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FOOD ADDITIVE SAFETY

August 1, 2014

VIA FEDERAL EXPRESS

Dr. Antonia Mattia
Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Glucanase Produced from *Streptomyces violaceoruber*

Dear Dr. Mattia:

On behalf of Nagase America Corporation ("Nagase"), we are submitting under cover of this letter three paper copies and one eCopy of Nagase's generally recognized as safe ("GRAS") notification for its enzyme preparation of glucanase, which is produced from *Streptomyces violaceoruber.* The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Nagase has determined through scientific procedures that its glucanase preparation is GRAS for use in the food industry as a processing aid to degrade the beta-glucans present in the yeast cell wall.

The use of glucanase increases yielding volume of yeast or mushroom extract, and improves residual filtration after extract abstraction by hydrolyzing β-D-glucan. The use of the glucanase can shorten the reaction time during yeast extract production, which could subsequently reduce manufacturing costs. It can also be used as a clarification and filtration aid in alcohol fermentation, such as wine, beer and sake, breaking down the beta-glucans which can clog filters and affect clarity.

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Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this its enzyme preparation of glucanase, produced from *Streptomyces violaceoruber,* is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require any additional information to aid in the review of Nagase's conclusion, please do not hesitate to contact me via email at gyingling@morganlewis.com or by telephone, (202)739-5610.

Sincerely,

(b) (6)

Gary L. Yingling

cc: Nagase America Corporation

GRAS NOTIFICATION FOR A GLUCANASE PREPARATION FROM *STREPTOMYCES VIOLACEORUBER*

"DENAZYME"

Submitted by:

Nagase America Corporation
546 5th Avenue 16th Floor
New York, NY 10036-5000

GRAS NOTIFICATION FOR GLUCANASE FROM *STREPTOMYCES VIOLACEORUBER*

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS 1

1.1. Name and Address of Notifier 2

1.2. Common or Usual Name of Substance 2

1.3. Applicable Conditions of Use 2

1.3.1. Substances Used In 3

1.3.2. Levels of Use 3

1.3.3. Purposes 3

1.4. Basis for GRAS Determination 4

1.5. Availability of Information for FDA Review 4

2. PRODUCTION MICROORGANISM 5

2.1. Name and designation 5

2.2. Source of the organism 5

2.3. Safety of the source organism 5

2.4. Strain improvement 6

2.5. The classical taxonomy 7

2.6. Enzyme Identity 8

2.8. Principal enzymatic Activities 8

MANUFACTURING PROCESS 10

2.10. Overview 10

2.11. Raw Materials 10

2.12. Purification process 11

2.13. Formulation and standardization process 11

2.14. Quality Control of Finished Product 11

COMPOSITION AND SPECIFICATIONS 12

2.2. Formulation 12

2.3. General Production Controls and Specifications 12

3. SAFETY EVALUATION 14

3.1. Safety of the Production Strain 14

3.1.1. Safety of the source and production organisms 14

3.2. Safety of the Beta-glucanase Enzyme Preparation 16

3.2.1. Fate of the food enzyme during food processing 17

3.2.2. Intended and unintended reaction products 17

3.2.3. Possible effects on the other components of the food 17

3.2.4. Allergenicity 18

3.3. Safety of the Manufacturing Process 19

3.4. Summary of toxicology studies 20

3.4.1. 13-week oral toxicity in rats 20

3.4.3. Genotoxicity: Bacterial mutation assay (Ames test) 22

3.4.4. *In vitro* mammalian chromosome aberration test 23

3.5. Estimates of Human Consumption and Safety Margin 24

3.5.2. Estimate of the glucanase dietary exposure based on the Budget method 25

3.6. Results and Conclusion 25

4. LIST OF REFERENCES 27

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

Nagase Chemtex Corporation ("Nagase") manufactures an enzyme preparation of glucanase, which is produced from *Streptomyces violaceoruber*. Nagase produces the glucanase preparation in a powder form and liquid form, standardized with sodium chloride (powder form) or glycerol (liquid form).

This enzyme preparation is for use in the food industry as a processing aid to degrade the beta-glucans present in the yeast cell wall. The use of glucanase increases yielding volume of yeast or mushroom extract, and improves residual filtration after extract abstraction by hydrolyzing β-D-glucan. The use of the glucanase can shorten the reaction time during yeast extract production, which could subsequently reduce manufacturing costs. It can also be used as a clarification and filtration aid in alcohol fermentation, such as wine, beer and sake, breaking down the beta-glucans which can clog filters and affect clarity. The FDA has reviewed beta-glucanase preparations in the past with similar intended uses, including GRN 149, GRN 195, GRN 479 and GRN 482, all of which received "No Questions" letters from the Agency.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, Nagase has determined that its glucanase preparation from *Streptomyces violaceoruber* is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme preparation and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme preparation, its applicable conditions for use, Nagase's basis for its GRAS determination and the availability of supporting information can be found here in this notification.

The production organism, *Streptomyces violaceoruber*, has a long history of safe use, and is discussed in Section 2. Section 2 also describes the production microorganism and strain improvement that was performed. The safety studies outlined in Section 6 indicate that the glucanase preparation from *Streptomyces violaceoruber* shows no evidence of pathogenic or toxic substances. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 6.

1.1. Name and Address of Notifier

NOTIFIER

Nagase America Corporation
546 5th Avenue 16th Floor
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1.2. Common or Usual Name of Substance

The common name for the enzyme that is the subject of this notification is glucanase. The trade name for the product will be DENAZYME GEL-L1 and DENAZYME GEL-P1.

1.3. Applicable Conditions of Use

The endo-1,3-beta-glucanase hydrolyses the (1->3)-beta-D-glucosidic linkages in (1->3)-beta-D-glucans. Beta-glucans occur commonly as cellulose in plants, the bran of cereal grains, and the

cell wall of baker's yeast, certain fungi, mushrooms and bacteria. The glucanase preparation can be used in a variety of ways. First, it is used as a processing aid in the production of glucan-rich ingredients, such as yeast extracts, where it hydrolyzes the cell wall to increase yield. Second, it can be used as a clarification and filtration aid in alcohol fermentation, such as wine, beer and sake, breaking down the beta-glucans which can clog filters and affect clarity.

The use of glucanase can be regarded as a processing aid because it has no function in the finished foodstuff.

1.3.1. Substances Used In

The glucanase preparation is used as a processing aid in the production of yeast extracts and alcoholic beverages.

1.3.2. Levels of Use

The glucanase preparation is intended in the manufacture of yeast extracts. The intended use level on yeast is:

- glucanase activity: 3 U/g corresponding to,
- 0.3% of powdered glucanase preparation (specific glucanase activity >1000 U/g)
- 0.6% of liquid glucanase preparation (specific glucanase activity >400 U/g)

1.3.3. Purposes

The Nagase glucanase preparation is intended for use in the manufacture of yeast products, such yeast extracts, by degrading the cell walls that contain β-glucans. The use of glucanase increases the yield of yeast or mushroom extract, and improves residual filtration after extract abstraction by hydrolyzing β-D-glucan. The yeast extracts are used in a variety of foods, such as soups and seasonings.

The glucanase preparation may be used as a processing aid in alcohol production, breaking down beta-glucans found in beer and wine production and enhacing clarification and filtration. Barley, a significant component of the mash used to manufacture fermented beverages, contains 4-7% beta-glucan. Beta-glucanase is able to degrade the beta-glucans into less viscous, smaller molecules, improving the filtration and haze that can result during the production of beer and fermented beverages. In wine production, beta-glucanase can degrade the beta-glucans produced by fungi and yeast which can infect the grapes, causing decreased clarity and poor filtration results. This intended use is consistent with the beta-glucanase preparations previously reviewed by the FDA, all of which recieved "No Questions" letters (GRN 149, GRN 195, GRN 479 and GRN 482).

The glucanase preparation presents the advantage of reacting in a temperature range of 50-60°C. The advantage of using the preparation is the reduction of bacterial contamination during yeast and mushroom extract manufacture given the higher operating temperatures, and a longer

reaction time. This results in a reduction in the amount of glucanase that must be used, and a decrease in manufacturing costs.

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, Nagase has determined, through scientific procedures, that its glucanase enzyme preparation from *Streptomyces violaceoruber* is GRAS for use as an enzyme for breaking down the beta-glucans present in yeast, and as a processing aid in alcohol production, breaking down beta-glucans found in beer and wine production and enhacing clarification and filtration, in levels not to exceed good manufacturing practices.

1.5. Availability of Information for FDA Review

The data and information that are the basis for Nagase's GRAS determination are available for the FDA's review and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW
Washington, DC 20004-2541

2. PRODUCTION MICROORGANISM

2.1. Name and designation

The strain used to produce the glucanase preparation is self-cloned and designated *Streptomyces violaceoruber* pGlu.

2.2. Source of the organism

The strain was constructed by transformation of a plasmid from *S. violaceoruber* ATCC35287 further inserted into the strain host *S. violaceoruber* 1326 (refer to section 2.4).

The strain host complies with the OECD GILSP criterions (Good Industrial Large Scale Practice microorganisms, OCDE, 1992[1]). It also complies with the criterions on the safety of food enzymes sets by Pariza and Foster (1983[2]) as well as Olempska-Beer, et al[3]. The strain host is known to be neither toxic nor pathogenic, as documented in the literature.[4]

2.3. Safety of the source organism

With reference to the Risk Classification of the European Community (Directive 2000/54/EC[5]) the host strain *Streptomyces violaceoruber* would be classified in Group 1: biological agent that is most unlikely to cause human disease.

The source organism is self-cloned. Then, an intensive bibliographic research, using several data bases such as Web of Science or MEDLINE was performed in order to determine published toxic, mutagenic or pathogenic effects and disease or infection which would be possibly exhibited by *Streptomyces violaceoruber*.

This search performed with no time limit (up to June 2014) failed to highlight any scientific publications or reports indicating any toxic, toxigenic or pathogenic effects for this strain. This is consistent with prior GRAS notifications 145 (filed 2004) and 212 (filed 2006), both of which used *Streptomyces violaceoruber* for phospholipase enzyme preparations. Both notifications, which received "No Questions" letters from the Agency, found no evidence of human pathogenic properties of *Streptomyces violaceoruber* in literature searches performed prior to submission. Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary

---

[1] Organization for Economic Co-operation and Development (1992) Safety Considerations for Biotechnology. Paris, France. 45 pages (available on June 2011 at at: http://www.oecd.org/dataoecd/8/3/2375496.pdf)

[2] Pariza MW, Foster EM (1983) Determining the Safety of Enzymes Used in Food Processing. *Journal of Food Protection.* 46(5):453-468

[3] Olempska-Beer, ZS, Merker, RI, Ditto, MD, DiNovi, MJ. (2006). Food-processing enzymes from recombinant microorganisms-a review. *Regulatory Toxicology and Pharmacology*. 45:144-158.

[4] Pariza, MW and Johnson, EA. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regulatory Toxicology and Pharmacology*. 33:173-186.

[5] Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC) (available on 19th April, 2011 at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:262:0021:0045:EN:PDF)

conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." We concluded that *Streptomyces violaceoruber* meets these criteria for nontoxigenicity and nonpathogenicity following analysis of the scientific literature. The safety of this strain is further supported by the FDA review and lack of objection to two prior GRAS notifications for enzyme preparations manufactured with *Streptomyces violaceoruber*, the first of which was reviewed over ten years ago.

2.4. Strain improvement

2.4.1. Construction of the self-cloned strain S. violaceoruber pGlu

The plasmid pIJ702 used to transform the *Streptomyces violaceoruber* 1326 strain, contains:

- A promoter sequence pro-SSMP obtained from *S. cinnamoneus* TH-2
- The structural gene of glucanase glu obtained from *S. violaceoruber* NBRC 15146 (or S. coelicolor A3) ,
- A terminator sequence pld-ter obtained from *S. cinnamoneus* NRBC 12852.

All DNA sequences used are derived from bacteria belonging to the genus Streptomyces.

pGlu plasmid contains an antibiotic (thiostrepton) resistance gene, tsr, which is indigenous to *Streptomyces azureus* (see Section 3.1.3.1 for further discussion). p Glu plasmid is removed tryrosinase gene MelC1 and MelC2 derived from pIJ702. This plasmid is Biosafety level 1, and commonly used to construct variants of *Streptomyces* species (Gusek and Kinsella, 1992).[6]

The host microorganism is *Streptomyces violaceoruber* 1326. This strain previously named *Streptomyces lividans* strain is non-pathogenic or toxigenic. The strain was obtained from the John Innes Center. This strain would be classified as group 1 biological agent that is unlikely to cause disease in humans and is not on the list of microbiological hazards (ANSES[7]).

2.4.2. Insertion of the pGlu plasmid into the host organism and transferability

The host strain *S. violaceoruber* 1326 is modified by means of the method of protoplast fusion. This method has been long been utilized for the incorporation of plasmids in actinomycetes. The plasmid is not considered transferable.[8]

2.4.3. Stability of the introduced gene sequence

As described by Kieser et al (1982), plasmid pIJ702 is non-conjugative and not transferable that maintains its autonomous form in the cell. Consequently, the frequency of the inserted vector's mobilization and the ability for genetic material transfer are considered as very low.
The stability of the introduced gene sequence was also assessed by culturing the production strain for several generations, including cultures in the absence of selective pressure.

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[6] Gusek, TW and Kinsella, JE. (1992). Review of the *Streptomyces lividans* vector pIJ702 system for gene cloning. *Critical Reviews in Microbiology*. 18(4):247-260.
[7] http://www.anses.fr/PN5701.htm (available on June 2011)
[8] "Practical Streptomyces Genetics." The John Innes Foundation: 2000.

Productivity of the enzyme was measured for each generation. The results indicate that the efficiency remains constant over several generations.

2.5. The classical taxonomy

Information on the taxonomy of *Streptomyces violaceoruber* was collected on the Genbank data base (http://www.ncbi.nlm.nih.gov/Taxonomy/).

The taxonomy of *Streptomyces violaceoruber* 1326 is a follow (Taxonomy ID: 1935 on GenBank):

Super Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria (class)
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Streptomycineae
Family	Streptomycetaceae
Genus	Streptomyces
Species	*violaceoruber*

Streptomyces is the most abundant gender of Actinobacterias. More than 570 species of *Streptomyces* were reported. These are Gram+ bacteria with high proportion of G + C in their DNA. 16S rRNA sequencing allowed identifying 39 families such as bifidobacteria or micrococcaceae (Ventura et al., 2007).

Streptomyces violaceroruber is a component of soil bacteria, and closely related to *Streptomyces lividans* and *Streptomyces coelicolor*. These *Streptomyces* species are used frequently as model systems for biochemical and physiologic studies, and have been used to produce multiple antibiotics (Duangmal et al., 2006).[9]

[9] Duangmal, K, Ward, AC, Goodfellow, M. (2006). Selective isolation of members of the *Streptomyces violaceroruber* clade form soil. *FEMS Microbiology Letters*. 245(2):321-327.

ENZYME IDENTITY

2.6. Enzyme Identity

Key enzyme and protein chemical characterizations of the enzyme are:

Trade name:	Denazyme GEL-L1 or Denazyme GEL-P1
Common name:	glucanase
IUBMB nomenclature:	3-beta-D-glucan glucanohydrolase
IUBMB Number:	EC3.2.1.39
CAS Number:	9025-37-0
Synonyms :	glucan endo-1,3-beta-D-glucosidase; endo-1,3-beta-glucanase; laminarinase; laminaranase; oligo-1,3-glucosidase; endo-1,3-beta-glucanase; callase; beta-1,3-glucanase; kitalase; 1,3-beta-D-glucan 3-glucanohydrolase; endo-(1,3)-beta-D-glucanase; (1->3)

2.7. Molecular Characterization of the Enzyme

The gene encoding an endo-1,3-β-glucanase from Streptomyces sp. was cloned and expressed in Escherichia coli by Shi et al. (2010[10]). The authors identified the biochemical properties of the purified recombinant enzyme. This study showed that the full-length gene contains 1,362 bp and encodes a protein of 453 amino acids with a calculated molecular mass of 42.7 kDa.

This endo-1,3-β-Dglucanase contains two functional domains, a glycosyl hydrolase family 16 domain and a C-terminal CBM13 domain, and one N-terminal leader sequence. Between the hydrolase and C-terminal CBM domain, there is a glycine rich region containing a stretch of six consecutive glycine residues.

2.8. Principal enzymatic Activities

The endo-1,3-beta-glucanase hydrolyses the (1->3)-beta-D-glucosidic linkages in (1->3)-beta-D-glucans according to equation 1. This reaction leads to the formation of fructose and β-glucan.

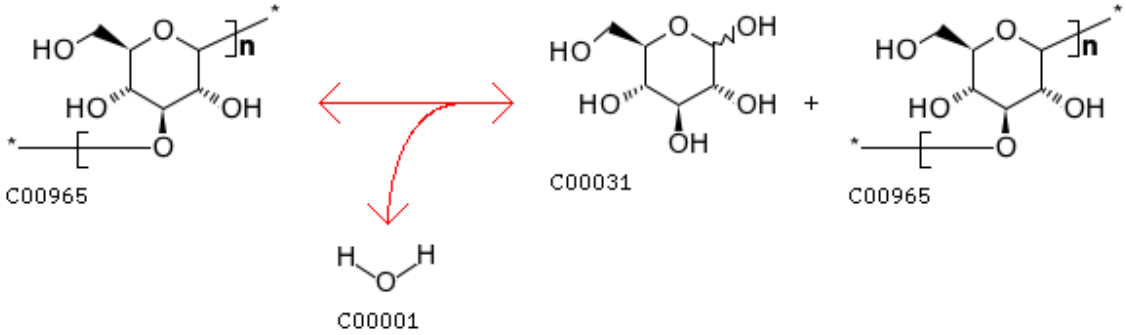
Equation 1:

1,3-beta-D-Glucan + H2O ↔ D-Glucose + 1,3-beta-D-Glucan

This reaction is characterized by Figure 1 below.

[10] Shi P, Yao G, Yang P, Li N, Luo H, Bai Y, Wang Y, Yao B. 2010. Cloning, characterization, and antifungal activity of an endo-1,3-beta-D: -glucanase from Streptomyces sp. S27. Appl Microbiol Biotechnol. 85(5):1483-1490.

Figure 1: Reactions catalyzed by endo-1,3-beta-glucanase (from Kegg database[11])



The glucanase preparation is active in a temperature range of 50-60°C. The enzyme is inactivated at higher temperatures, and therefore no functional enzyme will be present in final foods or beverages due to subsequent heating steps (such as wort boiling, in fermented beverage applications).

2.9. Glucanase activity: Nagase method

In the literature (Murray, P. et al., 2001) the beta-glucanase activity is expressed in international units IU units. One IU unit of endo-beta-glucanase activity is defined as the amount of enzyme that yields 1 micromole reducing sugar (as glucose equivalence) in 1 min under the conditions of the assay.

The glucanase activity is defined as follows: one unit of enzymatic activity catalyzes the formation of one micromole of glucose per min at 37°C. A calibration curve is prepared using d-glucose. This is a method where enzymes react with substrate curdlan, a linear β-1, 3- glucan high-molecular-weight polymer of glucose, and a colorimetric determination of the water-soluble oligosaccharides and monosaccharides produced is measured using the phenol-sulfuric acid method.

This method is consistent with published methods for assessing beta-glucanase activity (Fontaine et al. 1997)[12] The Nagase method employs curdlan as the substrate, in contrast to many methods which use laminarin. While both substrates are linear β-glucans, curdlan consists almost entirely of β-1, 3 linkages, whereas laminarin has β-1,6 linkages.[13] Given that the proposed enzyme preparation is effective on β-1,3-linkages, curdlan is an appropriate substrate. The phenol-sulfuric acid method is also a commonly used, highly specific and very reliable method for quantifying carbohydrates (Dubois, et al., 1956 and Masuko, et al., 2005).[14,15]

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[11] Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M.(2014). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res*. 42, D199–D205.

[12] Fontaine, T, Hartland, RP, Beauvais, A, Diaquin, M, and Latge, JP. (2004). Purification and characterization of an endo-1,3-β-glucanase from Aspergillus fumigatus. *European Journal of Biochemistry*. 243:315-321.

[13] McIntosh, M, Stone, BA, and Stanisich, VA. (2005). Curdlan and other bacterial (1→3)-beta-D-glucans. *Applied Microbiology and Biotechnology*. 68(2):163-172.

[14] Dubois, M, Gilles, KA, Hamilton, JK, Rebers, PA and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28(3): 350-356

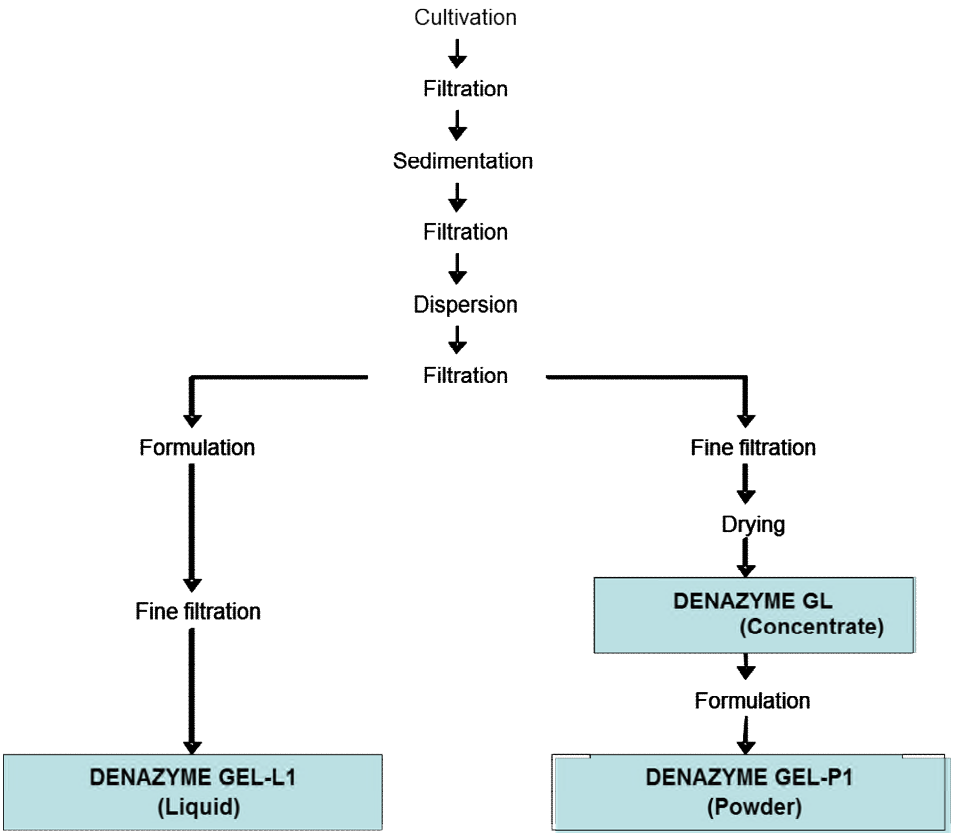
MANUFACTURING PROCESS

2.10. Overview

The glucanase preparation is produced under ISO 9001 certification. Each lot is controlled according to Nagase's specifications (refer to section 2.3).

The manufacturing process it outlined in the figure below:

Figure 2: Manufacturing Process



2.11. Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of Nagase ChemteX Corporation. The raw materials used for the formulation are of food grade quality. All raw materials used for the production of the glucanase preparation are food, food additives, or

[15] Masuko, T, Miniami, A, Iwaski, N, Majima, T, Nisimura, AI, Lee, YC. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry*. 339:69-72.

food processing aids listed in the Food Chemical Codex (9th edition) and/or the Code of Federal Regulations ("21 CFR"). The antifoam used in the fermentation is used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998.

2.12. Purification process

At the end of the fermentation, the producing strain is removed by different steps of filtration as detailed in figure 2. A study was performed to demonstrate the efficiency of the strain removing process. As *Streptomyces violaceoruber* pGlu has a resistance gene to thiostrepton, yeast extracts treated with Denazyme GL were incubated in a thiostrepton rich medium at 30°C for 5 days. No colonies appeared on the plates indicating the absence of the production strain in the finished foodstuff (Annex A).

2.13. Formulation and standardization process

The concentrates of glucanase preparation are standardized to 400 U/mL or 1000 U/g by addition of sodium chloride (Powder form) or glycerol (liquid form) to produce the commercial glucanase preparation. Both sodium chloride and glycerol are food ingredients present in numerous natural or processed foodstuffs.

2.14. Quality Control of Finished Product

The glucanase preparation is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO in 2006 and the Food Chemical Codex (9th edition). These specifications are described below.

Parameter	**Norm**
Lead	< 5 mg/kg
Coliforms	< 30 CFU/g
Salmonella	0/25 g
Escherichia coli	0/25 g
Antimicrobial activity	Absent by test
Mycotoxins	Not detected

COMPOSITION AND SPECIFICATIONS

2.2. Formulation

The concentrates of glucanase preparation are standardized to 400 U/mL or 1000 U/g by addition of sodium chloride (Powder form) or glycerol (liquid form) to produce the commercial glucanase preparation. For the purpose of TOS determination, three samples of glucanase preparation representative of scale production were analyzed for water and ash contents. The analyses were performed at the Nagase Chemtex Corporation. The results are summarized in the Table 1.

Based on these results, the TOS percentage was calculated as follows: TOS = 100 – (A + W + D) (Where A = ash content, W = water content and D = content of diluents).

Based on these results, the mean TOS of the glucanase preparation is: 3.39% for the liquid form and 5.1% for the powder one.

Table 1: TOS determination on different lots of glucanase preparation

	Glucanase liquid			Glucanase powder		
Batch n°	T2	1102231	1102232	1102181	1102182	1102183
Ash (g/100)	0.06	0.05	0.07	94.68	94.55	94.59
Moisture (g/100g)	42.02	40.07	40.76	0.28	0.28	0.38
Diluent (g/100g)*	55.6	55.6	55.6	94	94	94
TOS (%)	2.32	4.28	3.57	5	5.2	5
Mean TOS (%)	**3.39**			**5.1**		

2.3. General Production Controls and Specifications

The glucanase production process comprises a cultivation step, followed by several filtration and purification steps. All raw materials used for the production of the glucanase preparation are food, food additives, or food processing aids listed in the Food Chemical Codex (9th edition) and/or the Code of Federal Regulations (“21 CFR”). The glucanase preparation is produced under ISO 90001 certification. The use of these materials ensures an optimum chemical purity of the preparation.

Each lot of glucanase is controlled according to Nagase’s specifications. These specifications include glucanase activities, heavy metals, microbial contaminants and quality measurements such as pH, colour or loss on drying. Details on specification are given in Table 2 below.

Table 2: General specifications of glucanase preparation

Parameter	Target	Glucanase preparation		Method
		liquid	powder	
Appearance		Brown liquid	Brown powder	Visual examination
Glucanase activity		>400 U/ml	>1000 U/g	In-house method*
Lead	not more than	< 5 µg/g	< 5 µg/g	Atomic absorption spectrometry
Arsenic (as As)	not more than	< 4 µg/g	< 4 µg/g	US Pharmacopoeia
Viable bacteria count	not more than	10000 CFU/ml	10000CFU/g	US Pharmacopoeia
Coliforms	Negative in 1g	Negative	Negative	US Pharmacopoeia
Salmonella	Negative in 25g	Negative	Negative	JECFA
Sulphur-reducing anaerobe	Less than	30CFU/g	30CFU/g	US Pharmacopoeia
Staphylococcus aureus	Negative in 1g	Negative	Negative	US Pharmacopoeia
Antibiotic activity	Negative	Negative	Negative	FAO/WHO method

*The glucanase activity is defined as follows: one unit of enzymatic activity catalyzes the formation of one micromole of glucose per min at 37°C. A calibration curve is prepared using d-glucose. This is a method where enzymes react with substrate curdlan, a linear β-1, 3- glucan high-molecular-weight polymer of glucose, and a colorimetric determination of the water-soluble oligosaccharides and monosaccharides produced is measured using the phenol-sulfuric acid method (See section 2.9 for further discussion of the method).

3. SAFETY EVALUATION

3.1. Safety of the Production Strain

3.1.1. Safety of the source and production organisms

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the IFBC, food or food ingredients are safe to consume if they have been produced, according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (IFBC, 1990).[16] A nontoxigenic organism is defined 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 nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983, Pariza and Johnson, 2001).

Streptomyces violaceoruber occurs in nature as a component of soil. The species was the subject of review in GRAS notices GRN 145 and GRN 212, submitted for phospholipase A2 preparations produced with *Streptomyces violaceoruber*.

With reference to the Risk Groups given in the NIH Guidelines, the November 2013 revision, the host and production strains of *Streptomyces violaceoruber* could be classified in Group 1: "Agents that are not associated with disease in healthy adult humans."

However, the source organism is self-cloned. An intensive bibliographic research, using several data bases such as Web of Science or MEDLINE was performed in order to determine published toxic, mutagenic or pathogenic effects and disease or infection which would be possibly exhibited by *Streptomyces violaceoruber*. This search performed with no time limit (up to June 2014) failed to highlight any scientific publications or reports indicating any toxic, toxicogenic or pathogenic effects for this strain, consistent with the prior GRAS notifications 145 and 212.

The Good Industrial Large Scale Practice published by the OECD summarizes concerning genetically manipulated: "The concept encompassed certain criteria which an r-DNA organism must meet in order to be given GILSP status. It stated that r-DNA GILSP organisms can be handled, on a large scale, under the same conditions of minimal controls and containment procedures as would be used for the host strains. The key principle for GILSP is that the r-DNA organism should be as safe as the low-risk organism from which it is derived." (OECD, 1992).

3.1.2. Adverse effects

A search was performed to determine if there was any evidence of adverse events due to the use of the strain in the publically available literature. The key-words used for this search were:

[16] IFBC (1990). Biotechnologies and food: assuring the safety of foods produced by genetic modification. *Regul. Toxicol. Pharmacol.* 12, S1-S196.

- *Streptomyces violaceoruber*, *Streptomyces lividans* and *Streptomyces coelicolor* because these species are considered belonging to the same taxon
- Toxic effects, mutagen, genotoxic, pathogenic and safety.

This search failed to identify any published study or report on human or animal adverse effects of *Streptomyces violaceoruber*.

Expanding the search to the genus Streptomyces, we identified a number of publications on the pathogenicity of certain species for plants. For example, data are available on Streptomyces scabies and S. acidiscabies that produce phytotoxins (thaxtomin A) and are responsible for the disease of potato scab. No publication covers toxigenic or pathogenic effects in mammals.
For humans, Kiezer et al. (2000) carried out a synthesis of the *Streptomycetes* pathogenic species. They showed that human diseases caused by actinomycetes are largely related to infections with Mycobacterium, Actinodura, Nocardia and Actinomyces. This has been confirmed by recent works which also identifies as pathogen agents *Streptomyces somaliensis*, *Streptomyces madurae* et *Streptomyces sudanensis*, and *Streptomyces cacaoi* (Boiron et al., 1998, Khatri et al., 2002, Dieng et al., 2003, 2005, Quintana et al., 2008, Pellegrini et al., 2012). Most of these studies were conducted in Africa and none are citing effects from *Streptomyces violaceoruber*, *Streptomyces lividans* or *Streptomyces coelicolor*.

3.1.3. Resistance to antibiotics

A literature search was performed to evaluate if antibiotic resistance by Streptomyces had been documented in the literature. The key-words used for this search were [antimicrobial or antibiotic resistance] and [*Streptomyces* or Actinobacteria]. This search gave a limited number of publications dealing with a very small number of species (*S. lividans*, *S. coelicolor*, *S. davawensis*, *S. viridochromogenes*, and *S. toyocaensis*). *Streptomyces violaceoruber* was not found in the publications. We note that numerous studies have used *S. coelicolor* as a model for evaluating resistance for other species.

Considering the high number of species in the *Streptomycetaceae* family and that the reported antimicrobial resistance differs from one specie to another and according the EFSA (2008), it could be conclude that the risk of transfer to other organisms can be considered as minimal.

3.1.3.1. Resistance to thiostrepton:

The introduced gene contains an antibiotic resistance gene (thiostrepton, tsr). This gene is already naturally present in *Streptomyces azureus* (Bibb et al. 1985). According to Pariza et Johnson (2001), the presence of antibiotic resistance genes is of concern only if they are transferable. These authors also indicate that the *tsr* gene, even under plamid form, is not transferable.

The FAO/WHO Consultation on Biotechnology and Food Safety (1996) suggested that "if the recombinant microorganism is not present in the food or if not deliberately released into the environment, then the presence of antibiotic resistance genes should not be a concern”. Considering the glucanase production procedures where exist several steps to eliminate the producing strain, it could be concluded that the presence of such a gene is unlikely.

The U.S. FDA (1998) has evaluated the occurence of antibiotic resistance genes in transgenic plants. It found that the likelihood of transfer of antibiotic resistance genes from plants to micro-organisms present in the digestive tract of humans or animals or to the environment is unlikely. In addition, it concluded that exposure to antibiotic resistance genes does not raise health concern because "most of the DNA is degraded in the intestine and therefore would not be transferable. Even if the DNA remained, it would not be integrated and expressed in the absence of selective pressure. In addition, because these cells are constantly renewed, a cell that incorporates a gene for antibiotic resistance could not be active for a long time and does not present a hazard to antibiotic therapies".

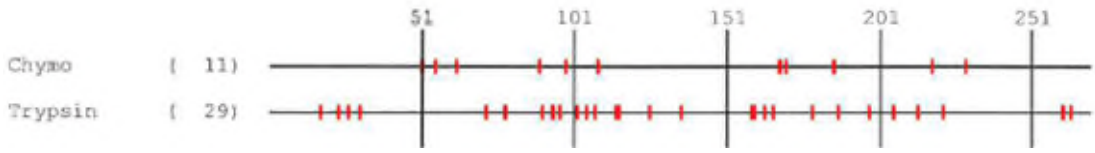
Knowing the amino acid sequence of the thiostrepton resistance protein, we studied its digestibility using the software Gene / Protein Analysis software (GENETYX Corporation). As shown in Figure 3, many parts can be recognized and bonds hydrolyzed by chymotrypsin and trypsin. Moreover, pepsin although less specific, may act on other residues and complete the catabolism. It can therefore be concluded that the thiostrepton resistance protein can be easily digested by enzymes in the human digestive tract.
In summary, considering that:

- The tsr gene (antibiotic resistance gene) is considered as non-transferable
- Resistance protein is readily hydrolyzed;

We believe that the transfer of antibiotic resistance is very unlikely.

Figure 3: Viewing zones of hydrolysis of peptide bonds by chymotrypsin and trypsin

The horizontal line represents the amino acid sequence of the protein resistance. The red areas indicate hydrolysis.



3.1.4. Work-associated allergenic and adverse effects

In 7 years of production by Nagase ChemteX, no adverse effects have been reported in workers exposed to *Streptomyces violaceoruber*. This is certified in Annex B.

3.2. Safety of the Beta-glucanase Enzyme Preparation

Beta-glucanases are widely distributed in nature. They have been isolated from a variety of sources, such as fungi, yeasts, bacteria, and plants (Müller, J.J. *et al.*, 1998; Wong, Y. and MacLachlan, G.A., 1980; McCarthy, T.C. *et al.*, 2005) . Since beta-glucanases are enzymes naturally present in nature and notably in plants and marine invertebrates consumed by human, Nagase expects it will be digested as would any other protein occurring in food. The FDA has

reviewed beta-glucanase preparations in the past, including GRN 149, GRN 195, GRN 479 and GRN 482, all of which received “No Questions” letters from the Agency. These notifications contained similar intended uses to those proposed by Nagase. The first notification, GRN 149, was reviewed over 10 years ago, and despite this long history of use, no safety issues were found in the literature. Several enzyme preparations of beta-glucanase have been evaluated by JECFA attributed an ADI ‘not specified’ for their use in several applications such as the preparation of fruit juices, beer and baking products (Joint FAO/WHO Expert Committee on Food Additives, 2006a, Joint FAO/WHO Expert Committee on Food Additives, 2006b).

3.2.1. Fate of the food enzyme during food processing

During the food processing, the glucanase preparation is either inactivated by heating or with pH. Glucanase activity is inactivated at temperature above 50°C which is low compared to sterilization processes. At pH lower 4 and above 7 the glucanase activity is also inactivated.

A study was performed on yeast extract treated by Denazyme GEL-L1. The glucanase activity was measured in samples diluted up to 20% for 2 reaction times.

Under this experimental condition, no glucanase activity was measured in the final product treated by Denazyme GEL-L1 (Table 3, Annex A).

Table 3: Residual glucanase activity in yeast extract treated by Denazyme GEL-L1

Yeast extract dilution	Glucanase activity (U／g)
	30 min
0.25%	ND
0.67%	ND
2%	ND
20%	ND

ND: not detected

From this information, it could be concluded that the glucanase preparation does not present any technological function in the final food products, and will be digested as any other naturally occurring protein.

3.2.2. Intended and unintended reaction products

As indicated in section 2.8, the glucanase preparation only hydrolyses β-glucans. The main intended reaction product is fructose. No unintended reaction products are expected.

3.2.3. Possible effects on the other components of the food

The glucanase preparation does not act on proteins and lipids. Further, the enzyme will likely be inactivated by heat processing during the manufacturing of food products using the enzyme

preparation, and should therefore have no impact on other components of foods, including microorganisms naturally present.

3.2.4. Allergenicity

To assess allergenicity, a sequence search on Allergen Database for Food Safety[17] was performed to identify allergenic sequence in the glucanase enzyme. No hit was found. The following amino acid sequence was used for the search:

Amino acid sequence of glucanase:

MLSRLRHRLLAVAAAAGLTGALLSFGAAPPADAAVPATIPLKITNNSARGDAVHIYN
LGTSLTTGQQGWADENGTFHAWPAGGNPPTPAPDASIPGPAAGQTKTIRIPKLSGRIY
FSYGQKLDFRLTTGGLVQPAVQNPSDPNRNILFNWSEYTLNDGGLWLNSTQVDMFS
APYTVGVQRADGGVTSAGQLKAGGYRGVFDALRAQPGWGGLIQTRPDGTVLRALA
PLYGVETGALPASVMDDYINRVWQKYTTTTLTVTPFGDRPDTKYFGRVSGNVMNF
TNTSGAVVTSFQKPDASSVFGCHRLLDAPNDQVRGPISRTLCAGFNRSTLLSNPNQP
DPSAANFYRDPVTNHYARIIHERMADGKAYAFAFDDVGNHESLVHDGNPAEARLTL
APLD

The absence of food allergenicity has been further evaluated by an extensive literature performed on Toxnet and Pubmed databases, attempting to identify allergenic reactions to glucanase enzymes. This intensive literature search failed to find any scientific work on this effect. Even among people who ingest high daily doses of enzymes as digestive aids for many consecutive years, there are no reports of gastrointestinal allergy to enzymes. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen, C. et al.,2006).

In addition, beta-glucanase has been used worldwide for a number of years without any known complaints from the end consumer, and has been previously reviewed on multiple occasions, and received “No Questions” letters from the FDA.

With regard to allergenicity of the fermentation media, Nagase has concluded that the data that it has and the public data and information allow it to conclude that there is no published or unpublished data that suggest there is an allergen causing protein from the fermentation media in the finished enzyme product. To reach that conclusion, Nagase relies on:

1. The Enzyme Technical Association (ETA) in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA’s website. The statement concludes that no allergens protein from the fermentation medium has been found in the finished enzyme, and states that regulatory bodies in both the EU and Japan have concluded that enzyme preparations do not pose an allergen risk that would require allergen labeling on the final product. Further, ETA points out that the typical manufacturing process of enzyme

[17] http://allergen.nihs.go.jp/ADFS/index.jsp?pagen=top

preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product purity and stability, and would likely remove most proteins present in the fermentation media. A copy of the public statement from the ETA website is attached in Annex C.

2. In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that because of the nature of enzymes as catalysts, they are used in very small amounts and the fermentation media is consumed during the enzymatic process. It is clear that any *de minimis* amount of fermentation media protein that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscores the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicates that there is no reliable assay that could be used to detect the presence of most allergen proteins in the final enzyme products, as the proteins would likely be degraded fragments that would not reach levels of quantitation available with current commercial ELISA assays. The full August 2013 statement, provided in Annex D, clearly concludes that that any protein allergen present in the final enzyme product would not be present at a level that requires labeling or present at a level that raises a public health concern.

Finally, it is our understanding that a search of the scientific literature will not result in a reported allergic reaction from an enzyme caused by the fermentation medium. The ETA has conducted similar literature searches in the past, with no findings of allergic reactions due to fermentation media. The fermentation media as noted above is consumed in the process, and is removed with subsequent purification and filtration steps used in the enzyme production process. There is no evidence to support that a level of protein from the fermentation media exists in the final enzyme product which would cause an allergic reaction.

It is therefore concluded neither the beta-glucanase protein nor the fermentation media are likely to produce any allergenic or sensitization reactions by oral consumption of the enzyme preparation.

3.3. Safety of the Manufacturing Process

The manufacture of the beta-glucanase is performed under the food GMP requirements. Moreover the ingredients used are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.[18,19]

[18] Aunstrup, K., Andersen,O., Falch, E.A., and Nielsen, T.K.(1979). Production of Microbial Enzymes in Microbial Technology, 2nd ed., Vol.1, Eds. Peppler, H.J. and Periman, D., Chapter 9.
[19] Aunstrup, K. (1979). Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp.28-68.

3.4. Summary of toxicology studies

This section provides a brief overview of the studies performed to evaluate the safety of using Nagase's glucanase preparation, and their results. Detailed descriptions of the assays and the results follow in the subsequent sections.

All studies were performed are in compliance with the principles of Good Laboratory Practice (FDA/ OECD).

Several toxicity studies were performed on the glucanase powder preparation.

A thirteen-week toxicity test of glucanase powder preparation in rats was performed by repeated oral administration at concentrations up to 1000 mg/kg/day. The no-effect level of glucanase was considered to be 200 mg/kg/day for both males and females (or 4404 U/kg/day or 190.66 mg TOS/kg/day).

An acute toxicity test by gavage in rats showed that the lethal dose of glucanase powder was above 2.0 g/kg.

A reverse-mutation assay in bacteria, demonstrated that the glucanase powder preparation is not mutagenic.

An *in vitro* chromosomal aberration test in cultured mammalian cells highlighted that the glucanase powder preparation induced structural chromosome aberrations but not numerical chromosome aberrations (polyploidy). To further investigate these results, an *in vivo* micronucleus test demonstrated no evidence of clastogenic potential in bone marrow. Because the glucanase was negative in the reverse mutation study in bacteria, showing no direct effect on DNA, and the micronucleus assay also showed no clastogenic potential, it can be concluded that there is no evidence of genotoxicity resulting from exposure to glucanase.

Detailed descriptions of the tests follow in the sections below.

3.4.1. 13-week oral toxicity in rats

Study Information: Nagase Chemtex Corporation. A 13-week oral gavage toxicity study of glucanase bulk powder in rats. Study Number: B-7068. Toyohisa Katsumata. Bozo Research Center Inc. Tokyo, Japan. March 30, 2011

GLPs and QA:
The study was performed under quality assurance and complies with GLPs.

Summary:
Thirteen-week toxicity test of glucanase bulk powder administered by repeated oral administration at levels of 0 (water for injection), 40, 200 and 1000 mglkg/day), was conducted in six-weeks old Sprague-Dawley strain SPF rats [Crl:CD(SD)] of both genders (10 animals of each sex per group). The study was conducted according to OECD Guideline for the Testing of Chemicals n° 408 (1998).

The test substance (Glucanase bulk powder, lot No T3, 22,020 U/g) was suspended in water and administered daily by gavage.

At the end of the administration and recovery period, the following parameters were measured: clinical observation, body weights, food consumption, water intake, ophthalmology, urinalysis, hematology, blood chemistry, pathology and histopathology. Detailed clinical observations were also performed: manipulative test, measurements of grip strength and motor activity.

During the test period, no deaths and no clinical signs (including detailed ones) were observed. No glucanase-related changes in body weight, food consumption, ophthalmological examination, urinalysis blood chemistry, and organ weight were detected.

Histopathological examination showed hyperplasia of the squamous epithelium in the forestomach in males and females in the 1000 mg/kg group. This change was judged to be of little toxicological significance as it was considered as a specific reaction of rodents to the test solutions at high dose concentration of the test article.

In conclusion, no treatment-related changes were found in the tested animals, except a hyperplasia of squamous epithelium in the forestomach in the high dose group, which were not considered as toxicologically significant.

The no-effect level of glucanase preparation in this sub-chronic study was thus considered to be 200 mg/kg/day for both males and females.

Considering a specific activity of 22,020 U/g and the TOS of 95.33% for the glucanase tox batch, the NOAEL could be defined as 4404 U/kg/day and 190.66 mg TOS/kg/day**.**

3.4.2. Acute Toxicity in Rats

Study Information:
Nagase Chemtex Corporation. Acute oral toxicity study of glucanase bulk powder in rats. Study Number: B-7067. Toyohisa Katsumata. Bozo Research Center Inc. Tokyo, Japan. December 26, 2011

GLPs and QA:
The study was performed under quality assurance and complies with GLPs

Summary:
The acute toxicity via oral route of an aqueous solution of glucanase bulk powder (Lot N° T3, 22,020 U/g) was evaluated at doses of 2.0 g/kg in eight-weeks old Sprague-Dawley strain SPF rats [Crl:CD (SD), (3 females per group, study duplicated) according to OECD Guideline for Testing of Chemicals n°423 (2001[20]).

[20] OECD (2002),*Test No. 423: Acute Oral toxicity - Acute Toxic Class Method*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. 14 pages

The animals were observed for general condition and mortality for the first 6 hours after the administration, and once daily thereafter for a period of continuous 14 days. Body weights were measured immediately before the administration and used to calculate the individual dose volume. Body weights were also measured on day 1, 3, 7 and 14 post-administration. At the end of the fourteen-day observation period, the rats were sacrificed by exsanguinations via the abdominal aorta under isoflurane anesthesia and autopsied.

No deaths and no abnormalities were observed throughout the study. No changes due to administration of the glucanase solution were observed in the general condition, body weight, body weight gain or gross pathology.

Therefore, the lethal dose by single oral administration of glucanase was estimated to be above 2.0 g/kg.

3.4.3. Genotoxicity: Bacterial mutation assay (Ames test).

Study Information: Nagase Chemtex Corporation. A bacterial reverse mutation test of glucanase powder. Study Number: T-0796. Yoshihiro Oguma. Bozo Research Center Inc. Tokyo, Japan. August 26, 2011

GLPs and QA:
The study was performed under quality assurance and complies with GLPs.

Summary:
Reverse mutation assays of glucanase (Lot No. T3, 22,020 U/g) were conducted using *Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537 and *Escherichia coli* strain WP2 uvrA, with and without metabolic activation according to the OECD Guidelines for Testing of Chemicals n°471 (1997).

The glucanase was dissolved in water. Water was used as negative control. Classical positive controls were realized. The preincubation method was applied. For the test without metabolic activation, 0.1M Na-phosphate buffer, 0.1 mL of glucanase solution and 0.1 mL of each bacterial medium was added to each test tube. For the test with metabolic activation, S9 mix was added to each tube instead of Na-phosphate buffer. The mixture was pre-incubated (37°C, 20 min.), 2.0 mL top agar was added and the completed mixture was spilled over on minimal glucose agar plate. All plates were incubated (37°C, 48 h), the bacterial growth inhibition of the tester strains was checked using a stereoscopic microscope and the number of revertant colonies was counted with an automatic colony counter.

The dose-finding preliminary tests did not demonstrate any growth inhibition at doses up to 5000 µg/plate in *S.typhimurium* and *E. coli* strains with or without metabolic activation. In the two main tests, the number of revertants with or without metabolic activation was not doubled by glucanase treatment. A dose-response increase was observed at higher dosage in S. typhimurium TA100 and TA98 with or without metabolic activation. However this increase was not reproducible.

It was concluded that glucanase was non-mutagenic when tested in bacterial mutation assays under the above experimental conditions.

3.4.4. *In vitro* mammalian chromosome aberration test

Study Information: Nagase Chemtex Corporation. Chromosome aberration test in cultured Chinese hamster cells treated with Glucanase. Study Number: T-G011. Takayuki Fukuda. Bozo Research Center Inc. Tokyo, Japan. October 14, 2011

GLPs and QA:
The study was performed under quality assurance and complies with GLPs.

Summary:
Chromosomal aberration tests of glucanase (Lot No. T3, 22,020 U/g) were carried out using Chinese hamster lung (CHL/IU) fibroblast cell line according to the OECD Guidelines for Testing of Chemicals n°473 (1997[21]).

The CHL cells were seeded and pre-cultured for three days in plastic plates and 0.5 mL of glucanase sample was added to the culture in duplicates. Solution without the test substance was used as a negative control. Standard positive controls were also assayed.
The cells were cultured in the test substance for 6h (short term treatment), 24 hours or 48 hours (continuous treatments).

After cultivation, the medium was discarded and the cells were washed with saline, fixed with methyl alcohol/acetic acid solution and stained in 0.1% crystal violet solution. Two plates of each group were observed for precipitation of the test article and the cell conditions under the inverted phase-contrast microscope. The cell density was measured with a monolayer culture densitometer. Two chromosome preparations per plate were made. The slides were air-dried and stained with 2% Giemsa solution.

In the short term and 24-hour continuous treatments, growth inhibition was observed at 2500 µg/mL with metabolic activation and at 5000 µg/mL without metabolic activation. In the 48-hour continuous treatment, inhibition was showed at 1250 µg/mL.

In the short term treatment with metabolic activation, the incidence of cells with structural aberrations was dose-related, but the incidence of polyploid cells was within the negative range at all dose concentrations. Without metabolic activation, neither potential to induce structural aberrations nor chromosome structural aberrations were demonstrated. Because the short-term treatment with metabolic activation was positive, observation of chromosome aberrations was not carried out in the continuous treatments.

[21] OECD (1997) Test No. 473: In vitro Mammalian Chromosome Aberration Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.

It was concluded that glucanase had potential to induce structural chromosome aberration but no potential to induce numerical chromosome aberrations (polyploidy) under the conditions of this study.

3.4.5. Micronucleus Test

Study Information: Nagase Chemtex Corporation. A Micronucleus Test of Glucanase Bulk Powder in Rats. Study Number: M-1497. Takayuki Fukuda. Bozo Research Center Inc. Tokyo, Japan. January 21, 2013-July 19, 2013

GLPs and QA:
The study was performed under quality assurance and complies with GLPs.

Summary:
To examine the clastogenic potential of glucanase bulk powder, a micronucleus assay was conducted in Crl:CD(SD)SPF rats.

Glucanase powder was administered twice orally to animals at 24 hour intervals. The doses chosen were 500, 1000 or 2000 mg/kg/day, with 5 animals per treatment group. Following administration, bone marrow smear specimens were prepared and evaluated approximately 24 hours following the second dose. A negative control received water and a positive control group received mitomycin C once, at a dose of 2 mg/kg.

The proportion of micronucleated immature erythrocytes showed no statistically significant increase compared to negative control, at all doses. No dose-dependent changes were reported. The proportion of immature erythrocytes in a total of 200 examined erythrocytes in each test substance dosage group did not show any statistically significant change when compared to the negative control group. The positive control group and negative control group were within the range of background materials historically used at the test facility.

Based on the above results, it was determined that glucanase bulk powder had no clastogenic potential in the bone marrow of Crl:CD(SD)SPF rats under the conditions of the study.

3.5. Estimates of Human Consumption and Safety Margin

3.5.1. Natural Occurrence

Glucanase has been found to be widely distributed in nature. A variety of glucanases have been discovered in many tissues and organs of plants and microorganisms.[22] Among the naturally occurring sources of glucanase noted above, many have been consumed by humans for centuries.

[22] D. Schomburg, I. Schomburg; 2003. Handbook of Enzymes. 2nd Edition,Vol. 12, P564-582.

3.5.2. Estimate of the glucanase dietary exposure based on the Budget method

Several authors and agencies recommend using the budget method as a first estimate of the human intakes of substances intentionally added to the foodstuffs. Indeed, this method is generally considered as conservative and protective for the general population, and has been utilized in previous GRAS notifications reviewed by the FDA, including GRN 274 and 476, both of which received "No Questions" letters (Hansen 1966; Hansen 1979; Douglass et al. 1997; Ilsi 1997; European Commission 1998; FAO/WHO 2008).

This method consists in determining a Theoretical Maximum Daily Intake (TMDI). It considers maximum intakes of 25g/kg BW/days for solid food and 100 ml/kg BW/days for liquids except milk. In an approximate, it is stated that 25% of solid or liquid foods contains the substance to be evaluated.

For the purpose of the glucanase preparation, we considered the maximum use level that is 3 U/g yeast corresponding to 153 mg TOS/kg (considering the highest TOS, 5.1%, of the powder glucanase). As regard to the technological function of the enzyme, its use is intended in both solid and liquid foodstuffs.

Table 4: Theoretical Maximum Daily Intake (TMDI) of glucanase preparation

TMDI	Calculation	Results (mg TOS/kg/day)
solid	= 0.051 x 3% / 160 x 1000 =	0.00956
liquid	= 0.051 x 3% /40 x 1000 =	0.03825
TMDI total diet	**= TMDI solid + TMDI liquid =**	**0.04781**

The Theoretical Maximum Daily Intake (TMDI) was for this worst case 0.04781 mg TOS / day. A thirteen-week toxicity test of glucanase powder preparation in rats found the no-effect level of glucanase was considered to be 200 mg/kg/day for both males and females (or 4404 U/kg/day or 190.66 mg TOS/kg/day).

According to the budget's intake estimate, the margin of exposure is 3988.

3.6. Results and Conclusion

On the basis of the information provided in this notification, Nagase has determined that its beta-glucanase enzyme preparation discussed in this notification, intended for use in the food industry as a processing aid to degrade the beta-glucans present in the yeast cell wall and as a clarification and filtration aid in alcohol fermentation, such as in the production of wine, beer and sake, is GRAS for the proposed uses.

The glucanase preparation is produced under good manufacturing practices and quality assurance. The host strain is not considered as pathogenic or toxinogenic and was not found in treated foodstuffs. The glucanase preparation is inactivated by heating or with acidic pH, and will therefore be inactive in the final food product. No residual glucanase activity was found in yeast extracts treated with the enzyme preparation. Therefore, the glucanase preparation has no

technological function in the food. And, no unintentional reactions with other food components or on the gut micro-flora are expected.

A full set of toxicity studies, conducted under GLP, was performed on the glucanase preparation, with no evidence of toxicity found. The NOAEL was set at 200 mg/kg Body Weight /day corresponding to 190.66 mg TOS/kg Body Weight/day, far below the estimated maximum daily intake, which Nagase had determined is 0.04781 mg TOS/kg Body Weight /day. This gives a margin of exposure of 3988.

Based upon these factors it is Nagase's conclusion that their glucanase preparation from *Streptomyces violaceoruber* is GRAS for the intended conditions of use.

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LIST OF ANNEXES

A. Absence of the Production Strain in the Final Product
B. Certification of Lack of Adverse Events in Employees
C. Public Statement from the ETA Regarding Allergenicity of the Fermentation Media
D. August 2013 Food Allergy Research and Resource Program (FARRP) Statement Regarding Fermentation Media

Annex A

Nagase ChemteX

Fate of the glucanase activity in final product

1. Sample
   ① Yeast extract EYP(treated by DENAZYME GEL-L1)
   ② Yeast extract EYP was distributed in distilled water so that it might become 20% from 0.25%.
2. Assay for residual activity
   It was measured according to assay method for enzyme activity.
3. Results

Sample		GLU／g	PUN/g
Yeast extract EYP	0.4%		*
	1%		*
	4%		*
	20%		*
	0.25%	*	
	0.67%	*	
	2%	*	
	20%	*	

*：Not Detected

*：LOQ(Limited of quantitation)：

Glucanase; 0.045U/g or mL, Protease; 2 PUN/g or mL,

4. Conclusion
   The residual activity of glucanase and protease is evaluated as "none"

(b) (6)

Naoki Shirasaka

Manager,
Product Development Section
Enzymes Division
Bio Chemicals Department
Nagase ChemteX Corporation

Annex B

Nagase ChemteX

18th, June, 2012

To whom it may concern:

We, at Nagase ChemteX Corporation, have been production Denazyme GEL-L1and Denazyme GEL-P1 from a microorganism named *Streptomyces violaceoruber* pGlu since 2005 in the following production site:

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The production so far has involved more than 20 people and no allergenic symptoms have been observed or any other adverse effect reported.

Liu Xiaoli
(b) (6)

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Annex C

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POSITION PAPER

ETA Position On Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials

It is the position of the Enzyme Technical Association (ETA) that microbially derived enzymes do not fall within the scope of the Food Allergy Labeling and Consumer Protection Act (FALCPA) and that labeling for food allergens is not triggered by the use of a microbially derived enzyme preparation. There may be other reasons why a manufacturer labels a food product with regard to allergen content, but the use of a microbially derived enzyme preparation is not a reason for such labeling.

Enzymes are not one of the eight major allergenic foods, often referred to as the big 8, so they do not fit within the first requirement of FALCPA. In addition, microbial enzymes are not byproducts of nor are they derived from the major food allergens. Although enzymes are not major food allergens,[1] many enzymes are produced with microorganisms and the nutrient media used to feed these microorganisms may contain protein from one or more of the major food allergens. The enzymes are not derived from raw materials containing major food allergens, but rather are obtained from the microorganisms which are used to produce the enzyme proteins. In other words, enzymes obtained from fermentation are directly derived from microorganisms fed on media that may include protein obtained from one or more of the major food allergens. Proteins and other nitrogenous material are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein. It is the intent of the enzyme manufacturer to supply enzymes, therefore it is critical that the ratio of nutrient to enzyme yield is carefully controlled. It is also the intent of the manufacturer that these raw materials are added to the fermentation as food to be consumed by the microorganism and are not added as formulation ingredients.

In arriving at its position ETA also considered that:

- The regulatory agencies in the EU and Japan have determined that enzyme preparations are not required to have allergen labeling for the raw materials used in the fermentation process. Indeed, the European Commission's Health & Consumer Protection Directorate General has clearly stated that enzymes

[1] To the extent the enzyme producer uses an allergenic material, such as wheat flour diluent in the final product formulation, labeling may be required.

are outside the scope of the Directive 2003/89/EC which amended the EU Food Labelling Regulations.

- Enzyme broths are normally processed to separate biomass and fermentation materials from the enzyme, to concentrate the enzymatic activity, and formulated to achieve a uniform and stable enzyme product.

- The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food.

- Many enzymes do not become a component of the food ingredient or final food. Some enzymes are used in an immobilized form or are denatured during processing. Further, processing of the food ingredient after the enzyme catalyst has performed the expected function often reduces or eliminates the enzyme from the product.

- ETA has made an extensive review of the published scientific literature and has found no reports that even suggest there has been an allergenic reaction to a component of the fermentation media which was used to feed the microorganism that produced the enzyme.

The above position paper and accompanying report were provided to FDA on September 12, 2005 and to date ETA has received no comment.

Annex D

UNIVERSITY OF Nebraska Lincoln

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM

EXPERT OPINION STATEMENT
FOOD ALLERGY RESEARCH & RESOURCE PROGRAM
UNIVERSITY OF NEBRASKA

Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials

August 13, 2013

Prepared by: Steve L. Taylor, Ph.D., Co-Director
and
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with assistance from Enzyme Technical Association

Microbially derived enzymes are used by food processors as additives and processing aids in a wide variety of foods. Enzymes obtained from microbial fermentation are directly derived from microorganisms fed on sterilized media[1] that may include protein sources obtained from one or more of the recognized commonly allergenic foods (e.g., milk, soybean) or from a cereal source of gluten (e.g., wheat, barley). This paper addresses the relevance of testing microbial enzymes for allergenic material from the fermentation growth media.[2]

It has been the long-standing position of the Food Allergy Research & Resource Program (FARRP) at the University of Nebraska that testing of the products of fermentation (with limited exceptions), including microbially derived enzymes is unreliable using enzyme-linked immunosorbent assays (ELISAs).

While various fermentation media may contain one or more of the major food allergens, the biochemical reactions that occur during fermentation result in the breakdown of the fermentation media proteins. The extent of proteolysis is dependent upon the fermentation culture and the resultant enzyme (e.g., some enzymes are proteases). As proteins are digested, the resulting amino acids, along with other

[1] Aunstrup, K., O. Andresen, E.A. Falch, and T.K. Nielsen (1979) *Microbial Technology*. (Perlman and Peppler, eds.) Academic Press, pp. 281-309.

[2] For this paper, FARRP's analysis is limited to microbially derived enzymes that are intended for additive and processing aid applications in food.

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nitrogenous material, are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein.

Upon completion of fermentation, remaining fermentation media that are not consumed by the microorganism are typically separated and/or purified from the enzyme in the recovery process. Enzymes are recovered from the fermentation broth by standard chemical engineering operations, such as filtration and centrifugation, broadly used in enzyme production.[3,4] (See Appendices for further information.) The recovery steps result in separation of microbial biomass and other fermentation solids from the enzyme, concentration of the enzyme, and removal of impurities prior to final formulation with food-grade ingredients.

Any potential residual fragments from the food allergen would be difficult to measure as there is no reliable assay. Commercial ELISAs are able to detect only intact proteins in most cases. Any peptides, even larger ones, would not likely be detected, although this possibility has not been well investigated. Results would typically be reported as below the limit of quantitation for the enzyme preparation. Further, if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven. Accordingly, testing of fermented product does not result in reliable or useful data.

In addition, due to the specific catalytic nature of enzymes, only very small amounts of enzymes are generally required and used by food processors to make the desired modifications to the property of a food, and therefore any *de minimis* amount of fermentation media protein that may survive the fermentation process will not pose a significant public health risk to the consumer.[5]

FARRP also notes that regulatory agencies in the European Union and Japan do not require allergen labeling of enzyme preparations for the raw materials used in the fermentation process.

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[3] Atkinson, B. and F. Mavituna (1991) *Biochemical Engineering and Biotechnology Handbook.* (Atkinson, B. and Mavituna, F., eds.) Stockton Press, Hampshire, pp. 1146-1158.

[4] Kroschwitz, J.I. (1994) *Enzyme Applications in Encyclopedia of Chemical Technology.* 4th edition, Volume 9. (Kroschwitz, J.I., ed.), pp. 567-620.

[5] To the extent the enzyme producer uses an allergen as diluent to formulate the final product, labeling for such allergen is appropriate and required under Food Allergen Labeling and Consumer Protection Act.

SUBMISSION END