Han Wistar rats at concentrations up to 5625 ppm (in terms of TOS) for 13 weeks was well-tolerated, with no evidence of any adverse finding at any of the administered dietary concentrations. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 5625 ppm TOS which achieved overall dosages of 372 mg TOS/kg bwt/day for males and 447 mg TOS/kg bwt/day for females, equivalent to enzyme activities of 4209 TGHU(A)/kg bwt/day for males and 5058 TGHU(A)/kg bwt/day for females.

Based on the presented toxicity data, the history of safe use of the enzyme and the safe strain lineage of the Bacillus licheniformis production strain, it can be concluded that the test preparations exhibit no toxicological effects under the experimental conditions described in the summary.

6.9 RESULTS AND CONCLUSION

Novozymes has reviewed the available data and information on transglutaminase from *Streptomyces mobaraensis* produced by *Bacillus licheniformis*, and 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, and the long history of safe use of transglutaminase in food products as noted in published literature and through the No Questions letters granted to multiple GRAS notifications for similar preparations, Novozymes concludes through scientific procedures that the subject of this notification; transglutaminase enzyme preparation, which meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices 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

APPENDIX

1. Summary of Toxicity Data, Transglutaminase Batch PPN66927 and Batch PPN81685 File: 2022-07788-01
2. Novozymes' Assessment of Literature Linking Transglutaminase and Celiac Disease.

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Toxicology

Date: March 10th, 2023
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Ref.: MITH

SUMMARY OF TOXICITY DATA

Transglutaminase, Batch PPN66927 and Batch PPN81685

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CONTENTS

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Transglutaminase, represented by batch PPN81685 and PPN66927.

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 Labcorp (UK) during the period May 2020 to April 2022.

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

- Transglutaminase, Batch PPN66927 did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Transglutaminase, Batch PPN66927 did not induce 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).
- In a 13-week oral toxicity study in rats Transglutaminase, Batch PPN81685 was well tolerated and did not cause any toxicologically significant changes at any dose level applied.

Based on the present toxicity data it can be concluded that Transglutaminase represented by Batch PPN66927 and PPN81685, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

2.1 Characterization

Transglutaminase toxbatch PPN81685 and PPN66927, were used in the toxicological studies. The characterization data of the toxbatches are presented in Table 1 and 2.

Table 1. Characterization data of Transglutaminase, batch PPN66927

Test substance ID and storage	
Test substance name/Product:	Transglutaminase
Batch/PP code:	PPN66927
Production strain:	*Bacillus licheniformis*
IUBMB/E.C.:	2.3.2.13
Storage conditions:	<÷ 10ºC (i.e. lower than minus 10°C)
Expiry date:	20-November-2029
Appearance:	Light brownish, transparent liquid that precipitate over time at room temperature
Analytical results	
Main enzyme activity:	478 TGHU(A)/g
N-Total:	0.74 % w/w
Water (**K**arl **F**ischer):	88.5 % w/w

Dry matter (Calculated [§]):	11.5 % w/w
Ash (600°C):	4.0 % w/w
Total **O**rganic **S**olids (Calculated [#]):	7.5 % w/w
Density:	1.060 g/mL
pH:	7.02
Total viable counts:	<1.0 x 10^2 CFU/g

[§] Dry matter = 100 - (Water (KF))
[#] **TOS** = 100 - (Water (KF) + Ash + *****diluent)

Initial Stability Assessment

The test substance has been examined for short term stability as a 10% and 100% solution in water at room temperature for up to 24 hours, in refrigerator for up to 24 hours and in the freezer for up to 168 hours. The stability was assessed based on enzyme activity analysis. It was concluded that the test substance was considered stable under the test conditions applied The result for sample '10% - 4 hours at room temperature' was 0.04% outside the acceptance criteria; however, as sample '10% - 24 hours at room temperature' was found to be stable this small deviance was concluded to be incidental.

The analytical results listed above are obtained from analysis performed in accordance with GLP regulations and standards. At the time of issue of the present certificate data has not yet been QA audited. Results will be verified in the final report of characterization, NZ Study No. 20208001.

Table 2. Characterization data of Transglutaminase, batch PPN81685

Test substance ID and storage	
Test substance name/Product:	Transglutaminase
Batch/PP code:	PPN81685
Production strain:	*Bacillus licheniformis*
IUBMB/E.C.:	2.3.3.13
Storage conditions:	<÷ 10ºC (i.e. lower than minus 10°C)
Expiry date:	10-March-2031
Appearance:	Light brown transparent liquid, with precipitate at room temperature
Analytical results	
Main enzyme activity:	1290 TGHU(A)/g
N-Total:	1.58 % w/w
Water (**K**arl **F**ischer):	87.3 % w/w
Dry matter (Calculated [§]):	12.7 % w/w
Ash (600°C):	1.3 % w/w
Total **O**rganic **S**olids (Calculated [#]):	11.4 % w/w
Density:	1.041 g/mL
pH:	6.80
Total viable counts:	<100 CFU/g

[§] Dry matter = 100 - (Water (KF))
[#] **TOS** = 100 - (Water (KF) + Ash + *****diluent)

The test item was examined for stability. All samples were exposed to two thaw-freeze cycles but under different conditions:

- As 100% solutions for 8 or 28 hours at room temperature or in a refrigerator followed by at least 192 hours on frost. Then another 8 or 28 hours at room temperature or in a refrigerator followed by up to 192 hours on frost.
- As 10% solutions for 8 or 28 hours at room temperature or in a refrigerator followed by at least 192 hours on frost. Then another 8 or 28 hours at room temperature or in a refrigerator followed by up to 192 hours on frost.

After these freeze-thaw cycles, the stability was assessed based on enzyme activity of the solutions.
It can be concluded that the test item is stable under test conditions applied.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

In this *in vitro* assessment of the mutagenic potential of Transglutaminase, Batch PPN66927, histidine-dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 *uvr*A (pKM101), were exposed to Transglutaminase, Batch PPN66927 diluted in water. Water was also used as a vehicle control.

The mutation tests were performed according to the treat-and-wash method. They were performed in the presence and absence of liver preparations (S9 mix) from rats treated with phenobarbital and 5,6-benzoflavone. Two independent mutation tests were performed.

Concentrations of Transglutaminase, Batch PPN66927 up to 5000 µg TOS/mL were tested. Other concentrations used were a series of *ca* half-$\log_{10}$ dilutions of the highest concentration.

No signs of toxicity towards the tester strains were observed in either mutation test following exposure to Transglutaminase, Batch PPN66927. No precipitate was observed on any plates exposed to Transglutaminase, Batch PPN66927.

No evidence of mutagenic activity was seen at any concentration of Transglutaminase, Batch PPN66927 in either mutation test.

The concurrent positive controls verified the sensitivity of the assay and the metabolizing activity of the liver preparations. The mean revertant colony counts for the vehicle controls were within the current historical control range for the laboratory.

It was concluded that Transglutaminase, Batch PPN66927 showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

3.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

This study was designed to assess the potential of Transglutaminase, batch PPN66927 to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes in vitro.

The study consisted of a preliminary toxicity test and a main micronucleus test. Human lymphocytes in whole blood culture, were exposed to Transglutaminase, batch PPN66927 for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 3000 µg TOS/mL, dosed at 10% v/v, in order to test up to the highest concentration which did not alter the osmolality by more than 50 mOsmol/kg when compared to the vehicle control. Vehicle (water; purified by reverse osmosis) and positive control cultures were included in all appropriate test conditions.

In both the absence and presence of S9 mix, following 3-hour treatment, and in the absence of S9 mix, following 20-hour treatment, Transglutaminase, batch PPN66927 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls. There was no evidence of any dose-concentration relationship and all the mean micronucleus frequencies for the vehicle and test item treated cultures were within the laboratory historical 95% confidence limits. These results all fulfilled the criteria for clearly negative results.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

It was concluded that Transglutaminase, batch PPN66927 did not show evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this in vitro test system under the experimental conditions described.

4. GENERAL TOXICITY

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

SUMMARY
The objective of this study was to assess the systemic toxic potential of Transglutaminase, batch PPN81685 (an enzyme used in the food industry) when administered in the diet to Han Wistar rats for 13 weeks.

Three groups, each comprising ten male and ten female Han Wistar rats, received Transglutaminase, batch PPN81685 in the diet at concentrations of 16448, 32895 and 49343 ppm (equivalent to 1875, 3750 and 5625 ppm in terms of TOS). The overall mean achieved dosages were 122, 244 and 372 mg TOS/kg bwt/day in males and 138, 292 and 447 mg TOS/kg bwt/day in females (equivalent to 122, 2761 and 4209 TGHU(A)/kg bwt/day in males and 1562, 3304 and 5058 TGHU(A)/kg bwt/day in females, respectively). A similarly constituted control group received the basal diet containing water at the same concentration as the high group (49343 ppm, or ~4.9%).

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

Results
The general appearance and behavior of the animals and sensory activity, grip strength and motor activity were unaffected by treatment. There were no premature deaths associated with Transglutaminase, batch PPN81685 (one high dose male died during the study but the death was not attributable to treatment).
There was no effect of treatment on body weight gain or food and water consumption.

There were no treatment-related ophthalmoscopic findings.

The haematological and blood chemistry investigations did not indicate any toxicologically significant findings.

Estrous cycles at the end of the treatment period were unaffected. There was also no effect of treatment on testicular pathology.

Serum triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations were unaffected by treatment.

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

Conclusion

It is concluded that the dietary administration of Transglutaminase, batch PPN81685 to Han Wistar rats at concentrations up to 5625 ppm (in terms of TOS) for 13 weeks was well-tolerated, with no evidence of any adverse finding at any of the administered dietary concentrations. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 5625 ppm TOS which achieved overall dosages of 372 mg TOS/kg bwt/day for males and 447 mg TOS/kg bwt/day for females, equivalent to enzyme activities of 4209 TGHU(A)/kg bwt/day for males and 5058 TGHU(A)/kg bwt/day for females.

5. REFERENCES

5.1 Study reports

Covance: Study No.: SM87XK; Novozymes Reference No.: 20206001: Transglutaminase, Batch PPN66927: Bacterial Reverse Mutation Test (Treat-and-wash method). LUNA file: 2020-10594.

Covance: Study No.: FW19LT; Novozymes Reference No.: 20206002: Transglutaminase, Batch PPN66927: In Vitro Micronucleus Test in Human Lymphocytes. LUNA file: 2020-16995.

Labcorp Study No.: 8439628; Novozymes Reference No.: 20206004: Transglutaminase, Batch PPN81685: Toxicity Study by Dietary Administration to Han Wistar Rats for 13 Weeks. LUNA file: 2022-06017.

Appendix 2 - Novozymes' Assessment of Literature Linking Transglutaminase and Celiac Disease

Basis of the Hypothesis:

As noted in Section 6(e) in the notification, the Lerner and Matthias' hypothesis is based on a correlation between the increase in mTGase use and the increase of celiac disease incidences. They propose two possible modes of action for TGase to contribute to celiac disease:

1) The mTGase crossbinding of gliadin molecules from gluten may give rise to degradation products which are resistant to enzymatic degradation and gastric acidity and these peptides might reach the immune cells of the intestinal mucosa in an intact and stimulatory form. And/or that these crosslinked gliadins may be more immunogenic than non-crosslinked gliadins.

2) mTGase itself may affect the permeability of the tight junctions in the intestinal epithelial barrier.

Assessment of the Hypothesis:

1) Lerner and Matthias' proposed correlation between the increase in mTGase use and the increase of celiac disease incidences is just an associative observation, and one for which no causality between the two has been found (1). Further, the increase in the incidence in celiac disease started around 1980-1990, however the use of mTGase, at least in the US and EU was later. The first FDA GRAS notification of mTGase was filed in 1998. Similarly, in Japan, commercial use was from 1993 but more widespread use was not until the end of the 1990s. With the difference in the start of these trends, it is highly unlikely that the industrial use of mTGase is causing this increase in celiac disease incidences.

2) Both human TGase and microbial TGase are ubiquitous in the small intestine, which is the main target for celiac disease. The mTGase is inactivated in the application and/or the mTGase is degraded in the stomach. Therefore no, or at most insignificant amounts of active mTGase from the food will reach the intestines. Consequently, ingested, inactive, and likely degraded mTGase is highly unlikely to affect the tight junctions in the intestinal epithelial barrier.

3) Antibodies to deamidated gliadin have higher accuracy in celiac disease diagnosis than antibodies to unmodified gliadin (2). However, several studies have shown that mTGase induced transamidation prevents the formation of immunodominant gliadin peptides, and that mTGase leads to reduced immune cross reactivity (3) (4). In addition, no difference was found between gliadin from native pasta and pasta treated with mTGase (5).

4) TGase crosslinked molecules are not foreign to the human body, and are natural in the human diet. Such isopeptide bonds are naturally occurring in a number of foods and they can be formed during preparation or cooking, by naturally occurring TGase or by extensive heat treatment.

5) Gliadins are in general poorly degraded in the human body due to the high content of proline and glutamine (3). Nevertheless, proteins crosslinked by TGase can be completely digested down to the ε-(γ-glutamyl)lysine crosslink. Once released from the protein chain, this crosslink can be absorbed and utilized (6) (7) (8). Transglutaminases occur in various muscle tissues, eg. in a study of transglutaminase in croaker, carp, pollack, salmon, makerel and trout transglutaminase was found in the muscles of all species in a range of 0.10-2.41 unit/g of muscle. For comparison rabbit muscles were also analyzed and found at 0.23 unit/g of muscle (9). Naturally occurring isopeptide bonds are found in amounts up to 1.35 micromol/g protein. Extensive heat treatment can result in isopeptide bonds in amounts up to 33 micromol/g protein. Consequently, industrial application of transglutaminase as a processing aid for modifying foods results in formation of isopeptide bonds in amounts similar to what can be found in existing natural foods.

Based on the information above and in Section 6(e) of the GRAS notification, it is apparent that the link between microbial transglutaminase used as proposed in this notice and celiac disease is unsubstantiated, and unlikely given the mechanism of action in the body and in disease pathogenesis.

In summary, a review of the literature, and the results of the toxicology studies reveal that microbial transglutaminase has a history of safe use in food and the proposed use of the microbial transglutaminase from *Streptomyces mobaraensis* in food products does not present a safety concern.

References

1. Lerner, A. and Matthias, T. Microbial Transglutaminase Is Immunogenic and Potentially Pathogenic in Pediatric Coeliac Disease. Front Pediatr. 11;6:389, 2018.

2. Rashtak, S., et al. Comparative usefulness of deaminated gliadin antibodies in the diagnosis of celiac disease. Clin Gastroenterol Hepatol. 6(4):426-370, 2008.

3. Luongo, D., Bonavita, R., Rossi, S., Rotondi Aufiero, V., Feliciello, N.R., Maurano, F.,Iaquinto, G., Mazzarella, G., Rossi, M. Tailoring the immune response to wheat gliadin by enzymatic transamidation cytokine. 117:23-29, Feb 2019.

4. Lombardi, E., Bergamo, P., Maurano, F., et al. Selective inhibition of the gliadinspecific, cell-mediated immune response by transamidation with microbial transglutaminase. J Leukoc Biol. 93(4):479-88, April 2013.

5. Ruh, T., Ohsam, J., Pasternack, R., Yokoyama, K., Kumazawa Y, Hils M. Microbial transglutaminase treatment in pasta-production does not affect the immunoreactivity of gliadin with celiac disease patients' sera. J Agric Food Chem. 62(30):7604-11,July 2014.

6. Motoki, M. and Seguro, K., Trends in Food Science & Technology 9 :204-210, 1998.

7. Romeih and Walker. Trends in Food Science & Technology 62:133-140, 2017.

8. Roos N, et al. Kieler Milchw Forsch. 55:261–276, 2003.

9. Araki H. and Seki N., Comparison of Reactivity of Transglutaminase to Various Fish Actomyosins, NIPPON SUISAN GAKKAISHI, 59, 4, 711-716, 1993.

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
001139	April 3, 2023
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	Regulatory Affairs

Company *(if applicable)*: Novozymes North America

Mailing Address *(number and street)*: P.O. Box 576, 77 Perrys Chapel Church Rd

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

Telephone Number	Fax Number	E-Mail Address
919-218-5934		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

Transglutaminase enzyme preparation from Bacillus licheniformis

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

☒ Electronic Submission Gateway

☐ Electronic files on physical media with paper signature page

☐ Paper

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 transglutaminase enzyme preparation is used as a processing aid. The enzyme can be used in any food application where the protein that is present can be linked by the TGase. TGase can be used in a wide range of applications such as processing of dairy foods (milk, fermented milk, yogurt, cheese) and plant-based dairy analogues including plant-based egg analogues, plant-based meat analogues, tofu, processed fish (non-Siluriformes only) and seafood and plant-based analogues. There are also uses in baking, cereal, and grain-based products. Finally, TGase can be used in brewing and other cereal-based or plant-based beverages. The enzyme preparation is used at minimum levels necessary to achieve the desired ressults. The "general" population is the target population for consumption.

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	Transglutaminase	EC	2.3.2.13		
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.

The transglutaminase enzyme preparation catalyzes the formation of an isopeptide bond between glutamine and lysine residues in proteins. This results in a protein gelling through an intra- and intermolecular crosslinking of proteins present in food matrices that contain milk, plant, or other proteins. The molecular weight is 38 kDa

3. Synonyms

Provide as available or relevant:

1	protein-glutamine:amine gamma-glutamyltransferase
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 Transglutaminase enzyme preparation from Bacillus licheniformis *(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.

(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: 2023.04.03 11:47:46 -04'00'	Lori Gregg, Regulatory Affairs	04/03/2023

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_Transglutaminase from Bacillus licheniformis_2023-04-03.pdf	Submission
	Supplement_Assessment of Literature Linking TGase and Celiacs_23-04-03.pdf	Submission
	Tox Summary_transglutaminase_2023-03-10.pdf	Administrative