GRN 714

Subtilisin Enzyme

Preparation Derived from

Bacillus subtilis

Expressing a Subtilisin Gene

from

Bacillus amyloliquefaciens

Is Generally Recognized As Safe

For Use in Food Processing

Notification Submitted by Danisco US Inc. (operating as DuPont Industrial Biosciences)

June 16, 2017

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §170. 225, Danisco US Inc. (operating as DuPont Industrial Biosciences) submits this GRAS Notice for subtilisin. The subtilisin enzyme preparation under consideration is produced by the submerged fermentation of *Bacillus subtilis* expressing the gene encoding the subtilisin enzyme from *Bacillus amyloliquefaciens*. As both *B. amyloliquefaciens* protease and *B. subtilis* protease were affirmed as GRAS by the US Food and Drug Administration (FR 64 (78) / April 23, 1999; codified in 21 C.F.R. §184.1150, see Appendix 1), the current assessment focuses on the molecular biology methods to transform and characterize the recombinant production organism to produce this enzyme that otherwise has a very long history of safe use.

The subtilisin enzyme product is intended for the hydrolysis of proteins with broad specificity for peptide bonds, a preference for a large uncharged residue in P1, hydrolysis of peptide amides, and is used for processing of proteins. In these applications, subtilisin will be used as a processing aid and be either not be present in the final food or will be present in insignificant quantities as inactive amino acid residues, having no function or technical effect in the final food.

The systematic name of the principal enzyme activity is subtilisin. This enzyme is also known as alcalase, bacillopeptidase, alkaline proteinase, protease, thermoase, and subtilopeptidase, as described in Section 2.2.1 of this submission.

The enzyme hydrolyzes proteins with broad specificity for peptide bonds and a preference for a large uncharged residue in P1 and the hydrolysis of peptide amides with the release of protein fragments of various lengths, peptides, and free amino acids.

The EC number of the enzyme is 3.4.21.62, and the CAS number is 9014-01-1.

The information provided in the following parts is the basis of our determination of GRAS status of this subtilisin enzyme preparation.

Notwithstanding the GRAS nature of *B. amyloliquefaciens* subtilisin protease, our safety evaluation was conducted in conformance with the outline in the recent publication by the Enzyme Technical Association (Sewalt *et.al.*, 2016, see Appendix 2), which includes an evaluation of the production strain, the enzyme, and the manufacturing process (see Part 6 of this submission), as well as a determination of dietary exposure (see Part 3 of this submission).

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster, 1983). The safety of the production organism (*Bacillus subtilis* for the subtilisin) is discussed in Part 2 and 6 of this submission. A particularly important part of the safety evaluation of this enzyme, as for all enzymes derived from genetically engineered microorganisms, is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; Pariza & Foster, 1983; IFBC, 1990; EU Scientific Committee for Food, 1991; OECD, 1993; Berkowitz and Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and

are described in Part 2 of this submission. The safety evaluation described in Part 3 and 6 shows no evidence to indicate that any of the introduced DNA for or express a harmful toxic substance.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. §170.36, Danisco US Inc. has determined that our subtilisin enzyme preparation from a genetically engineered strain of *Bacillus subtilis* expressing the subtilisin enzyme from *Bacillus amyloliquefaciens* is a Generally Recognized As Safe ("GRAS") substance for the intended food applications and is, therefore, exempt from the requirement for pre-market approval.

1.2 Proposed §170.36 (c)(l)(i) Name and Address of Notifier

Danisco US Inc.
(operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

1.3 Proposed §170.36 (c)(l)(ii) Common or Usual Name of Substance

The subtilisin enzyme preparation is produced in a *Bacillus subtilis* strain expressing the gene encoding a subtilisin from *Bacillus amyloliquefaciens.*

1.4 Proposed §170.36 (c)(l)(iii) Applicable Conditions of Use

The subtilisin is used as a processing aid in the processing of proteins at 58-369 mg TOS/kg substrate) to facilitate protein hydrolysis.

Protein hydrolysates are produced by hydrolysis of proteins and peptides or protein containing raw materials from different origins, for example:

- plant (derived) raw materials, such as soy, wheat, maize, rice, etc.,
- animal (derived) raw materials, such as milk and milk derived products (whey proteins, caseins), meat, fish/seafood, collagen, gelatin, etc., and
- microbial such as yeast and microalgae.

1.5 Proposed §170.36 (c)(l)(iv) Basis for GRAS Determination

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §§170.30 (a) and (b). It is of note that both the subtilisin from the gene donor and the native subtilisin from the host were affirmed as GRAS by US FDA (21 CFR §184.1150) based on documented pre-1958 history of use as published in the Federal Register (Vol 64 No 78 / April 23, 1999).

1.6 Proposed § 170.36 (c)(l)(v) Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 during normal business hours or will be sent to the US Food and Drug Administration upon request.

1.7 Disclosure and Certification

This GRAS notice does not contain any data and or information that is exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C §552).

We confirm that the data and information in this GRAS notice satisfactorily addresses Parts 2- 7 of a GRAS notice per 21 C.F.R. §§170.230 to 170.255 as copied below:

170.230 Part 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect.
170.235 Part 3 of a GRAS notice: Dietary exposure.
170.240 Part 4 of a GRAS notice: Self-limiting levels of use.
170.245 Part 5 of a GRAS notice: Experience based on common use in food before 1958.
170.250 Part 6 of a GRAS notice: Narrative.
170.255 Part 7 of a GRAS notice: List of supporting data and information in your GRAS notice.

Danisco US Inc. certifies that to the best of our knowledge this GRAS notice is complete, representative, and a balanced submission that includes unfavorable and favorable information known to us as well as relevant to the evaluation of the safety and GRAS status of the use of the notified substance.

(b) (6)

June 16, 2017

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Date

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATION AND PHYSICAL OR TECHNICAL EFFECT

2.1 Production microorganism

2.1.1 Production Strain

The production organism is a non-sporulating strain of *Bacillus subtilis*, which has been genetically engineered through the inactivation of the endogenous genes encoding the neutral protease (*npr*), introduction of endogenous mutations to enhance protease production, removal of sporulation capacity by introduction of a deletion, and the expression of a heterologous engineered *apr* gene for the production and secretion of subtilisin from the donor *Bacillus amyloliquefaciens*. An intermediate strain in this construction, *B. subtilis* BG3594-3, was recognized by the Dutch authorities as Risk Class 1.

The production strain expresses the subtilisin gene under the regulation of the *Bacillus subtilis* subtilisin gene. An expression cassette only consisting of the engineered subtilisin gene and a chloramphenicol resistance marker gene, which has been reported in *B. subtilis,*[1] was finally integrated into the chromosome of the host strain. The final production strain was characterized by Southern blotting analyses to confirm that only the intended genetic modifications to the *B. subtilis* strain had been made.

2.1.2 Host Microorganism

The host microorganism *Bacillus subtilis* strain BG125, a previously described laboratory strain (Dedonder *et al.*, 1977) which was obtained as strain 1A10 from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio. *Bacillus subtilis* strain BG125 was derived from the well-known *Bacillus subtilis* strain 168 via classical genetics (Dedonder *et al.*, 1977).

B. subtilis is a non-toxigenic and non-pathogenic gram-positive bacterium that has a long history of safe use as a common host microorganism for food enzymes production as described in FDA's GRAS affirmation for protease and carbohydrase from *B. subtilis* (Federal Register Vol 64 Issue 78 of April 23, 1999) and subsequent GRAS Notices.[2] *B. subtilis* is considered safe as a viable probiotic product for human oral consumption (Hong *et al.*, 2008; Sorokulova *et al.*, 2008). Authors associated with the US FDA reviewed the safe use of food-processing enzymes from well-characterized recombinant microorganisms, including *B. subtilis* (Olempska-Beer et al. 2006). An extensive environmental and human risk assessment of *B. subtilis*, including its history of commercial use was published by the US Environmental Protection Agency (1997). It was concluded that *B. subtilis* is not a human pathogen nor it is toxigenic. It is also considered as Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001).

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[1] University of Goettingen, GenBank CP015975, nucleotides 1697805 to 1698840; Shaanxi Normal University, GenBank: CP014473.1, nucleotides 56598 to 57548.

[2] There are nine FDA GRN that use *B. subtilis* as the host microorganism, all of which have received a positive "FDA has no questions" letter (GRN 649, 592, 579, 476, 406, 274, 205, 114, and 20).

2.1.3 Subtilisin Expression Vector

The *apr* gene encoding the engineered *B. amyloliquefaciens* subtilisin was ligated to the chloramphenicol resistance marker gene and placed under the expression signals of the *B. subtilis* subtilisin. An expression cassette only consisting of the engineered *apr* gene, the chloramphenicol resistance gene, and two short non-expressed linker sequences, was stably integrated into the chromosome of the host strain. There is no plasmid vector replication sequence in the final production organism.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information, and the final construct was verified by Southern blot analysis.

2.1.4 Stability of the Introduced Genetic Sequences

The production strain proved to be genetically stable after at least 60 generations of fermentation, judged by chloramphenicol resistance, subtilisin production, and Southern blot analysis.

2.1.5 Antibiotic Resistance Gene

The chloramphenicol gene has been reported in the genome of *B. subtilis* strains[1] and integrated into the chromosome of the host microorganism. No new antibiotic resistance trait was conferred to the production strain.

2.1.6 Absence of Production Microorganism in Product

The absence of the production microorganism in the final product is an established specification for the commercial product and utilizes an analytical method with a detection limit of 1 CFU/g. As the production organism is not present in the enzyme preparation, it cannot be carried over into the finished.

2.2 Enzyme identity and substantial equivalence

2.2.1 Enzyme Identity

IUBMB Nomenclature:	Subtilisin
IUBMB Number:	3.4.21.62
CAS Number:	9014-01-1
Reaction catalyzed:	hydrolysis of proteins with broad specificity for peptide bonds and a preference for a large uncharged residue in P1 and the hydrolysis of peptide amides.

[1] University of Goettingen, GenBank CP015975, nucleotides 1697805 to 1698840; Shaanxi Normal University, GenBank: CP014473.1, nucleotides 56598 to 57548.

Other Names:	Alcalase; Bacillopeptidase; Alkaline proteinase; Protease; Thermoase; Subtilopeptidase
Systematic Name:	Subtilisin

Further information on subtilisin can be found in Appendix 3 and at IUBMB website[1].

2.2.2 Amino Acid Sequence

The amino acid sequence of the engineered subtilisin enzyme from *B. amyloliquefaciens* is shown in Appendix 4. It is 1 amino acid different from the native *B. amyloliquefaciens* subtilisin that was affirmed as GRAS by US FDA. The enzyme preparation is therefore substantially equivalent to the GRAS affirmed protease from both *B. amyloliquefaciens* and *B. subtilis.*

2.3 Manufacturing process

This section describes the manufacturing process for this subtilisin enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup *et al.*, 1979; and Aunstrup 1979). For a diagram of the manufacturing process, see Appendix 5. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

2.3.1 Raw Materials

The raw materials used in the fermentation and recovery process for this subtilisin concentrate are standard ingredients used in the enzyme industry (Kroschwits 1994; Aunstrup 1979; and Aunstrup *et al.*, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex ("FCC"), 10th edition (US Pharmacopeia, 2016), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco US Inc. uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam (also known as defoamers) and flocculants used in the fermentation and recovery are used in accordance with cGMP per the FDA correspondence to ETA acknowledging the listed antifoam dated September 11, 2003. Therefore, the maximum use level of these antifoam in the production process is $\leq$1.0%, cationic polymer flocculants < 1% and anionic polymer flocculant at $\leq$0.025%.

In regard to potential major food allergens, glucose (which may be derived from wheat), soy meal and milk (including lactose) will be used in the fermentation process and is expected to be consumed by the microorganism as nutrients. Therefore, the final enzyme preparation is not expected to contain any major food allergens from the fermentation medium. No other major

[1] http://www.chem.qmul.ac.uk/iubmb/enzyme/

allergen substances will be used in the fermentation, recovery processes, or formulation of this product.

2.3.2 Fermentation Process

The subtilisin enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *Bacillus subtilis* described in Part 2 of this submission. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

2.3.2.1 Production organism

A new lyophilized stock culture vial of the *Bacillus subtilis* production organism as described in Part 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed, and main fermentor) before inoculation, at regular intervals during growth, and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$ CFUs/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed necessary. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

2.3.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process.

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

1. Primary separation –centrifugation or filtration;
2. Concentration – ultrafiltration;
3. Addition of stabilizers/preservatives; and
4. Polish filtration.

2.3.4 Formulation and standardization process

The ultra-filtered concentrate is stabilized by final formulation to contain 8-12% sodium acetate and 35-45% propylene glycol at pH 5.8-6.2. The remaining portion of the formulation is water.

The final subtilisin liquid concentrate from *Bacillus subtilis* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA") in 2006 and the FCC (US Pharmacopeia, 2016). These specifications are set forth in Section 2.4.

2.4 Composition and specifications

2.4.1 Quantitative Composition

The liquid concentrate is stabilized with formulation ingredients listed below and tested to demonstrate that it meets the specification.

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition:

- Enzyme activity 2750-3490 U/g
- Enzyme protein 5-10% (w/w)
- Sodium acetate 8.00-12.00% (w/w)
- Water 33-52% (w/w)
- Propylene glycol 35-45% (w/w)

2.4.2 Specifications

The subtilisin meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex (FCC) 10th edition (US Pharmacopeia, 2016). 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 (JECFA, 2006).

The results of analytical testing of the 3 lots of product is given in Appendix 6 verifying that the product meets the FCC (2016) and JECFA (2006) specifications for enzyme preparations.

2.5 Application

2.5.1 Mode of Action

The subtilisin catalyzes the hydrolysis of proteins with broad specificity for peptide bonds and a preference for a large uncharged residue in P1 and the hydrolysis of peptide amides.

2.5.2 Use Levels

This subtilisin preparation is intended for use in protein processing. The product contains 22.0% Total Organic Solids (TOS).

	Maximum Use Rate (kg Product/MT Substance)	**Resulting Exposure** (mg TOS/kg Substance)
Protein processing	5	369

2.5.3 Enzyme Residues in the Final Foods

The subtilisin enzyme will be deactivated or removed during the subsequent production and refining processes for all applications. In the rare case that inactive subtilisin enzyme is present in the processed food and is ingested; it will not be absorbed intact. Instead, the enzyme is broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which poses no human health risk.

3. DIETARY EXPOSURE

Subtilisin is used in protein processing. While we expect no active subtilisin to remain in the processed proteins, and the following calculations assume that 100% of the TOS remains in the processed food.

The processed protein can be used in a wide variety of food, food ingredients and beverages. The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, *e.g.,* snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	**Total solid food** (kg)	**Total non-milk beverages** (l)	**Processed food (50% of total solid food)** (kg)	**Soft drinks (25% of total beverages)** (l)
	0.025	0.1	**0.0125**	**0.025**

In Section 2.5.2, the recommended use levels of the enzyme subtilisin are given, based on the raw materials used in the food process. The calculation takes into account how much food or beverage

is obtained per kg raw material (see Appendix 7 for further explanation on how these ratios were obtained), and it is assumed that all the TOS will end up in the final product.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Ratio RM/FF	Maximal level in FF (mg TOS/kg food)
Beverages	Protein processing	Proteins from various sources	369	Sport drinks	0.30	**110.7**
Solid food	Protein processing	Proteins from various sources	369	Protein hydrolysates used in e.g. soups, bouillons, dressings.	0.17	**62.7**
				protein bar	0.30	**110.7**

HUMAN EXPOSURE ASSESSMENT

In this assessment, the Budget method is used. This method was previously used by JECFA (FAO/WHO, 2001) and uses the following assumptions:

The Total Theoretical Maximum Daily Intake (TMDI) can be calculated on basis of the **maximal** values found in food and beverages (in the above case, protein processing) multiplied by the average consumption of food and beverage/kg body weight/day. It is assumed that individuals will not chronically consume both high-protein sports drinks and protein bars in the stated amounts (25% of beverage + 50% of solid food consumption). Consequently, as the potential exposure to subtilisin via high-protein sports drinks exceeds that via solid protein bars, the former is used in the TDMI calculations, in addition to exposure via protein hydrolysates in generic food categories. The Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
62.7x0.0125=0.78	110.7x0.025=2.77	**3.55**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a worst-case value because of the following reasons:

- It is assumed that all producers of the above-mentioned foodstuffs and beverages use the specific enzyme subtilisin from *Bacillus subtilis*;
- It is assumed that all producers apply the highest use level per application;
- For the calculation of the TMDI's in foodstuffs as well as in beverages, only those foodstuffs and beverages were selected containing the highest theoretical amount of TOS as the worst case. Thus, foodstuffs and beverages containing lower theoretical amounts were not considered;
- It is assumed that the amount of TOS does not decrease because of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily;
- Assumptions regarding food and beverages intake of the general population are overestimates of the actual average levels (Douglass *et al.*, 1997).

The worst-case estimated TDMI of 3.55 mg TOS/kg bw/day is consistent with the estimate made by the FDA (200 mg/person/day) as reported in its GRAS affirmation for *B. subtilis* and *B. amyloliquefaciens* protease.

4. SELF-LIMITING LEVELS OF USE

As the enzyme will be used as processing aid in the food manufacturing process according to cGMP, the intake by humans will be insignificant. Therefore, self-limiting levels of use attributable to enzyme properties (flavor, etc.) are not applicable.

In processing aid uses of enzymes, economics drive self-limitation, as customers are unlikely use more enzyme than is needed to achieve the technical effects in order to minimize production costs.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Subtilisin protease from non-toxigenic and non-pathogenic strains of *B. amyloliquefaciens* and *B. subtilis* is GRAS affirmed based on common use before 1958. The statutory conclusion of this GRAS determination is based on scientific procedures to assess substantial equivalence of the same *B. amyloliquefaciens* subtilisin enzyme produced with a genetically engineered strain of *B. subtilis*.

6. SAFETY EVALUATION

6.1 Safety of the production strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the

organism, using current Good Manufacturing Practices, are safe to consume. (IFBC, 1990). Pariza and Foster (1983) 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. *Bacillus subtilis* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity. As stated above, US FDA affirmed as GRAS the native subtilisin protease produced by both *B. subtilis* and *B. amyloliquefaciens* (Appendix 1).

6.1.1 Safety of the host

Bacillus subtilis is a Gram positive, rod shaped bacteria that is commonly found in soil. It was originally named "*Vibrio subtilis*" when it was discovered in 1835 by Christian Gottfried Ehrenberg. It was renamed "*Bacillus subtilis*" in 1872 by Ferdinand Cohn. This bacterium is also known by the names hay bacillus, grass bacillus or *Bacillus globigii.* Numerous species that appeared in the early literature are no longer recognized as official species. Former species designations that are now considered to be members of the species *Bacillus subtilis* include *Bacillus aterrimus*, *Bacillus mesentericus*, *Bacillus niger*, *Bacillus panis*, *Bacillus vulgarus*, *Bacillus nigrificans*, and *Bacillus natto* (Gibson, 1944 and Smith *et al.*, 1946 as cited by Gordon, 1973). Until 1967 strains currently identified as *Bacillus amyloliquefaciens* were also comprised in this species (Welker and Campbell, 1967). Since 1987 the separate status of *Bacillus amyloliquefaciens* has been made official in the approved lists of bacterial names (Priest *et al.*, 1987). More recently some strains have been regrouped in the species *Bacillus atrophaeus* (Nakamura, 1989). Lastly, *Bacillus mojavensis* (Roberts *et al.*, 1994), and *Bacillus vallismortis* (Roberts *et al.*, 1996), were identified from *Bacillus subtilis*-like strains isolated from soil. Together with *Bacillus amyloliquefaciens* and *Bacillus licheniformis* these species form the "*Bacillus subtilis* group" (Chun and Bae, 2000), differing by few or no phenotypic characters and having very high similarities of their 16S rRNA sequences.

Synonyms[1]: *Vibrio subtilis, Bacillus uniflagellatus, Bacillus natto,* and *Bacillus globigii.*

Bacillus subtilis has been used for many decades to produce food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991).

In accordance with the procedures described in §170.35 (21 CFR §170.35), the Ad Hoc Enzyme Technical Committee (now the Enzyme Technical Association) submitted a petition (GRASP 3G0016) to FDA requesting that, amongst various other enzyme preparations, mixed carbohydrase and protease from *Bacillus subtilis*, var. be affirmed as GRAS for use in food. FDA published a notice of filing of this petition in the Federal Register of April 12, 1973 (38 FR 9256). The petition was amended by several Federal Register notices, the last one of which on August 5, 1996 (61 FR 40648), proposed affirmation that carbohydrase and protease enzyme preparations from *B. amyloliquefaciens* are also GRAS for use in food. FDA published its final GRAS affirmation Rule on April 23, 1999 (FR 64 (78)) as follows: (1) carbohydrase enzyme preparation from *B. subtilis*; (2) protease enzyme preparation from *B. subtilis*; (3) carbohydrase enzyme

[1] Reference: Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

preparation from *B. amyloliquefaciens*; and (4) protease enzyme preparation from *B. amyloliquefaciens*. The associated enzyme activities covered in the GRAS affirmation included: α-amylase (EC 3.2.1.1), β-glucanase (EC 3.2.1.6), subtilisin (EC 3.4.21.62), and neutral proteinase (EC 3.4.24.28).

For its safety evaluation, FDA relied largely on the history of safe use in food. Based on several published literature reports (Underkofler and Ferracone, 1957; Underkofler et al., 1958, and references therein) FDA concluded that carbohydrase and protease enzyme preparations derived from *B. subtilis* and *B. amyloliquefaciens* were in common use in food prior to January 1, 1958. In addition, FDA considered the following corroborating evidence of safety:

FDA concluded that "carbohydrase enzyme preparation and protease enzyme preparation derived from either *B. subtilis* or *B. amyloliquefaciens* are GRAS under conditions of use consistent with cGMP. The agency is basing its conclusion on evidence of a substantial history of safe consumption of the enzyme preparations in food by a significant number of consumers prior to 1958, corroborated by the other evidence summarized in section IV.B of this document."

The corroborative evidence established the substantial equivalence to enzymes that are known to have been safely consumed in the diet for many years, the non-toxigenicity and non-allergenicity of the enzymes when ingested, and the low likelihood of any health concerns resulting from added substances or impurities, if any, due to the low exposure via food manufactured in accordance with cGMP. FDA estimated the highest level expected in the human diet to be 200 mg/person/day (3.3 mg/kg body weight per day for a 60kg person).

FDA affirmed that the use of these bacterially-derived carbohydrase and protease enzyme preparations in food is GRAS with no limits other than cGMP (21 CFR §184.1(b)(1)). Conditions to GRAS affirmed status include the following: that the enzyme preparations not contain antibiotics; that the bacterial strains used as a source of these enzyme preparations be nontoxigenic and nonpathogenic; and that the enzyme preparations are manufactured in accordance with cGMP using the controlled fermentation conditions, methods, and substances described in section III.B of the affirmation, as this meets the general requirements and additional requirements in the monograph on enzyme preparations in the Food Chemicals Codex, 4th ed. (Ref. 3).

The role of spore-forming Bacillus species in foodborne illness was reviewed recently by Logan (2012). *Bacillus cereus* is well known agent of food poisoning, and much more is now understood about its toxins and their involvement in infections and intoxications. It is distinct from members of the *B. subtilis* group, of which *B. licheniformis, B. subtilis* and *B. pumilus* have occasionally been isolated from cases of food-associated illness, while their roles were usually uncertain. Much more is now known about the toxins that strains of these species may produce, such as surfactin (From *et al.*, 2005; Heerklotz *et al.*, 2007) and amylosin (Apetroaie-Constantin *et al.*, 2009), so that their significances in such episodes are clearer. Given that spores of *Bacillus* species are so widely distributed and that they so commonly contaminate our food and survive processing, it is surprising that they are not isolated from cases of foodborne illness more frequently. It is also not clear why episodes involving *B. licheniformis, B. subtilis*, and *B. pumilus* are so rarely reported. However, the pre-eminence of *B. cereus* as an endospore-forming, food-poisoning organism may be explained in

part by the ability of strains of this species to grow faster than members of the *B. subtilis* group in order to out-compete them.

The ability of some spore-forming *bacilli* to play a role in food-borne illnesses is not very relevant to *Bacillus subtilis* used as production organism of subtilisin enzyme, as 1) the spore-forming ability of the strain is deleted, 2) the manufacture process removes the production organism from the enzyme preparation, and 3) the production strain was demonstrated to be non-toxigenic (Appendix 8 for the subtilisin toxicological study summary and Appendix 9 for the safe strain lineage).

The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. The safety of *Bacillus subtilis* as a production organism has been assessed by EFSA and been accorded QPS status (EFSA, 2007). The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ) and *B. subtilis* continues to be on the list (EFSA, 2017). If a newly defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. Note that, contrary to the *B. subtilis* group, both EFSA and FDA's Center for Veterinary Medicine discourage the use of strains from the *B. cereus* group, because of their potential to cause illness in humans and animals.

The US Food and Drug Administration reviewed the safe use of food-processing enzymes from well-characterized recombinant microorganisms, including *B. subtilis* (Olempska-Beer et al. 2006). An extensive environmental and human risk assessment of *B. subtilis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. subtilis* is not a human pathogen nor is it toxigenic. It is also considered part of Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001).

B. subtilis is a known safe host for enzyme production and widely used by enzyme manufacturers around the world to produce enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. This also applies to the DuPont Industrial Biosciences *B. subtilis* host strain, which has been demonstrated to be non-pathogenic, non-toxigenic, and non-cytotoxic. Various *B. subtilis* strains have been approved to produce commercial enzyme products internationally, for example, in the Canada List of Permitted Food Enzymes[1], incorporated by reference into the Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes, in the United States (21 CFR §§§ 184.1148, 184.1150 and 173.115), Mexico, Brazil, France, Denmark, Australia/New Zealand, and China. To date, nine enzymes produced in *Bacillus subtilis* have been notified to FDA/CFSAN as GRAS for their intended uses and all received a "no questions" letter.[2]

The production organism of the subtilisin enzyme preparation, the subject of this submission, is *Bacillus subtilis* strain, which was produced from strain BG125 using recombinant DNA methods.

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[1] http://www.hc-sc.gc.ca/fn-an/securit/addit/list/5-enzymes-eng.php
[2] http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=subtilis

The purpose of this genetic modification is to express the subtilisin from *Bacillus amyloliquefaciens* in *Bacillus subtilis*. BG125, a commercial production strain, is derived, because of several classical mutagenesis steps, from the well-known *Bacillus subtilis* strain 168. DuPont Industrial Biosciences has safely used strain BG125 and its derivatives for research and production purposes for over 15 years in many fermentations at a scale up to 180 tons.

DuPont has developed many production strains from *B. subtilis* using recombinant DNA techniques. All the food/feed grade products produced by this lineage were determined to be safe for their intended uses and are the subject of numerous GRAS determinations based on the Pariza and Johnson (2001) and Pariza and Cook (2010) decision tree analysis, including repeated toxicological testing (see Appendix 9). Two previous GRAS Notices were filed for the products from this strain lineage, in which FDA issued "no questions" letters (please refer to GRN592 and GRN579).

From the information reviewed, it is concluded that the organism and this specific *Bacillus subtilis* strain provides no specific risks to human health and is safe to use as the production organism of subtilisin.

6.1.2 Safety of the donor source

The donor microorganism for the gene encoding for subtilisin used in construction of the new production microorganism *Bacillus subtilis* was *Bacillus amyloliquefaciens* ATCC 23844 (previously identified as *Bacillus subtilis*).

Bacillus amyloliquefaciens ATCC 23844 is a non-pathogenic micro-organism with a long history of safe use in food production. *Bacillus amyloliquefaciens* is taxonomically very closely related to the host organism *Bacillus subtilis,* indeed, so close that prior to 1967, *Bacillus amyloliquefaciens* was called *Bacillus subtilis* and was, only after 1967, separated from that species by taxonomists (De Boer & Biderichsen 1991). Accordingly, the history and safety of *Bacillus subtilis* discussed under Section 6.1.1 of this submission is also applicable to *Bacillus amyloliquefaciens*. The food enzyme industry has extensively used *B. amyloliquefaciens* as a safe production organism for decades. Both carbohydrase (alpha-amylase and beta-glucanase) from *Bacillus amyloliquefaciens* and protease enzyme from *Bacillus amyloliquefaciens* (subtilisin and neutral protease) are affirmed as GRAS by FDA (21 C.F.R. §184.1148 and §184.1150, respectively).

In addition, food enzymes from *Bacillus amyloliquefaciens,* as well under the name *B. subtilis*, have been subjected to significant number of toxicological tests as part of their safety assessment for the use in food products manufacturing processes including a 90-day toxicological tests. These studies show that fermentation products as produced by use of *B. amyloliquefaciens* were safe for their intended uses.

6.2 Safety of the manufacturing process

In its GRAS affirmation of *B. subtilis* protease and *B. amyloliquefaciens* protease (see Appendix 1), FDA stipulated the manufacture process to utilize bacterial strains to start from a pure

laboratory culture and grown in a sterile liquid nutrient medium or sterile moistened semisolid medium. Accepted microbiological techniques were to be used to exclude contaminating organisms and to avoid development of sub-strains from within the culture itself. FDA indicated as common acceptable fermentation procedures (1) submerged culture, which uses closed fermenters equipped with agitators, aeration devices, and jackets or coils for temperature control; and (2) semisolid culture, which uses horizontal rotating drums or large chambers fitted with trays. During fermentation by either method, the pH, temperature, appearance or disappearance of certain ingredients, purity of culture, and level of enzyme activity must be carefully controlled. The fermentation is harvested at the point where laboratory tests indicate that maximum production of enzyme activity has been attained.

FDA's GRAS affirmation publication acknowledges the processes by which microbial-derived enzyme preparations are produced to vary widely, due to the large number of enzymes produced by a single strain, the marked variation in levels and types of individual enzymes produced among species and even among strains of the same species, and further dependent upon the composition of the growth medium and the fermentation conditions, which are each optimized to maximize the desired enzyme activity. FDA further states:

> The carbohydrase and protease enzymes from *B. subtilis* and *B. amyloliquefaciens* are excreted into the fermentation medium (Refs. 9 through 11). In the semisolid culture method, an enzyme that is present in the fermentation medium is extracted either directly from the moist material, or later after the cualture mass has been dried. In the submerged culture method, the microorganisms and other insolubles are removed from the fermentation medium by decanting, filtering, or centrifuging, and therefore an extraction step is not required. In either method, further processing steps may involve clarification, evaporation, precipitation, drying, and grinding (Refs. 6 and 9 through 12).

The manufacturing process for the subject of this GRAS Notice, our *B. amyloliquefaciens* subtilisin expressed in *B. subtilis* will be conducted in a manner consistent to that described above, as it is for most enzymes for use in food and feed production processes. It consists of a pure-culture submerged fermentation process, cell separation, concentration, and formulation. The process is conducted in accordance with current food good manufacturing practice (cGMP) as set forth in 21 C.F.R. Part 110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 10th Edition (US Pharmacopeia, 2016) and the general specifications for enzyme preparations used in food processing proposed by JECFA (2006).

The fermentation process may utilize a wheat-derived source of glucose, soy meal and milk including lactose that may contain trace amount of protein. This feedstock is expected to be consumed by *B. subtilis* as nutrients. The final enzyme preparation does not contain any major food allergens from the fermentation medium.

6.3 Safety of Subtilisin

Traditionally, protein hydrolysis was carried out using substances such as hydrochloric acid or through the process of boiling meat and fish pieces. Since the 1950s, proteases came into use because of their effective hydrolysis activity, leading to increased yield and enhanced flavors (Whitehurst and Law, 2010). Initially, a limited number of proteases were used (Criswell *et al.*, 1964; Sripathy *et al.*, 1962), but currently a larger range of proteases (animal, plant, fungal or bacterial origin and alkaline, acid, neutral, heat-resistant, etc.), including modified subtilisin, are in use (Hale, 1969; Feldman *et al.*, 1974; Koury and Spinelli, 1974; Shimono and Sugiyama, 2010). In general, enzymatic hydrolysis has been utilized for over a century as evidenced by publications in 3 different journals dating back as far as 1917[1], and can be stopped at any time through heating hereby providing more control of the hydrolysis process. Protease from both *B. subtilis* and *B. amyloliquefaciens* were affirmed as GRAS by FDA (21 C.F.R. §184.1150), and such protease includes subtilisin, in addition to neutral protease.

6.3.1 Allergenicity

According to Pariza and Foster (1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Subtilisin proteases have been in commerce since before 1958 as outlined in the GRAS affirmation for protease from *B. subtilis* and *B. amyloliquefaciens* (FR 64 (78), April 23, 1999) without any publically available reports of oral allergenicity.

In 1998, the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy" (Bindslev-Jensen *et al.*, 2006).

Despite this lack of general concern, the potential that subtilisin could be a food allergen was assessed by comparing the amino acid sequence with sequences of known allergens in a public database, which is described in more detail below. The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.*, (2011) recommend the use of FASTA or BLASTP bioinformatic searches (Pearson, 1996) for matches of $\geq$ 35% identity over 80 or greater amino acids of a subject protein and a known allergen. To conduct the bioinformatic analysis of subtilisin, three FASTA searches were performed: 1) a full length amino acid sequence search and 2) a sliding 80-amino acid window search and 3) an 8-amino acid search

[1] Wallersteein, L. 1997. Enzymes in the Fermentation Industries. Journal of The Franklin Institute 183 (5): 531-556 and 715-734. Also reprinted in: The Western Brewer; and Journal of the Barley, Malt and Hop Trades; Journal of the American Association of Cereal Chemists: various short papers on enzymes in wheat, brewing and baking.

The *Bacillus amyloliquefaciens* subtilisin (mature) amino acid sequence is given in Appendix 4. The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[1] containing 2035 peer-reviewed allergen sequences released on January 18, 2017[2] revealed 26 allergens by identity across 80 amino acids exceeding the threshold of 35 % identity.

Most matches included other serine proteases from various microorganisms however, none of them are food allergens. Matches were also found to other subtilisins from various sources. In fact, bacterial subtilisins are well-known for their ability to elicit sensitization via inhalation and respiratory allergies, yet this is irrelevant for food allergenicity as also asserted in FDA's GRAS affirmation for bacterial protease.

One of the 80-mer matches was identified as Cuc m 1 (muskmelon, cucumisin, an alkaline serine protease), an IUIS recognized food allergen (IUIS; www.allergen.org), (Appendix 10) with a %-identity of 37.8% over 80 amino acids. The ≥ 35% match of our subtilisin to Cuc m 1 from muskmelon was identified using the sliding 80 amino acid window analysis. In contrast, the full-length FASTA sequence analysis did not reveal the Cuc m 1 match above the 35% threshold (*i.e.*, only 27.5% identity in a 189-amino acid overlap was identified). Importantly, the conduct of a full length FASTA analysis is the more appropriate search as it results in less false positive findings (Ladics *et al.*, 2007; Cressman and Ladics, 2009).

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al.,* (2011) and on AllergenOnline.com, there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and missed by the conservative 80 amino acid match (35%). The AllergenOnline database, however, does allow for isolated identity matches of 8-contiguous, identical amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8-contiguous identical amino acid search produced 23 of the 26 full FASTA alignment results, however, Cuc m 1 was not included in the 23 matches. No additional sequence matches with known allergens were revealed.

Cuc m 1 and its structural similarities with microbial subtilisins are discussed in several publications. Yamagata *et al.,* (1994) state that cucumisin (Cuc m 1) has several features in common with the microbial proteases of the subtilisin family and that the highly-conserved sequences to the proximal regions of the catalytic triad amino acids (Asp, His, and Ser), together with the substrate binding site in subtilisin, can be found within the amino acid sequence of the protease domain of the cucumisin precursor. Cuesta-Herranz *et al.,* (2003) discusses in detail the structural homology of the Cuc m 1 with plant subtilisin-like serine proteases.

In addition, we have found weak homology for Cuc m 1 with another bacterial subtilisin from *B. lentus,* with 30% identity and 5.3 x 10^{-6} as the E-score. Moreover, results from an allergen sequence screen comparison between the *B. amyloliquefaciens* subtilisin to other wild-type,

[1] http://www.allergenonline.org/index.shtml
[2] http://www.allergenonline.org/databasebrowse.shtml

GRAS-affirmed subtilisins showed the same weak homology to Cuc m 1, see table below. The amino acids from all the strains are shown in Appendix 4.

Table 1. Allergen screen results comparing *B. amyloliquefaciens* subtilisin produced in *B. subtilis* to three subtilisin wild-type strains using AllergenOnline.org.

Name	8-mer*	80-mer*	FASTA*
B. amyloliquefaciens subtilisin produced in *B. subtilis*	No	Yes**	No
B. subtilis subtilisin (Wild-Type) 21 C.F.R. §184.1150	No	No***	No***
B. amyloliquefaciens subtilisin (Wild-Type) 21 C.F.R. §184.1150	No	Yes**	No***
Bacillus licheniformis subtilisin (Wild-Type) 21 C.F.R. §184.1027	No	Yes**	No***

*Indicates whether any food allergen hits from found through AllergenOnline.org.
** "Yes" response indicates that the food allergen (Cuc m1) hit is above the threshold of 35%. The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.*, (2011) recommend the use of FASTA or BLASTP bioinformatic searches (Pearson, 1996) for matches of ≥ 35% identity over 80 or greater amino acids of a subject protein and a known allergen.
***Cuc m 1 listed but falls below the threshold of 35%.

Results from the allergen sequence screen comparison illustrates the similarities between previously GRAS-affirmed subtilisins and the production strain in question. The weak homology to the potential allergen Cuc m 1 (*Cucumis melo*) is shared by two of the wild-type and GRAS affirmed subtilisins (*B. amyloliquefaciens* 21 C.F.R. §184.1150 and *Bacillus licheniformis* 21 C.F.R. §184.1027).

As part of the weight-of-evidence approach, Codex Guidelines (2009) further recommend testing for resistance of a protein to pepsin digestion since "a correlation exists between resistance to digestion by pepsin and allergenic potential." As part of a parallel investigation on the highly similar subtilisin protease from *Bacillus lentus* also produced in *B. subtilis*, a pepsin resistance test was conducted on the *B. lentus* protease. A protein sequence comparison between the two proteases using the National Center for Biotechnology Information (NCBI) protein BLAST search tool revealed that the two protease amino acid sequences are 60% identical and have a similarity of 76% (Appendix 10). Results showed that this protease is readily degraded within one minute upon pepsin administration at pH 1.2 to peptides that are < 3 kDa. Therefore, it is highly unlikely that IgE binding epitopes would be present long enough in the GI tract to be of concern (Appendix 10). The high structural similarity of the modified *B. amyloliquefaciens* subtilisin with the *B. lentus* subtilsin is predictive of the subject subtilisn also being readily degraded.

It is of note that the human study by Bindslev-Jensen *et al.*, (2006) included subtilisin from *B. amyloliquefaciens* as one of the test articles. That study failed to indicate positive reactions to 19 orally challenged commercial enzymes, including subtilisin, in a double-blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens.

Taken together, these data indicate a lack of concern regarding the food allergy potential for the modified *B. amyloliquefaciens* subtilisin enzyme expressed in *B. subtilis*.

6.3.2 Safety of use in food

Subtilisin from *Bacillus amyloliquefaciens* expressed *in B. subtilis* has been determined to be Generally Recognized as Safe (GRAS) by scientific procedures by Danisco US Inc. Subtilisin[1] from *Bacillus amyloliquefaciens* has also been evaluated by many other regulatory bodies around the world, including those in Brazil, Canada, France, China, and Mexico and determined to be safe for use in food processing.

In addition to the allergenicity assessment described above, the safety of this subtilisin has also been established using the Pariza and Johnson (2001) decision tree:

1. **Is the production strain[2] genetically modified[3, 4]?** Yes. Go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes. Go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA[5, 6] have a history of safe use in food[7]?** Yes, subtilisins with the designation EC# 3.4.21.62, CAS# 9014-01-1, have been widely and safely used in many food applications, for decades. Subtilisin has been used for years in food processing with history of safe use. US FDA affirmed the GRAS status of mixed carbohydrase/protease enzyme preparation derived from *B. licheniformis* and protease *from B. licheniformis* or *B. amyloliquefaciens* for use in food with GMP as the only limitation (21 C.F.R. §§184.1027 and 1150, respectively). In addition, proteases were GRAS notified to FDA, including protease from *B. licheniformis* (GRN 564), and the agency issued "no Question" letters in response. Go to 3c.

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[1] Most positive lists mention the general name 'protease' and do not differentiate between the various proteases.
[2] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this.
[3] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (*e.g.*, UV or chemically-induced mutagenesis) or rDNA technologies.
[4] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.
[5] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.
[6] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.
[7] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

3c. Is the test article free of transferable antibiotic resistance gene DNA[1]?
Yes. No transferable antibiotic resistance gene DNA is present in the enzyme preparation. 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-grade products?
Yes. Inserted DNA is well characterized and free of unsafe attributes. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
No, the DNA was integrated by homologous recombination into the chromosome at the site of the *apr* promoter, where it stably maintained. Go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[2]? Yes. The *Bacillus subtilis* production strain pertains to the *Bacillus subtilis* safe strain lineage (Appendix 9). *Bacillus subtilis* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing.

Conclusion: This test article is **accepted.**

Notwithstanding the long-standing safety record and GRAS-affirmed status of subtilisin from both *B. amyloliquefaciens* and *B. subtilis*, and the outcome of the Pariza and Johnson (2001) decision tree, toxicological studies are also available to corroborate the safety of the subject subtilisin enzyme for its intended use.

6.3.3 Safety Studies

B. amyloliquefaciens subtilisin is an enzyme preparation produced from *B. subtilis* which will be used as a processing aid in protein processing to facilitate protein hydrolysis.

Genencor International Inc (later acquired by Danisco US Inc.) conducted toxicological studies on the subject subtilisin preparation in 1994. These studies predate current OECD guidelines from 1997/98, however the summary of those studies is provided in Appendix 8, as supportive but not pivotal safety studies. Also, these studies were not used to set the No Observed Adverse Effect Level (NOAEL) in support of our safety determination.

Danisco US Inc. determined by scientific procedures that the *B. subtilis* production strain pertains to a DuPont Industrial Biosciences' *B. subtilis* safe strain lineage. A review of all toxicology

[1] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm).

[2] In determining safe strain lineage one should consider the host organism, all the introduced DNA, and the methods used to genetically modify the host (see text). In some instances, the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage.

studies conducted with enzyme preparations produced by different strains of the DuPont Industrial Biosciences *Bacillus subtilis* safe strain lineage indicates that, regardless of the production *Bacillus subtilis* strain, all enzyme preparations were found to have the following conclusions:

1) Negative as a dermal irritant;
2) Negative as an ocular irritant;
3) Negative as a mutagen, clastogen, and aneugen in genotoxicity studies; and
4) Not observed to adversely affect any specific target organs.

Therefore, due to the consistency of the findings supporting the safety of enzyme preparations derived from different *Bacillus subtilis* strains, it is reasonable to expect that any other common with a history of safe use enzyme preparation produced with strains from the same *Bacillus subtilis* lineage would have a similar toxicological profile (Appendix 9).

Hence, the safety of this subtilisin produced in *Bacillus subtilis* can be supported using toxicology studies conducted on enzymes from other strains from the DuPont *Bacillus subtilis* Safe Strain Lineage.

Based on strain lineage, the production strain most closely related to, the *B. subtilis* production strain of the subtilisin is *B. subtilis* strain producing *Bacillus lentus* subtilisin, hereafter referred to as "*B. lentus* subtilisin". A 90-day oral toxicity study with the *B. lentus* subtilisin produced in *B. subtilis* has been conducted, and the data can be extrapolated to the *B. amyloliquefaciens* variant subtilisin, the subject of this document. This approach is in line with the Safe Strain Lineage concept (Pariza and Johnson, 2001) endorsed by the Enzyme Technical Association (Sewalt *et al.*, 2016) and accepted by regulatory agencies including US FDA and the Danish Food Safety Authority. The study was conducted in accordance with OECD Test 408 (1998), OECD Principles of Good Laboratory Practice (GLP) (1998), and all subsequent OECD consensus documents. The results are evaluated, interpreted, and assessed in this document.

DuPont Industrial Biosciences has conducted a 90-day oral (gavage) study at CiTox LAB Scantox (Denmark) on the *B. lentus* subtilisin enzyme produced with *Bacillus subtilis* strain. The test material, Ultra-Filtered Concentrate (UFC), used in all toxicology investigations has the following characteristic:

Lot No.:	6202401
Physical:	Fermentation liquid, brown
pH:	6.77
Specific gravity:	1.08 g/ml
Total protein:	193.4 mg/ml
TOS:	22.13 %
	(1 mg Total Protein (TP) = 1.144 mg TOS)

A 13-week Oral (Gavage) Toxicity Study in Rats, Citox Lab Scantox, Report No. 73796, November 23, 2011

Procedure

The objective of this study was to investigate the potential of *B. lentus* subtilisin to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 105, 210 or 420 mg total protein/kg body weight in a constant volume of 5 ml/kg body weight corresponding to 120.2, 240.3, or 480.6 mg TOS/kg bw/day, respectively.

Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity, and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted at study termination. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for the control and high dose groups. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed, and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals.

Results

Four animals were found dead - two males and one female in the low dose group and one high dose male. Blood, blood clots or reddish watery fluid was observed in the chest cavity at necropsy indicating mis-dosing of fluid into the chest cavity. One mid-dose female was killed in a moribund condition and at the microscopic examination inflammation of the lungs and larynx was observed, correlating well with the suspicion of a dosing accident. These mortalities were therefore considered as procedural errors (gavage errors) and not as treatment related.

A slight decrease in body weight gain was observed for the high dose males. However, as this finding was within the normal historical range, it was not considered of toxicological importance. Administration of *B. lentus* subtilisin for 90 consecutive days did not result in any treatment related effects on clinical examination, feed consumption, water consumption, ophthalmoscopic examination, urinalysis, clinical chemistry, hematology and coagulation parameters. No treatment related effects were noted in the functional observation battery and stimuli-induced tests. At necropsy, at the organ weight analysis and at the histopathologic examination, no treatment related findings were recorded.

In conclusion, daily administration by oral gavage of *B. lentus* subtilisin produced in *Bacillus subtilis* to Sprague Dawley rats for 13 weeks at dosages of 0, 105, 210, and 420 mg total protein/kg/day did not cause any test item related changes.

Consequently, in this study, the NOAEL (No Observed Adverse Effect Level) was 420 mg total protein/kg/day (corresponding to 480.6 mg TOS/kg bw/day).

Evaluation and conclusion
In this study, five animals died. However, all five mortalities were not considered as treatment-related but rather due to gavage error. Therefore, daily administration of subtilisin by oral gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 420 mg total protein/kg bw/day corresponding to 480.6 mg TOS/kg bw/day.

6.4 Overall safety assessment

6.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in Sprague Dawley rats, a NOAEL was established at 480.6 mg TOS/kg bw/day. The study was designed based on OECD guideline No. 408 (1998) and conducted in compliance with both the FDA Good Laboratory Practice Regulation (21 CFR Part 58) and the OECD (1998) Good Laboratory Practice Principles. Since human exposure to this subtilisin is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 480.6 mg TOS/kg bw/day

6.4.2 Conclusion

Determination of the margin of safety

Notwithstanding the outcome of the Pariza and Johnson (2001) decision tree analysis (Article ACCEPTED), it is prudent to ascertain that the most suitable available NOAEL is sufficiently high to accommodate the intended use. The margin of safety was calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst case scenario) assessed in Part 3. As the margin of safety was determined to be greater than 100 (*i.e.*, 135), it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of safety} = \frac{\text{No observed adverse effect level}}{\text{Maximum daily exposure}}$$

$$\text{Margin of safety} = \frac{480.6 \text{ mg TOS/kg bw/day}}{3.55 \text{ mg TOS/kg bw/day}} = 135$$

6.5 Basis for general recognition of safety

As noted in the Safety sections above, *Bacillus subtilis*, and enzyme preparations produced there with, including beta-glucanase, lactase xylanase, proteases, and alpha-amylases enzyme preparations, are well recognized by qualified experts as being safe for their intended uses. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA (1992), as well as Dansico US Inc.'s (operating as DuPont Industrial Biosciences) own unpublished safety studies, support such a conclusion.

Bacillus subtilis is widely used by enzyme manufacturers around the world for production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is generally recognized as a safe host for enzyme production. In addition, the *Bacillus subtilis* lineage used by Danisco US Inc. has been demonstrated to be safe based on repeated testing and evaluation using the Pariza and Johnson (2001) decision tree.

The safety of *Bacillus amyloliquefaciens* subtilisin expressed in *Bacillus subtilis* strain as a processing aid to be used in protein processing at the maximum recommended application rates is supported by toxicological data.

The margin of safety was calculated to be 135 based on a NOAEL of 480.6 mg TOS/kg bw/day based on the toxicological studies from *B. lentus* subtilisin (obtained from the cumulative maximum daily exposure to subtilisin of 3.55 mg TOS/kg bw/day). In the rare case of ingestion of the subtilisin enzyme preparation, it is not expected to pose safety or health concerns to humans, based on maximum recommended application rates which are supported by existing toxicology data for this enzyme. Based on a margin of safety greater than 100 even in the worst-case, the uses of subtilisin as a processing aid in the protein processing application are not of human health concern.

Based on the publicly available scientific data from the literature and additional supporting data generated by Danisco US Inc. (operating as DuPont Industrial Biosciences), the company has concluded that subtilisin from *Bacillus amyloliquefaciens* produced in *Bacillus subtilis* is safe and suitable for use in the protein processing application. Therefore, it can be considered Generally Recognized as Safe (GRAS). In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by the external experts in the field, Drs. Michael Pariza and Joseph Borzelleca, who concurred with the company's conclusion that the enzyme in the product (referred to in the GRAS letter as Multifect P3000) is GRAS for its intended uses (see Appendix 11).

7. SUPPORTING DATA AND INFORMATION

7.1 List of references

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7.2 List of appendices

Appendix 1: GRAS Affirmation 21 C.F.R. §184.1150

Appendix 2: ETA Publication by Sewalt *et al.,* 2016

Appendix 3: IUBMB Nomenclature Record on Subtilisin

Appendix 4: Amino Acid Sequences

Appendix 5: Manufacturing Process

Appendix 6: Certificates of Analyses (3 batches)

Appendix 7: Application Process and Assumptions behind Ratios of Raw Materials to Final Food

Appendix 8: Toxicology Study Summary of *Bacillus amyloliquefaciens* Subtilisin Produced in *B. subtilis*

Appendix 9: *Bacillus subtilis* Strain Lineage and Summary of Safety Studies

Appendix 10: Supporting Allergenicity Risk Assessment Data.

Appendix 11: GRAS Opinion Letter

Appendix 1: GRAS Affirmation 21 C.F.R. §184.1150

ELECTRONIC CODE OF FEDERAL REGULATIONS

e-CFR data is current as of May 30, 2017

Title 21 → Chapter I → Subchapter B → Part 184 → Subpart B → §184.1150

Title 21: Food and Drugs
PART 184—DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE
Subpart B—Listing of Specific Substances Affirmed as GRAS

§184.1150 Bacterially-derived protease enzyme preparation.

(a) Bacterially-derived protease enzyme preparation is obtained from the culture filtrate resulting from a pure culture fermentation of a nonpathogenic and nontoxigenic strain of *Bacillus subtilis* or *B. amyloliquefaciens.* The preparation is characterized by the presence of the enzymes subtilisin (EC 3.4.21.62) and neutral proteinase (EC 3.4.24.28), which catalyze the hydrolysis of peptide bonds in proteins.

(b) The ingredient meets the general requirements and additional requirements in the monograph on enzyme preparations in the Food Chemicals Codex, 4th ed. (1996), pp. 128-135, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or may be examined at the Food and Drug Administration's Main Library, 10903 New Hampshire Ave., Bldg. 2, Third Floor, Silver Spring, MD 20993, 301-796-2039, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.* In addition, antibiotic activity is absent in the enzyme preparation when determined by an appropriate validated method such as the method "Determination of antibiotic activity" in the Compendium of Food Additive Specifications, vol. 2, Joint FAO/WHO Expert Committee on Food Additives (JECFA), Food and Agriculture Organization of the United Nations, Rome, 1992. Copies are available from Bernan Associates, 4611-F Assembly Dr., Lanham, MD 20706, or from The United Nations Bookshop, General Assembly Bldg., rm. 32, New York, NY 10017, or by inquiries sent to *http://www.fao.org.* Copies may be examined at the Center for Food Safety and Applied Nutrition's Library, 5001 Campus Dr., College Park, MD 20740.

(c) In accordance with §184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in §170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

[64 FR 19895, Apr. 23, 1999, as amended at 81 FR 5593, Feb. 3, 2016]

Need assistance?

Appendix 2: ETA Publication by Sewalt *et al.*, 2016

Pages 000039-000046 have been removed in accordance with copyright laws. The removed citation is:

Sewalt, V, Shanahan, D, Gregg, L, La Marta, J, and Carrillo, R. 2016. The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology. 12(5): 295-302

Appendix 3: IUBMB Nomenclature Record on Subtilisin

IUBMB Enzyme Nomenclature

EC 3.4.21.62

Accepted name: subtilisin

Reaction: Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1. Hydrolyses peptide amides

Other names: alcalase; alcalase 0.6L; alcalase 2.5L; ALK-enzyme; bacillopeptidase A; bacillopeptidase B; *Bacillus subtilis* alkaline proteinase bioprase; bioprase AL 15; bioprase APL 30; colistinase; (see also comments); subtilisin J; subtilisin S41; subtilisin Sendai; subtilisin GX; subtilisin E; subtilisin BL; genenase I; esperase; maxatase; alcalase; thermoase PC 10; protease XXVII; thermoase; superase; subtilisin DY; subtilopeptidase; SP 266; savinase 8.0L; savinase 4.0T; kazusase; protease VIII; opticlean; Bacillus subtilis alkaline proteinase; protin A 3L; savinase; savinase 16.0L; savinase 32.0 L EX; orientase 10B; protease S

Comments: Subtilisin is a serine endopeptidase, type example of peptidase family S8. It contains no cysteine residues (although these are found in homologous enzymes). Species variants include subtilisin BPN' (also subtilisin B, subtilopeptidase B, subtilopeptidase C, Nagarse, Nagarse proteinase, subtilisin Novo, bacterial proteinase Novo) and subtilisin Carlsberg (subtilisin A, subtilopeptidase A, alcalase Novo). Formerly EC 3.4.4.16 and included in EC 3.4.21.14. Similar enzymes are produced by various *Bacillus subtilis* strains and other *Bacillus* species [1,3]

Links to other databases: BRENDA, EXPASY, KEGG, MEROPS, Metacyc, PDB, CAS registry number: 9014-01-1

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[EC 3.4.21.62 created 1992 (EC 3.4.21.14 created 1961 as EC 3.4.4.16, transferred 1972 to EC 3.4.21.14, modified 1986, part incorporated 1992)]

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Appendix 4: Amino Acid Sequences of Subtilisin

B. amyloliquefaciens subtilisin expressed in *B. subtilis*

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQ
DNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIAN
NMDVINMSLGGPSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVI
AVGAVDSSNQRASFSSVGPELDVMAPGVSIQSTLPGNKYGALNGTSMASPHVAGAAAL
ILSKHPNWTNTQVRSSLENTTTKLGDSFYYGKGLINVQAAAQ

B. lentus alkaline protease express in *B. subtilis*

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDG
NGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGM
HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATD
QNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKN
PSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR

B. subtilis subtilisin (Wild-Type) 21 C.F.R. §184.1150

MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVI
SEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQI
KAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAG
TIAALNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPT
GSTALKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASF
SSAGSELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVR
DRLESTATYLGNSFYYGKGLINVQAAAQ

B. amyloliquefaciens subtilisin (Wild-Type) 21 C.F.R. §184.1150

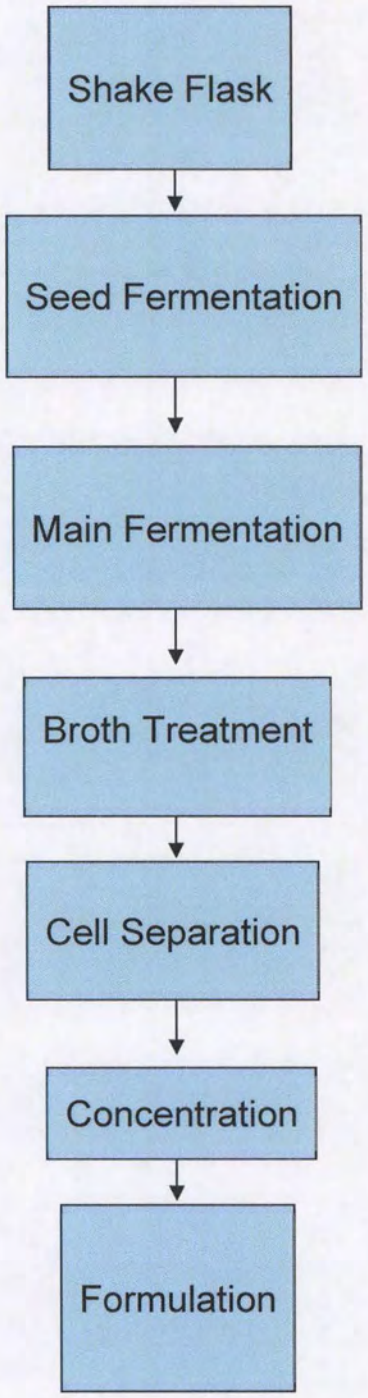
MRGKKVWISLLFALALIFTMAFGSTSSAQAAGKSNGEKKYIVGFKQTMSTMSAAKKKD
VISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAHAYAQSVPYGV
SQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSHGTH
VAGTVAALNNSIGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMS
LGGPSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSS
NQRASFSSVGPELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNW
TNTQVRSSLENTTTKLGDSFYYGKGLINVQAAAQ

Bacillus licheniformis subtilisin (Wild-Type) 21 C.F.R. §184.1027

MMRKKSFWLGMLTAFMLVFTMAFSDSASAAQPAKNVEKDYIVGFKSGVKTASVKKDII
KESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHALAQTVPYGIPLI
KADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASFVAGEAYNTDGNGHGTHV
AGTVAALDNTTGVLGVAPSVSLYAVKVLNSSGSGTYSGIVSGIEWATTNGMDVINMSL

GGPSGSTAMKQAVDNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSN
SNRASFSSVGAELEVMAPGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLS
ASQVRNRLSSTATYLGSSFYYGKGLINVEAAAQ

Appendix 5: Manufacturing Process of Subtilisin



Appendix 6: Certificates of Analyses (3 batches)

DANISCO.

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro® 30L**

BATCH: **4882822292**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alkaline Protease	GSU/g	2750-3490	3305
PHYSICAL PROPERTIES			
pH		5.8-6.2	6.1
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

6-Jun-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

DANISCO.

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro® 30L**

BATCH: **4882937561**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alkaline Protease	GSU/g	2750-3490	2803
PHYSICAL PROPERTIES			
pH		5.8-6.2	5.9
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

6-Jun-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

DANISCO.

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro® 30L**

BATCH: **4882964925**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alkaline Protease	GSU/g	2750-3490	3137
PHYSICAL PROPERTIES			
pH		5.8-6.2	6.0
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

6-Jun-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 7: Application processes and assumptions behind ratio's of raw material to final food

- **Protein processing:**

  Food and food ingredients produced with the use of Protein processing products fall in the category of solid foods and liquid food.

  Dietary proteins are found in variable proportions in different foods resulting in variability of dietary protein intake within and between populations. Data from dietary surveys show that the average protein intakes in European countries vary between 67 to 114 g/day in adult men and 59 to 102 g/day in women, wich represents about 12 to 20 % of total energy intake (E %) for both sexes (EFSA scientific Opinion on Dietary Reference Values for protein[1]).

  Protein processing leads to a variety of products such as, but not limited to peptides and amino acids. These products are applied in food processing, food ingredients and various types of final foods.

  Solid food: Examples (not exhaustive) of solid foods are processed foods, meat derived products, bread, snacks, soups and bouillons, dressings etc.
  The assumption used for calculation of dietary exposure is an average intake of 17% of the total diet. The corresponding RM/FF ratio will therefore be 0.17 kg protein / kg final food.

  Liquid food: Examples (not exhaustive) of liquid foods are sports drinks
  The assumption used for calculation of dietary exposure for Protein processing products in liquid food is from the example of sports drink set at a higher of 2.7 g protein/kg bw/day[2] which account for 33 % of total energy intake (E %) in a 70 kg adult male.

[1] http://www.efsa.europa.eu/fr/efsajournal/doc/2557.pdf
[2] Ref: Phillips, ST & Vanloon, LJC (2011), Dietary protein for athletes: From requirements to optimum adaptation, Journal of Sports Sciences, 29(S1): S29–S38

The assumption used for calculation of dietary exposure is an average intake of 30 % of the total diet. The corresponding RM/FF ratio for sports drinks will therefore be 0.30 kg protein/kg final food.

Application		**Raw material (RM)**	**Maximal recommended use level (mg TOS/kg RM)**	**Final food or beverage**	**Ratio RM/final food**	**Maximal level in final food (mg TOS/kg food)**
Beverages	Protein processing	Proteins from various sources	Y	Sports drinks	0.30	Yx0.30
Solid food	Protein processing	Proteins from various sources	Y	Protein hydrolysates used in e.g. soups, bouillons, dressings.	0.17	Yx0.17

Appendix 8: Toxicology Study Summary of *Bacillus amyloliquefaciens* Subtilisin Produced in *B. subtilis*

Toxicology Test Summaries for Subtilisin derived from *B. amyloliquefaciens* produced in *B. subtilis*

Toxicological testing was performed on the subtilisin, which is the subject of this GRAS notification, were performed in 1994, and therefore were not performed under internationally accepted guidelines (*i.e.,* not compliant with OECD guidelines). Nonetheless, we have provided a summary of the toxicological studies as supportive, but not pivotal, evidence of safety.

A review of all the following toxicology studies conducted with the subtilisin enzyme preparation indicate that it is not a strong irritant, not mutagenic or clastogenic in genotoxicity assays and does not adversely affect any specific target organ.

90-Day Oral Feeding Study, Pharmakon, Report No. PH-470-GNC 001-94. 1994.

Procedure:
In a 90-day oral feeding study, groups of rats were fed with a concentrated subtilisin enzyme solution produced from *B. subtilis* production strain at 0, 5000, 15000 or 50000 ppm in the diet for 90 consecutive days.

Evaluation and Results:
Feed consumption and body weight gain were significantly decreased during the first few weeks of treatment and were related to poor palatability. By study termination, all treated groups had comparable weight gain and feed consumption as the control group. There were no overt signs of toxicity throughout the entire study. One control animal died and the cause of death was not known. At necropsy, dose-related increases in salivary gland weights were noted in both male and female treated groups. These findings were related to hypertrophy of the serous acinar cells of the sub-mandibular salivary glands in both male and female treated rats. The presence of hypertrophy of the sub-mandibular salivary glands was determined not as an adverse effect but rather as a physiological adaptation and local response to continuous exposure to a subtilisin in the diet. The effect was expected due to the irritant property of protease.

Conclusion:
There were no other abnormal histopathologic findings found in the treated groups. No treatment related effects were noted in hematology, clinical chemistry, ophthalmology, and urinalysis. Consequently, the systemic NOAEL was established at 50,000 ppm (5% in the diet).

Subchronic Oral toxicity study in rats, Pharmakon, No. 470-GNC-001-94, 1994.

Procedure:
The test article subtilisin was incorporated into animal's diet and fed *ad libitum* to three groups of Sprague Dawley rats (20 animals/sex/group) seven days a week for 90 consecutive days at dose levels of 5000, 15000, and 50000 ppm. The vehicle, propylene glycol/water was incorporated into rat diet a concentration of 50000 ppm and fed *ad libitum* to a fourth group of rats (20

animals/sex/group) which served as the vehicle control group. During the study, individual animals were observed and clinical signs recorded daily, while individual animal body weights and food consumption were recorded weekly. Evaluation of clinical chemistry, hematology, and urinalysis parameters was performed on 10 animals per sex/group, selected at random, on Day 30 for males, and Day 31 for females, and just prior to the terminal sacrifice. All animals were sacrificed and necropsy performed.

Evaluation and Results:
There were no clinical signs of systemic toxicity observed during the study. Dermal scaling of the tail tip was observed for a few animals in all dose groups. Other observations which were low in incidence included scab formation on the dorsal cranial region, and red and/or missing tail tips. Chromodacryorrhea related to the retro-orbital bleeding procedure or malocclusion was also observed for a few animals in all dose groups. All of these findings were incidental and unrelated to the toxicity of the subtilisin.

Evaluation of the clinical chemistry data, hematology and urinalysis data obtained at interim blood sampling and just prior to the terminal sacrifice did not reveal any biologically significant test article related effects. There were no findings from the ophthalmic examination which were attributable to the test article.

Dietary administration of 5000, 15000, and 50000 ppm of subtilisin concentrate for 90 days resulted in a dose related enlargement (hypertrophy of the serous acinar cells) of the submandibular salivary glands of the male and female rats. No treatment-related changes were present in the other tissues evaluated from the male and female rats receiving 50000 ppm of the subtilisin concentrate. A few incidental findings occurred in both the control rats and the rats receiving 50000 ppm of subtilisin concentrate at essentially comparable incidences and were of the usual type and incidence commonly seen in Sprague Dawley rats. The presence of the incidental lesions did not interfere in the evaluation of the test substance as used in this study. The primary change in the enlarged salivary glands was hypertrophy without degenerative or inflammatory changes. These changes were considered to be a physiologic response and not a toxic effect on this organ.

Conclusion:
Because the changes in the salivary glands were a physiologic effect and not a toxic effect, a "No observed adverse effect level" of 5000 ppm was established for the subtilisin concentrate.

Mammalian-Microsome reverse mutation assay with a confirmatory assay, Corning Hazleton No. 16834-0-409R, 1995.

Procedure:
A Mammalian Microsome Reverse Mutation Assay with a confirmatory assay was performed using the subtilisin enzyme preparation to evaluate its ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains (TA98, TA100, TA1535, TA1537) and at the tryptophan locus of *Escherichia coli* tester strain *WP2uvrA*, both in the presence and absence of an exogenous metabolic activation system of mammalian

microsomal enzymes derived from Aroclor induced rat liver (S9) with five doses of test article along with concurrent vehicle, negative, and positive controls, using three plates per dose. The results of the initial assay were confirmed in an independent experiment. The doses to be tested, as well as the exposure method to be used in the mutagenicity assay were selected based on the results of a dose range-finding study conducted with the test article, and on the test article formulation ingredients alone. Both the test article and the formulation ingredients were checked for cytoxicity and for possible interference with the selective conditions of the test system up to maximum dose of 5000 µg per plate using the standard plate incorporation method of exposure. This experiment was performed with tester strains TA100 and *WP2uvrA*, and ten doses ranging from 6.67 to 5000 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

Evaluation and Results:
In the initial dose range-finding experiment, the test article caused a dose-responsive enhancement of overgrowth of the bacterial background lawn with *S. typhimurium* tester strain TA100 and *E. coli* tester strain *WP2uvrA,* only in the presence of S9 mix. This enhancement indicated that the test article was interfering with the selective conditions of the assay system. No enhancement of the bacterial lawn was observed with either tester strain in the absence of S9 mix. In addition, no enhancement of the bacterial lawn was observed with the formulation ingredients with either tester strain in either the presence or absence of S9 mix.

Due to the interference with the test system observed with test article with tester strains TA100 and *E. coli* tester strain *WP2uvrA* in the presence of S9 mix, a second dose range-finding experiment was conducted in which the 'treat and plate' method of exposure was used rather than the standard 'plate incorporation' method. The treat and plate exposure method allows the test article to be separated from the tester strain following a defined exposure period. This experiment was performed with tester strains TA100 and *WP2uvrA,* and ten doses ranging from 10.3 to 7690 µg per ml both in the presence and absence of S9 mix using three plates per dose. For the treat and plate exposure method, the doses were expressed as µg of test article per ml of treat and plate reaction mixture (0.5 ml of S9 mix or phosphate buffer, 0.1 ml of tester strain, and 0.05 ml of test article dose). The dose range covered was equivalent to 6.67 to 5000 µg per plate using the plate incorporation exposure.

In the dose range finding study with subtilisin and with its formulation ingredients alone, using the treat and plate exposure method, no interference with the selective conditions of the test system were observed and no cytotoxicity was observed up to the maximum dose tested, 7690 µg/ml. For this reason, the treat and plate exposure method was used in both the initial and confirmatory mutagenicity assays. The doses of subtilisin tested in the mutagenicity asays were 154, 512, 1540, 5120, and 7690 µg/ml (equivalent to 5000, 3330, 1000, 333, and 100 µg per plate).

Conclusions:
The results of the *Salmonella-E.coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, in both an initial and confirmatory assay, the test article subtilisin did not cause a positive increase in the number of

revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver (S9).

Chromosome aberration study in Human whole blood lymphocytes with a confirmatory assay with multiple harvests, Corning Hazleton No. 16834-0-449CO, 1995.

Procedure:

The objective of this *in vitro* assay was to evaluate the ability of subtilisin to induce chromosomal aberrations in cultured whole blood human lymphocytes with and without metabolic activation. The maximum concentration of 20.0 µl/ml was used in the activation assay and 4.00 µl/ml for the non-activation assay. Dosing was achieved using a 2% (20 µl/ml) dosing volume for the activation assay and using a 1% (10 µl/ml) dosing volume for the non-activation assay. In the initial trial of the aberrations assay, replicate cultures were incubated with 0.0625, 0.125, 0.250, 0.500, 1.00, 2.00, and 4.00 µl/ml of subtilisin without metabolic activation and with 0.625, 1.25, 2.50, 5.00, 10.0, 15.0, and 20.0 µl/ml with metabolic activation and harvested 22.1 hours after initiation of treatment. The highest dose level tested in the activation assay was achieved using the neat test article. The diluent for preparing the dilutions of the test article for the subsequent dose levels in the assay with metabolic activation and all dose levels in the assay without metabolic activation was phosphate buffered saline (PBS). The solvent control cultures were treated with 20.0 µl/ml of formulation ingredient in the activation assay and 10.0 µl/ml of formulation ingredient for the non-activation assay. Chromosomal aberrations were analyzed from the cultures treated with 0.500, 1.00, 2.00 and 4.00 µl/ml without metabolic activation and with 5.0, 10.0, 15.0, and 20.0 µl/ml with metabolic activation.

In the confirmatory assay, replicate cultures were incubated with 0.250, 0.500, 1.0, 2.00, and 4.00 µl/ml of subtilisin without metabolic activation and with 2.50, 5.00, 10.0, 15.0 and 20.0 µl/ml with metabolic activation in 21.9 and 45.8 hour aberrations assays. The highest dose level tested in the activation assay was achieved using the neat test article and using the same experimental conditions as in the initial trial of the aberrations assay. Chromosomal aberrations were analyzed from the cultures treated with 0.500, 1.00, 2.00 and 4.00 µl/ml without metabolic activation and with 5.0, 10.0, 15.0, and 20.0 µl/ml with metabolic activation.

Evaluation and Results:
No significant increase in cells with chromosomal aberrations or in polyploidy was observed at the concentrations analyze in the activation assay or the confirmatory assay.

Conclusions:
The test article subtilisin was considered negative for inducing chromosomal aberrations in cultured whole blood human lymphocytes both in the presence and absence of an exogenous activation system. These results were confirmed in independently conducted confirmatory trials with two harvest times.

Primary Dermal Irritation Test, IRDC, Report No. 001-94. 1994.

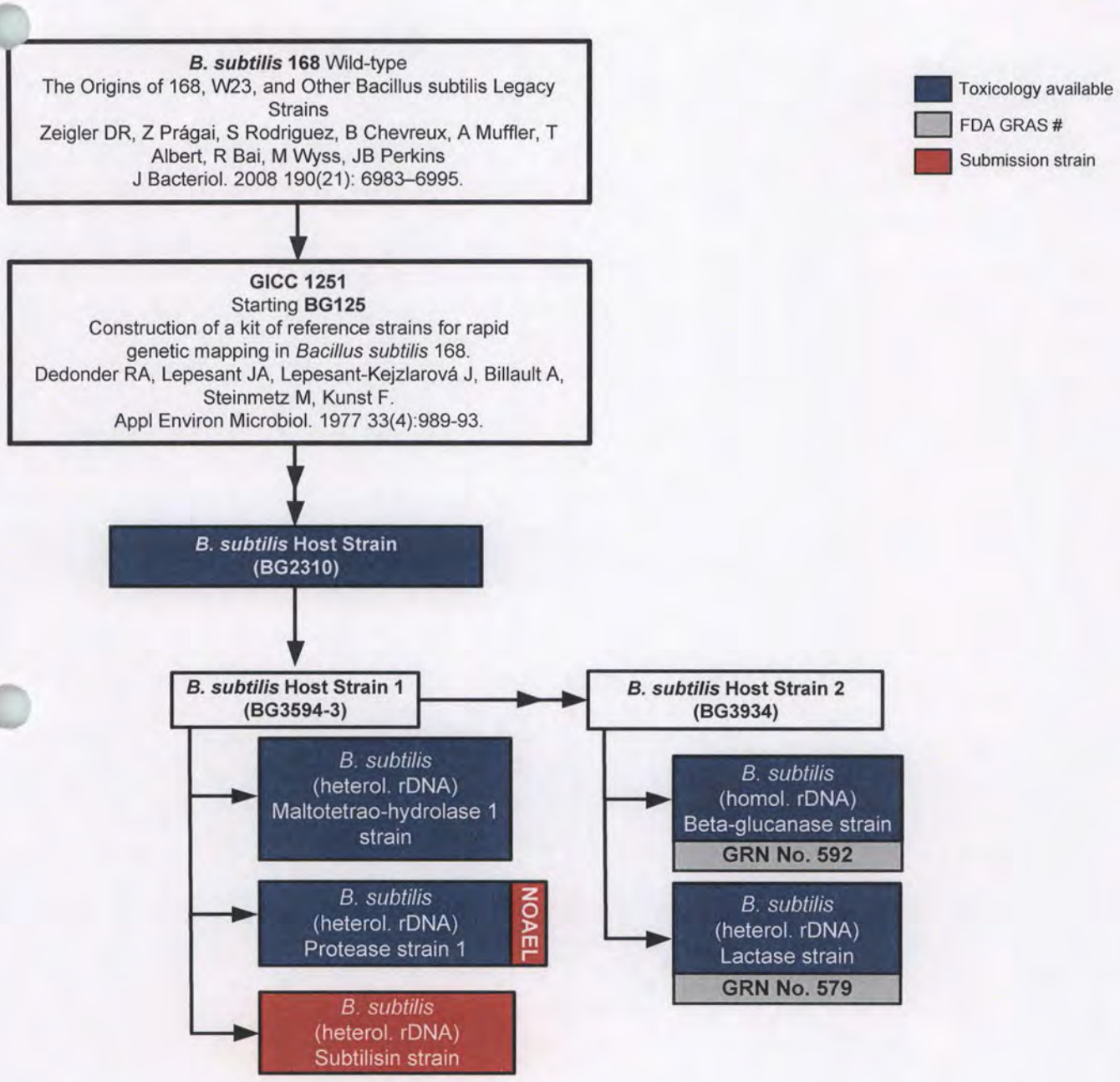
Procedure:
In a dermal irritation test, rabbits were dosed dermally with the test article, subtilisin (Lot No. 88027), undiluted as received. The test article was applied to one intact and one abraded site on the back of each rabbit under one-inch square gauze patches secured with Dermiform tape. The test sites were then wrapped with gauze bandaging, Saran wrapped and secured with Dermiform tape. A collar was also attached to each animal. The test article remained in contact with the test site skin for 4 hours. Following exposure period, the bandaging materials and collar were removed and the test sites were washed and dried. The test sites were evaluated for dermal irritation, in accordance with the Draize method, approximately 30 minutes after bandage removal, at 24, 48, and 72 hours after dosing and at day 7.

Evaluation and Results:
Very slight erythema was initially exhibited by very few of the animals 30 minutes after bandage removal. As time progressed, there was a slight increase in animals showing signs of irritation at 24 hours, and at 48 hours. Erythema persisted in a couple of the animals at 72 hours. Slight degrees of edema were additionally observed in a couple of the rabbits at one or both tests sites at 0.5, 24, and 48 hours, persisting in one to 72 hours. All rabbits cleared of dermal irritation by day 7. There were no remarkable differences in irritation between the intact and abraded skin sites.

Conclusion:
Based on the dermal irritation properties, the classification of the test article would be mildly irritating.

Appendix 9: *Bacillus subtilis* Strain Lineage and Summary of Safety Studies

Bacillus subtilis Safe Strain Lineage



Most enzymes derived from this Safe Strain Lineage were determined to be GRAS for their intended use, with GRAS Notices reviewed by the US FDA for enzymes from strains designated with gray horizontal banners indicating the GRAS Notice number.

The subject strain of this submission is the Subtilisin producing strain highlighted in red.

The safety of the Subtilisin enzyme is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The blue-colored boxes indicate strains for which we conducted toxicology tests.

According to the Safe Strain Lineage concept, the NOAEL for the subtilisin from the closely related production strain is used to support the safety of the subject protease enzyme in the intended use, as indicated with red flag labeled "NOAEL".

Toxicology Test Summaries

The safety of 6 Genencor *Bacillus subtilis* strains and 5 enzyme preparations derived from recombinant production strains were assessed in a number of toxicology tests as shown in the table below. All strains tested were found to be non-cytotoxic/pathogenic and all enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
B. subtilis **(homol. rDNA) Protease strain**	**Protease**	Cytotoxicity study, Chinese hamster ovary	Non-cytotoxic
		90-day subchronic study in rats	No adverse effects detected
B. subtilis **Host Strain 1 (BG3594-3)**	**Host Strain**	Cytotoxicity Study, Chinese hamster Ovary	Non-cytotoxic
B. subtilis **(heterol. rDNA) Maltotetrao-hydrolase Strain**	**Maltotetraohydrolase**	Acute Oral toxicity in rats	No signs of toxicity at 2000 mg total protein/kg bw
		91-day subchronic study in rats	No adverse effects detected at higher dose
		Ames test	Non-mutagenic
		In vitro chromosome assay (Human lymphocytes)	Non-clastogenic
B. subtilis **(heterol. rDNA) Subtilisin Strain**	**Protease**	90-day subchronic oral study in rats	NOAEL established at highest dose
B. subtilis **(homol. rDNA) Beta-glucanase Strain**	**Beta-glucanase**	Dermal irritation	Non-irritant
		Eye irritation	Non-irritant
		Ames assay	Non-mutagenic
		Chromosomal aberration	Non- clastogenic

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
B. subtilis **(heterol. rDNA) Lactase Strain**	**Lactase**	Dermal Irritation study	Non-irritant
		Eye Irritation study	Non-irritant
		Acute oral toxicity in rats	No signs of toxicity at 5000 mg total protein/kg bw
		Ames test	Non-mutagenic
		In vitro chromosome assay, Human lymphocytes	Non-clastogenic
		90-day subchronic oral study in rats	No adverse effects detected, NOAEL established at highest dose, 1000 mg total protein/ kg bw/ day or 1416. 4 mg TOS (total organic solid)/ kg bw/ day

Appendix 10: Supporting Allergenicity Risk Assessment Data.

ALLERGEN NOMENCLATURE
WHO/IUIS Allergen Nomenclature Sub-Committee

Member Login

Username or Email: Password:

Login

Plantae Magnoliopsida Cucurbitales *Cucumis melo* Cuc m 1

Allergen Details:

Allergen name:	Cuc m 1
Lineage:	Source: Plantae Magnoliopsida Order: Cucurbitales Species: Cucumis melo (Muskmelon)
Biochemical name:	Alkaline serine protease (cucumisin)
MW(SDS-PAGE):	67
Allergenicity:	N.A
Allergenicity ref.:	12801320
Food allergen:	Yes
Date Created:	2003-06-19 12:00:00
Last Updated:	2010-04-29 16:57:55
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Cuc m 1.0101	D32206	BAA06905	Q39547	

DuPont Industrial Biosciences 70

000070

Sequence comparison between *B.amyloliquefaciens* subtilisin vs. *B. lentus* subtilisin using NCBI BLAST (http://blast.ncbi.nlm.nih.gov)

BLAST® » blastp suite-2sequences » RID-CJYSKD3T113

BLAST Results

Blast 2 sequences

Job title: Protein Sequence (275 letters)

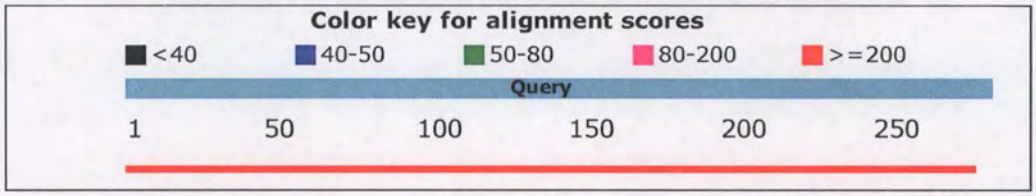
RID CJYSKD3T113 (Expires on 03-17 01:34 am)

Query ID	lcl|Query_181431	**Subject ID**	lcl|Query_181433
Description	None	**Description**	None
Molecule type	amino acid		See details
Query Length	275	**Molecule type**	amino acid
		Subject Length	269
		Program	BLASTP 2.6.1+

New Analyze your query with SmartBLAST

Graphic Summary

Distribution of the top 1 Blast Hits on 1 subject sequences



Dot Matrix View

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
unnamed protein product	316	316	100%	7e-114	60%	Query_181433

Alignments

unnamed protein product

Sequence ID: Query_181433 Length: 269 Number of Matches: 1
Range 1: 1 to 269

Score	Expect	Method	Identities	Positives	Gaps	Frame
316 bits(810)	7e-114()	Compositional matrix adjust.	165/275(60%)	211/275(76%)	6/275(2%)	

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GENENCOR®
A Danisco Division

***Bacillus lentus* subtilisin ALLERGENICITY RISK ASSESSMENT**
Part 2: Pepsin resistance Test

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By: Yoko Kinoshita
Date: April 18, 2012

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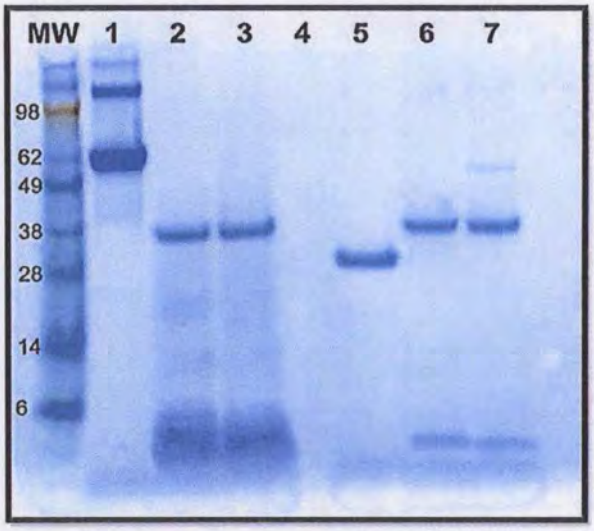
Vincent J Sewalt, PhD
Sr. Director, Regulatory Affairs

The current assessment strategy, as outlined by the Ad Hoc International Task Force on Foods Derived from Biotechnology (Codex Alimentarius Commission, 2009), focuses on a weight-of-evidence approach that recognizes that no single endpoint can be used to predict human allergenic potential. In this context, the following factors are considered: the source of the gene, the similarity of the amino acid sequence of the protein of interest to that of known allergens, the stability of the protein in an in vitro pepsin digestibility assay, and, when necessary, in vitro human sera testing or in vivo clinical testing.

As discussed in the *B. lentus* subtilisin (FN3) allergenicity risk assessment dated February 8, 2012, FASTA alignment of the *B. lentus* subtilisin sequence resulted in 29.6% identity match with a food allergen (cuc m 1, muskmelon). Although the biological relevance of using pepsin-resistance as part of a safety assessment of novel proteins has been challenged (e.g., Schnell and Herman, 2009), the lack of reproducible and consistent correlation between pepsin resistance and allergenicity may be due to the absence of a standardized digestibility protocol (Thomas et al., 2004). Hence, in order to further investigate the potential allergenicity of the *B. lentus* subtilisin protein, a pepsin resistance test was conducted adapted from a validated pepsin assay (Thomas et al., 2004) and performed by the analytical group in R&D, Palo Alto. See Appendix I for the test protocol used.

The SDS-PAGE gel below shows that the *B. lentus* subtilisin is degraded by pepsin within the first 10 minutes at pH 1.2 (lane 6). There are several remaining peptides < 6 kDa on lanes 6 and 7; the exact sizes of these bands are difficult to determine due to the lowest MW standard marker being 6 kDa. In order to better estimate the sizes of these peptides, a second gel was run with two additional MW standards and a shorter electrophoresis run time. T = 1 minute samples were also prepared to measure the degradation rate of the proteins.

Figure 1: 4-12% Bis-Tris gel, run time = 30 min.

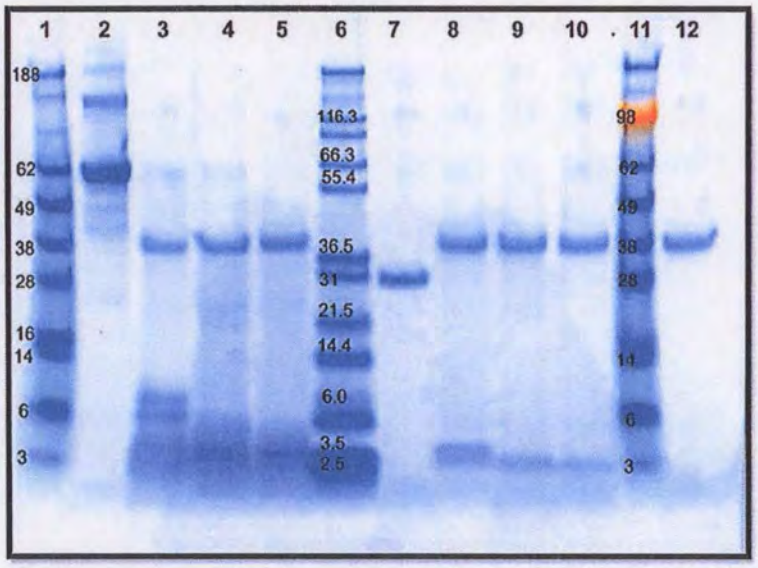


Lane	Description
MW	SeeBlue Plus2 std
1	BSA + Gcon*, t = 0 min
2	BSA + SGF**, t = 10 min
3	BSA + SGF, t = 60 min
4	Empty
5	FN3 + Gcon, t = 0 min
6	FN3 + SGF, t = 10 min
7	FN3 + SGF, t = 60 min

*Gcon = gastric ctrl solution

** SGF = Gcon + pepsin

Figure 2: 4-12% Bis-Tris gel, run time = 25 min.



Lane	Description
1	SeeBlue Plus std
2	BSA + Gcon*, t = 0 min
3	BSA + SGF**, t = 1 min
4	BSA + SGF**, t = 10 min
5	BSA + SGF, t = 60 min
6	Mark12 unstained std
7	FN3 + Gcon, t = 0 min
8	FN3 + SGF, t = 1 min
9	FN3 + SGF, t = 10 min
10	FN3 + SGF, t = 60 min
11	SeeBlue Plus2 std
12	SGF solution (with pepsin)

As clearly shown in figure 2, the *B. lentus* subtilisin protein is readily degraded by t = 1 min. With the three lanes of MW standards as reference, the smaller peptides on lanes 8, 9, and 10 are estimated to be < 3.5 kDa for lane 8 (t = 1 min) and < 3 kDa for lanes 9 and 10 (t = 10 min or 60 min respectively). The pepsin susceptible BSA control results on lanes 3, 4, and 5 validate the test and the conditions used.

In summary, *B. lentus* subtilisin is not resistant to pepsin degradation at pH 1.2 and no peptides > 3 kDa that could serve as IgE biding epitopes remain after 10 minutes. Using a weight-of-evidence approach, *B. lentus* subtilisin is unlikely to pose a risk of food allergenicity.

References

Codex Alimentarius Commission. 2009. Foods Derived from Modern Biotechnology, Annex 1, Assessment of Possible Allergenicity, Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Rome, Italy, http://www.fao.org/docrep/011/a1554e/a1554e00.htm, pp. 52-55.

Huby RDJ, Dearman RJ, Kimber I. 2000. Why are some proteins allergens? *Toxicological Sciences* **55:** 235-246.

Schnell S, and Herman RA. 2009. Should digestion assays be used to estimate persistence of potential allergens in tests for safety of novel food proteins? *Clinical and Molecular Allergy* 7:1.

Thomas K, M Aalbers, GA Bannon, M Bartels, RJ Dearman, DJ Esdaile, TJ Fu, CM Glatt, N Hadfield, C Hatzos, SL Hefle, JR Heylings, RE Goodman, B Henry, C Herouet, M Holsapple, GS Ladics, TD Landry, SC MacIntosh, EA Rice, LS Privalle, HY Steiner, R Teshima, R van Ree, M Woolhiser, and J Zawodnyk. 2004. A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology* **39:** 87-98.

Appendix I: Pepsin resistant test protocol

Stimulated Gastric Fluid (SGF) Digestion Worksheet (Simplified)

STUDY:

Reagents: in blue = may need to purchase

Reagent	Manufacturer	Item #	Lot #	Expiration	Storage
NaCl					RT
6N HCl					RT
Gastric control (G-con) = NaCl + HCl, store at RT					
Pepsin (Purity 95%)	Sigma-Aldrich	P6887	091M7020V	11/17/16	≤ - 10°C
Stimulated Gastric Fluid (Gcon + pepsin), store at RT					
Sodium Carbonate (Na_2CO_3)					RT
NaOH (if applicable to adjust pH)					RT
HCl (if applicable to adjust pH)					
FN3 – use QC std from Process Analytical lab, Lot # 101-02057-001, 13.5 mg/mL (12.6 mg/g)					
BSA (Control Substance)	Sigma-Aldrich	A0281	020M74001V	10/31/12	2-8°C
LDS Sample Buffer (4X)					2-8°C
Sample Reducing Agent (10X)					2-8°C

Solution Preparation:

NaCl solution: 100 mg /ml

Stop Solution: 0.2 M Na_2CO_3 (*i.e.* 2.12 g of Na_2CO_3 in 100 ml water)

BSA Control Protein Solution: 5 mg/ml in DI water

Test Protein Solution: The target concentration is ~5 mg/ml in DI water

Gastric Control (G-Con) Solution (pH 1.2), 10 ml:

200 µl of 100 mg/ml NaCl
140 µl of 6N HCl
9 ml of DI water

- Adjust to pH 1.2

Stimulated Gastric Fluid (SGF) Solution (G-con containing pepsin*, *e.g.*, 2500 units/ml):
* Pepsin activity in the powder is _____units/mg. In the final digestion mixture, there should be approximately 10 pepsin activity units per µg test or control protein.

Revised by Marina Chow 3/23/2012
Reference Document Name: SGF Worksheet Page 1 of 2 Initial/ Date
Document #: 196198
Pioneer Hi-Bred International, Inc., Regulatory Science

Stimulated Gastric Fluid (SGF) Digestion Worksheet (Simplified)

STUDY:

Protein Digestion:

Prepare STOP MIXTURE tubes (0, 10, 60 mins)

- For test protein digestion and time "0" samples: Add **48µl** Stop solution, **65 µl** 4X LDS sample buffer and **26 µl** 10X reducing agent to pre-labeled tubes. Cover tubes to minimize evaporation.

Digestion reaction (pepsin + FN3, and pepsin + BSA, no pepsin+FN3)

- Add 1)**1900** µl of SGF Solution and 2) water (for FN3 + no pepsin) to vials containing a stir bar, place in a 37°C water bath with a submersible magnetic stirrer and incubate for ~1- 2 minutes.
- Start reaction by addition of **100** µl Test Protein. Start timer to count up.
- At each of the following pre-set time points (10 and 60 mins), take out **120 µl** reaction mixture, add to the pre-labeled tubes containing STOP MIXTURE (48 µl stop solution, 65 µl 4XLDS sample buffer and 26 µl 10X sample reducing agent) and then mix by vortex.
- Heat samples (*e.g.*, at 70°C for ~10 min) and then store on ice prior to electrophoresis.

Time "0" samples: Prepare with DI water:

- Mix 100 uL Test Protein (5 mg/mL FN3 and BSA) + 1900 uL water.
- Add 120 uL of diluted Test Protein to tubes containing STOP MIXTURE.
- Heat samples (*e.g.*, at 70°C for ~10 min) and then store on ice prior to electrophoresis.

Electrophoresis

Suggested electrophoresis procedure:

4 – 12% Bis Tris, MES buffer, ~ 30 mins run time.
Sample Load: 20 µl /well
NOTE: bands down to 3kDa are of our special interest. Use appropriate MW std. and run time.

B. lentus subtilisin: Pepsin resistant test (page 5)

Appendix 11: GRAS Opinion Letter

7102 Valhalla Trail
Madison, WI 53719
October 1, 1994

Alice J. Caddow
Vice President of Regulatory
& Environmental Affairs
GENENCOR International
180 Kimball Way
South San Francisco, CA 94080

Dear Ms. Caddow:

We reviewed the material you provided on Genencor International's Multifect P3000. In this evaluation we considered the biology of the production organism and the toxicity of the enzyme product which was fed to rats for 90 days at levels ranging from 5,000 ppm to 50,000 ppm.

Based on this information, we conclude that the organism is safe to use for the manufacture of food- and feed-grade protease. We conclude further that the protease enzyme manufactured from this organism by the process you described is safe to use at the proposed concentrations as a direct food or feed ingredient, and to prepare protein hydrolysates for addition to foods or feeds.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of your product by you or others.

Sincerely,

(b) (6)

Michael W. Pariza, Ph.D.
Wisconsin Distinguished Professor

(b) (6)

Joseph F. Borzelleca, Ph.D.
Professor, Pharmacology & Toxicology
Medical College of Virginia