A Subtilisin Enzyme

Preparation Produced By

Bacillus subtilis

Expressing a Subtilisin Gene

From

Bacillus clausii

Is Generally Recognized As Safe

For Use in Food Processing

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

August 12, 2020

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §170. 225, Danisco US Inc. submits this GRAS Notice for subtilisin produced by submerged fermentation of *Bacillus subtilis* carrying the gene encoding a variant of the subtilisin enzyme from *Bacillus clausii*.

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 will 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.

Our safety evaluation is consistent with the recent publication by the Enzyme Technical Association (Sewalt *et. al.*, 2016),[1] which includes an evaluation of the production strain, the enzyme, and the manufacturing process, as well as a determination of dietary exposure. This generally recognized methodology, based on the decision tree by Pariza and Johnson (2001) and inclusive of published safety information, provides the common knowledge element of the GRAS status of this subtilisin enzyme notified to the FDA (Sewalt *et al.,* 2017).[2]

The safety of the production organism is considered to be 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 (*B. subtilis*) is discussed in Part 2 and 6 of this submission. The other essential aspect of the safety evaluation of enzymes derived from

[1] https://doi.org/10.1089/ind.2016.0011
[2] http://www.enzymeassociation.org/?p=595

genetically engineered microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; Pariza & Foster, 1983; IFBC, 1990; SCF, 1991; OECD, 1993; Berkowitz & Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and described in Part 2. The safety evaluation described in Part 3 and 6 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code or express a harmful toxic substance.

1.1 § 170.225 (c)(2) Name and Address of Notifier

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

1.2 § 170.225 (c)(3) Common or Usual Name of Substance

The subtilisin enzyme preparation is produced by a *Bacillus subtilis* strain expressing the gene encoding a variant of the subtilisin enzyme from *Bacillus clausii.*

1.3 § 170.225 (c)(4) Applicable Conditions of Use

The subtilisin is used as a processing aid in the processing of proteins at 569 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.,*
- Milk and seafood (derived) raw materials, such as milk and milk derived products (whey proteins, caseins), fish/seafood, collagen, gelatin, *etc*., and
- Microbial sources, such as yeast and microalgae.

1.4 §170.225 (c)(5) 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 the native subtilisin from the host was 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).

1.5 §170.225 (c)(6) Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in 21 C.F.R. §170.225, Danisco US Inc. has determined that its subtilisin enzyme preparation from a genetically engineered strain of *B. subtilis* expressing the subtilisin gene from *B. clausii* is a Generally Recognized As Safe ("GRAS") substance for the intended food applications and is, therefore, exempt from the requirement for premarket approval.

1.6 §170.225 (c)(7) 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 can be sent to the Food and Drug Administration upon request.

1.7 §170.225 (c)(8) and (c)(9) 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 Part 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 a complete, representative, and 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.

August 12, 2020

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

2.1 PRODUCTION ORGANISM

2.1.1 Production Strain

The production organism is a non-sporulating strain of *Bacillus subtilis*, which has been genetically engineered to introduce multiple copies of the *B. clausii* subtilisin gene, into the chromosome of the *B. subtilis* host. An intermediate strain in this construction, *B. subtilis* BG3594-3, was recognized by the Dutch authorities as Risk Class 1. Strain BG3594 also served as intermediate strain for the production strain of another subtilisin protease previously notified as GRAS to the FDA (GRN00714) as also reviewed in Ladics and Sewalt (2018).

The production strain expresses a subtilisin gene derived from *B. clausii*. The expression cassette only consisting of the subtilisin gene and a chloramphenicol resistance marker gene from plasmid pC194 (originally isolated from *S. aureus* but widely recognized to be naturally present in *Bacillus*), which has been reported in *B. subtilis,*[1] was finally integrated into the chromosome of the host strain. Whole Genomic Sequencing confirmed the genetic stability and only the intended genetic modifications to the *B. subtilis* strain had been made.

2.1.2 Recipient Organism

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). This organism was further optimized into expression host strain BG3594-3, which also served as recipient strain for expression of another subtilisin enzyme that was previously GRAS notified (see GRN00714).

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). Experts from the US FDA reviewed the safe use of food-processing enzymes from well-characterized recombinant

[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).

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).

2.1.3 Subtilisin Expression Plasmid

The genetic modification of the *B. subtilis* host involved recombinant DNA techniques to introduce multiple copies of the gene encoding the *B. clausii* subtilisin into the chromosome of *B. subtilis* host strain BG3594-3.

The modification employed a method by which only an expression cassette, consisting of the *B. clausii* subtilisin gene and the chloramphenicol resistance marker gene from plasmid pC194 (originally isolated from *S. aureus* but widely recognized to be naturally present in *Bacillus*), is introduced into the host genome, at the site of the endogenous alkaline protease *aprE* gene, without any vector sequences remaining in the final production strain.

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 genomic sequencing.

2.1.4 Stability of the Introduced Genetic Sequences

The introduced subtilisin gene in the production strain proved to be 100% stable after industrial scale fermentation judged as chloramphenicol resistance subtilisin production.

2.1.5 Antibiotic Resistance Gene

The chloramphenicol gene, which has been reported in the genome of *B. subtilis* strains[1], was 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.

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

The production organism does not end up in the finish food and therefore, the first step in the safety assessment as described by the International Food Biotechnology Council (IFBC)[1] is satisfactorily addressed.

2.2 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

2.2.1 Enzyme Identity

Classification:	Subtilisin
IUB Nomenclature:	Subtilisin
IUB Number:	3.4.21.62
CAS Number:	9014-01-1
Other Names:	Alcalase; Bacillopeptidase; Alkaline proteinase; Protease; Thermoase; Subtilopeptidase
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.

Further information on subtilisin can be found in Appendix 1 and at IUBMB website.[2]

2.2.2 Amino Acid Sequence

The amino acid sequence of the protein engineered *B. clausii* subtilisin is known and included in Appendix 2. It is 3 amino acids different from the native *B. clausii* amino acid sequence and is therefore 99% identical to the native *B. clausii* subtilisin, 62% identical to alkaline protease produced by *B. licheniformis* which was GRAS affirmed (21 C.F.R. § 184.1027), and 60% identical to alkaline protease produced by *B. subtilis* and *B. amyloliquefaciens* which was GRAS affirmed (21 C.F.R. § 184.1150). and is also 60% identical to *B. amyloliquefaciens* subtilisin produced by *B. subtilis* (GRN 714).

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; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 3. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is

[1] https://ac.els-cdn.com/S0273230005800807/1-s2.0-S0273230005800807-main.pdf?_tid=c89f62ce-5402-4e18-a3be-68ddbf116b10&acdnat=1530898844_165c4c45e811723d34f8db3e1878c745
[2] http://www.chem.qmul.ac.uk/iubmb/enzyme/

also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. §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, 12th edition, 2020 ("FCC"), 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 antifoams (also known as defoamers) and flocculants used in the fermentation and recovery are used in accordance with cGMP per the September 11, 2003 FDA correspondence to ETA acknowledging the listed antifoams and flocculants. Therefore, the maximum use level of the antifoams in the production process is <1.0%, and cationic polymer flocculants < 1.

In regard to potential major food allergens, soy meal will be used in the fermentation process and is hydrolyzed and consumed by the microorganism as nutrients. Therefore, the final enzyme preparation does not contain any major food allergens from the fermentation medium. No other major 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 *B. subtilis* described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

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 55% glycerol and 10% sodium acetate at pH 5. The remaining portion of the formulation is water.

The final subtilisin liquid concentrate 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 FCC, 12th edition (USP, 2020). These specifications are set forth in Section 2.4.

2.4 COMPOSITION AND SPECIFICATIONS

2.4.1 Quantitative Composition

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

- Enzyme protein ~ 5%
- Glycerol ~55%
- Sodium acetate[1] ~10%
- Remaining is water
- pH 5

[1] As stated in 21CFR §184.1721, sodium acetate has maximum allowable limits in certain foods; "the ingredient is used in food at levels not to exceed cGMP which results in a maximum level, as served, of 0.007 percent for breakfast cereals as defined in 170.3(n)(4) of this chapter; 0.5 percent for fats and oils as defined in 170.3(n)(12) of this chapter; 0.6 percent for grain products and pastas as defined in 170.3(n)(23) of this chapter and snack foods as defined in 170.3(n)(37) of this chapter; 0.15 percent for hard candy as defined in 170.3(n)(25) of this chapter; 0.12 percent for jams and jellies as defined in 170.3(n)(28) of this chapter and meat products as defined in 170.3(n)(29) of this chapter; 0.2 percent for soft candy as defined in 170.3(n)(38) of this chapter; 0.05 percent for soups and soup mixes as defined in 170.3(n)(40) of this chapter and sweet sauces as defined in 170.3(n)(43) of this chapter." Although use of FN3 alkaline protease at less than 1% (according to cGMP) will result in sodium acetate concentrations in the resulting protein hydrolysate that are expected to be less than 0.01%, it is the food processor's responsibility to ensure that the maximum allowable limits for specific foods are not exceeded.

2.4.2 Specifications

As mentioned, subtilisin preparation meets the purity specifications for enzyme preparations set forth in FCC, 12th edition (USP, 2020). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA (2006).

The results of analytical testing of the 3 lots of product is given in Appendix 4 verifying that it meets USP (2020) 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 5.69% Total Organic Solids (TOS).

	Maximum Use Rate (kg Product/MT Substance)	**Resulting Exposure** (mg TOS/kg Substance)
Protein processing	10	569

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 expected to be broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which is not expected to pose any human health risk.

3. DIETARY EXPOSURE

The subtilisin will be used as a processing aid in protein processing. While we expect the subtilisin to be not present in the final food or present as inactive residue in negligible amounts, the following conservative calculations assume that 100% of the enzyme remains in the processed food, as total organic solids (TOS).

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. This method was previously used by JECFA (FAO/WHO, 2001) and contains the following assumptions:

1) Level of consumption of foods and beverages:

For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day. For non-milk beverages, a daily consumption of 100 ml/kg bw is used corresponding to 6 liters per day for a 60-kg adult.

2) Concentration of enzymes in foods and beverages:

The concentration of enzyme in foods and beverages is the maximum application rate.

3) Proportion of foods and beverages that contain the enzymes:

   a) A default of 50% of all solid foods is used to represent processed foods (*i.e.,* 12.5 g/kg bw/day).

   b) A default of 25% is used to represent non-milk beverages that may contain the enzyme (*i.e.*, 25 ml/kg bw/day).

4) Estimation of the theoretical maximum daily intake (TMDI)

To represent a worst-case scenario, TMDI for solid foods will be combined with the TMDI for beverages in the risk assessment.

The Budget Method is based on the following assumed consumption of targeted important foodstuffs and beverages (for less important foodstuffs, *e.g.,* snacks, lower consumption levels are assumed). The regular assumption is for processed food (50% of total solid food) and for soft

drinks (25% of total beverages). For the consumption of the high protein products such as sport drink and protein bar, the following assumptions are used.

Average consumption over the course of a lifetime/kg body weight/day	**Total solid food (kg)**	**Total non-milk beverages (l)**	**Processed food (25% of total solid food) (kg)**	**Soft drinks (10% of total beverages) (l)**
	0.025	0.1	**0.006**	**0.01**

The recommended use levels of the enzyme subtilisin are given, based on the raw materials used in the food process. The calculation considers how much solid or liquid food is obtained per kg raw material, and it is assumed that all the TOS will end up in the final product. Therefore, the concentration of TOS from subtilisin in the applications can be calculated/summarized as in the table below:

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	569	Sport drinks	0.30	**170.7**
Solid food	Protein processing	Proteins from various sources	569	Protein hydrolysates used in e.g. soups, bouillons, dressings.	0.17	**96.7**
				protein bar	0.30	**170.7**

The Total Theoretical Maximum Daily Intake (TMDI) can be calculated on basis of the **maximum** 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. 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)**
170.7x0.006=1.02	170.7x0.01=1.71	**2.73**

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 this specific enzyme subtilisin produced by *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).

4. SELF-LIMITING LEVELS OF USE

As the enzyme will be used as processing aid in the food manufacturing process, there is no notable oral intake for humans. Therefore, self-limiting levels of use are not applicable.

In addition, as a processing aid the self-limited levels of use are primarily economical as customers 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

Information regarding this enzyme's common use in food before 1958 is not provided as the statutory basis of our GRAS conclusion, which is based on scientific procedures rather than common use before 1958.

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. US FDA affirmed as GRAS the native subtilisin and neutral protease produced by *B. subtilis* (21 C.F.R. § 184.1150).

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 (Ehrenberg, 1835). It was renamed "*Bacillus subtilis"* in 1872 by Ferdinand Cohn (Cohn, 1872). 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 21 C.F.R. §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 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 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

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

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 C.F.R. §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 5 for the subtilisin toxicological study summary and Appendix 6 for the safe strain lineage information).

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.

Scientists from 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 Nutrition & 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. 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 Nutrition & Biosciences has safely used strain BG125 and its derivatives for research and production purposes for over 15 years in many fermentations in volumes up to 300m^3.

Danisco US. Inc. 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) decision tree analysis, including repeated toxicological testing (see Appendix

[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

9). Three previous GRAS Notices were filed for the products from this strain lineage, in which FDA issued "no questions" letters (please refer to GRN 714, GRN592 and GRN579, see also Ladics and Sewalt, 2018).

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 strain is *Bacillus* sp. strain O-4 (Horikoshi & Ikeda, 1977), also known as ATCC21536. The strain was at that time not further identified to species level, due to the complex taxonomical situation, as exemplified *e.g*. in 1977 by Gordon *et al.* (1977) and by Priest *et al.* (1988). The strain was identified as *B. lentus* in 1990 by Dr. R.C.W. Berkeley of the University of Bristol. Later, Nielsen *et al.* (1995) proposed to change the taxonomy of several strains previously identified as *B. lentus* and other species to new species. Among these was ATCC21536, which was assigned to the new species *B. clausii*. The donor strain, *Bacillus clausii* ATCC21536, was only used as the donor of the subtilisin gene, which obtained from the American Type Culture Collection.

6.2 SAFETY OF THE MANUFACTURING PROCESS

In its GRAS affirmation of *B. subtilis* protease (21 C.F.R. §184.1150), FDA stipulated the manufacture process to utilize bacterial strains 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. clausii* 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 in the Food Chemicals Codex, 12th Edition (US Pharmacopeia, 2020) and the general specifications for enzyme preparations used in food processing proposed by JECFA (2006).

The fermentation process may utilize a soy meal that may contain trace amount of protein. This feedstock is 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 (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, 1998) 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 the 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. 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. Based on the sequence homology alone, it was concluded that the subtilisin is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of

[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.

aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity *(i.e.*, in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The *Bacillus clausii* subtilisin (mature) sequence is given in Appendix 2. The full allergenicity analysis is given in Appendix 7.

A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database,[1] February 10, 2020 V20, containing 2129 peer-reviewed allergen sequences listed in the database[2] (using E-value <0.1) 26 protease matches, with a peptide sequence of more than 35% identity to the mature *Bacillus clausii* (alkaline) protease sequence. However, none of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database.[3]

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 (February 10, 2020 V20), identified 28 matches from various *Bacilli* and *Fungi*, all fungal serine proteases. None of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database.

Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org, there is no evidence that a short identical contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (>35%). The AllergenOnline database allows isolated identity matches of eight contiguous identical amino acids to satisfy demands by

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

some regulatory authorities for this precautionary search. Performing the eight-contiguous identical amino acid search produced 28 sequence matches with known allergens, all fungal serine proteases. None of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database.

As noted above, none of the identified fungal serine proteases were identified as food allergens in the WHO/IUIS Allergen Nomenclature Database. Rather, the identified fungal serine proteases are known respiratory or contact allergens. Furthermore, microbial derived enzymes acting as environmental allergens have not been demonstrated to be active via the oral route. This concept was evaluated by Bindslev-Jensen *et al.* (2006) in which 19 microbial derived enzymes, including proteases, used in the food industry were tested for allergenicity in a double-blind placebo-controlled food challenge study in subjects with positive skin prick tests to inhalation allergens, food allergens, and bee and wasp allergens. None of the 19 microbial derived food enzymes were found to be food allergens and the authors concluded that ingestion of microbial derived food enzymes in general are not considered to be a concern regarding food allergy. In addition, a protease was found to be rapidly digested in an in vitro pepsin digestibility assay. Moreover, enzymes are processing aids and per definition, they are not present or active in the final food. Consequently, there is no exposure of the enzyme to the consumer. Taken together, these data indicate that the *Bacillus clausii* protease is unlikely to pose a risk of food allergenicity.

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

As for all enzyme products, a Safety Data Sheet (SDS) for the protease product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

6.3.2 Safety of Use in Food

As noted in the Safety section 6.1, *B. subtilis*, and enzyme preparations derived there from, including alpha-amylase, cellulase, beta-glucanase, and protease enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as Danisco US Inc.'s own unpublished safety studies, support such a conclusion.

B. subtilis is widely used by enzyme manufacturers around the world to produce enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

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[1] genetically modified[2,3]? 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[4,5] have a history of safe use in food[6]? 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. subtilis* 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 *B. subtilis* (GRN 714), and the agency issued "No Question" letters in response. The safety of the subject subtilisin is further supported by the allergenicity analysis and lack of sequence similarity with known oral toxins. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[7]? Yes. No transferable antibiotic resistance gene DNA is present in the enzyme preparation. Go to 3e.

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[1] 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.
[2] 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.
[3] 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.
[4] 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.
[5] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.
[6] 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.
[7] 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 (https://www.gpo.gov/fdsys/pkg/FR-1998-09-08/pdf/98-24072.pdf)

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, as it is stably integrated by homologous recombination into the chromosome at the *aprE* locus. Go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[1]? Yes. The *Bacillus subtilis* production strain pertains to the *Bacillus subtilis* safe strain lineage (Appendix 5). *Bacillus subtilis* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing.

Conclusion: The test article is ACCEPTED, once it has been verified that the NOAEL derived from existing toxicological studies is sufficiently high to provide adequate margin of exposure.

6.3.3 Safety Studies

B. clausii subtilisin is an enzyme preparation produced by *B. subtilis* that can be used as a processing aid in protein processing to facilitate protein hydrolysis.

Danisco US Inc. has determined by scientific procedures that this production organism *B. subtilis* pertains to a safe strain lineage. A review of all toxicology studies conducted with enzyme preparations produced by different strains of Danisco US Inc.'s *B. subtilis* (Appendix 5) indicates that, regardless of the production organism 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 in any of the 90-day oral toxicity studies performed on enzymes produced with members of this *B. subtilis* lineage.

[1] In determining safe strain lineage, one should consider the host organism, all of 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

Therefore, due to the consistency of the findings supporting the safety of enzyme preparations derived from different *B. subtilis* strains, it is reasonable to expect that most enzyme preparation produced from *B. subtilis* strains would have a similar toxicological profile (Appendix 6).

In addition to the decision tree analysis and the availability of multiple toxicology studies for the safe strain lineage, A 90-day oral toxicity study of the subject subtilisin was conducted as part of our safety program to satisfy international and external requirements globally. A study summary was previously included in GRN00714 to support the safety of a related subtilisin also produced by *B. subtilis*. In GRN00714, this subtilisin is referred to as *B. lentus* subtilisin produced with *B. subtilis* instead of *B. clausii* subtilisin produced with *B. subtilis*.

The results are evaluated, interpreted, and assessed in this document. 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 90-days Oral Toxicity (Gavage) Study in Rats. Citox Lab Scantox, Report No. 73796, November 23, 2011

a. Procedure

The objective of this study was to investigate the potential of *B. clausii* 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.

b. 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. clausii* 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. clausii* 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).

c. 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 rats, a NOAEL was established at 480.6 mg Total Organic Solids (TOS) /kg bw/day. The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to *B. clausii* 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

The margin of safety is 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. If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{No Observed Adverse Effect Level (NOAEL)}}{\text{Maximum Daily Exposure}}$$

$$\text{Margin of Safety} = \frac{480.6 \text{ mg TOS/kg bw/day}}{2.73 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 175

6.5 BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *B. subtilis*, and enzyme preparations derived there from, 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 Danisco US Inc.'s (operating as DuPont Nutrition & Biosciences) own unpublished safety studies, support such a conclusion.

B. 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 *B. subtilis* lineage used by Danisco US Inc. has been demonstrated to be safe safe based on repeated testing and evaluation using the Pariza and Johnson (2001) decision tree (Ladics and Sewalt, 2018).

The exposure of *B. clausii* subtilisin from *B. subtilis* as a processing aid in protein processing to facilitate protein hydrolysis is assessed based on a 90-days oral toxicology study and daily administration of *B. clausii* subtilisin for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 480.6 mg TOS/kg bw/day.

Based on a worst-case scenario that a person is consuming *B. clausii* subtilisin from the products with protein containing the subtilisin, the cumulative daily exposure of 2.73mg TOS/kg bw/day.

The margin of safety was calculated to be 175 based on a NOAEL of 480.6 mg TOS/kg bw/day based on the toxicological studies from *B. clausii* subtilisin (obtained from the cumulative maximum daily exposure to subtilisin of 2.73 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 Nutrition & Biosciences), and the decision tree analysis using generally recognized evaluation methodology (Pariza and Johnson, 2001; Sewalt *et al.*, 2016), the company has concluded that the *B. clausii* subtilisin from *B. subtilis* strain is safe and suitable for use as processing aid protein processing. Collectively, the use of published information and evaluation methods provide a strong common knowledge element, based upon

which this lipase can be considered Generally Recognized as Safe (GRAS) for its intended uses. In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by an external expert in the field, Dr. Michael Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 8).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES

Appendix 1: IUBMB Nomenclature Record on Subtilisin

Appendix 2: The Amino Acid Sequence of the Subtilisin and GRAS Affirmed Protease

Appendix 3: The Manufacturing Process

Appendix 4: Certificate of Analysis (3 lots)

Appendix 5: *Bacillus subtilis* Strain Lineage

Appendix 6: Summary of Safety Studies of Bacillus subtilis Safe Strain Lineage

Appendix 7: Allergenicity Analysis on the Subject Subtilisin

Appendix 8: External Expert Opinion Letter from Dr. Michael Pariza

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Appendix 1: 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 2: Amino Acid Sequences of Subtilisin

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

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDG
NGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGM
HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATD
QNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKN
PSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR

B. amyloliquefaciens subtilisin expressed in *B. subtilis*

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQ
DNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIAN
NMDVINMSLGGPSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVI
AVGAVDSSNQRASFSSVGPELDVMAPGVSIQSTLPGNKYGALNGTSMASPHVAGAAAL
ILSKHPNWTNTQVRSSLENTTTKLGDSFYYGKGLINVQAAAQ

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

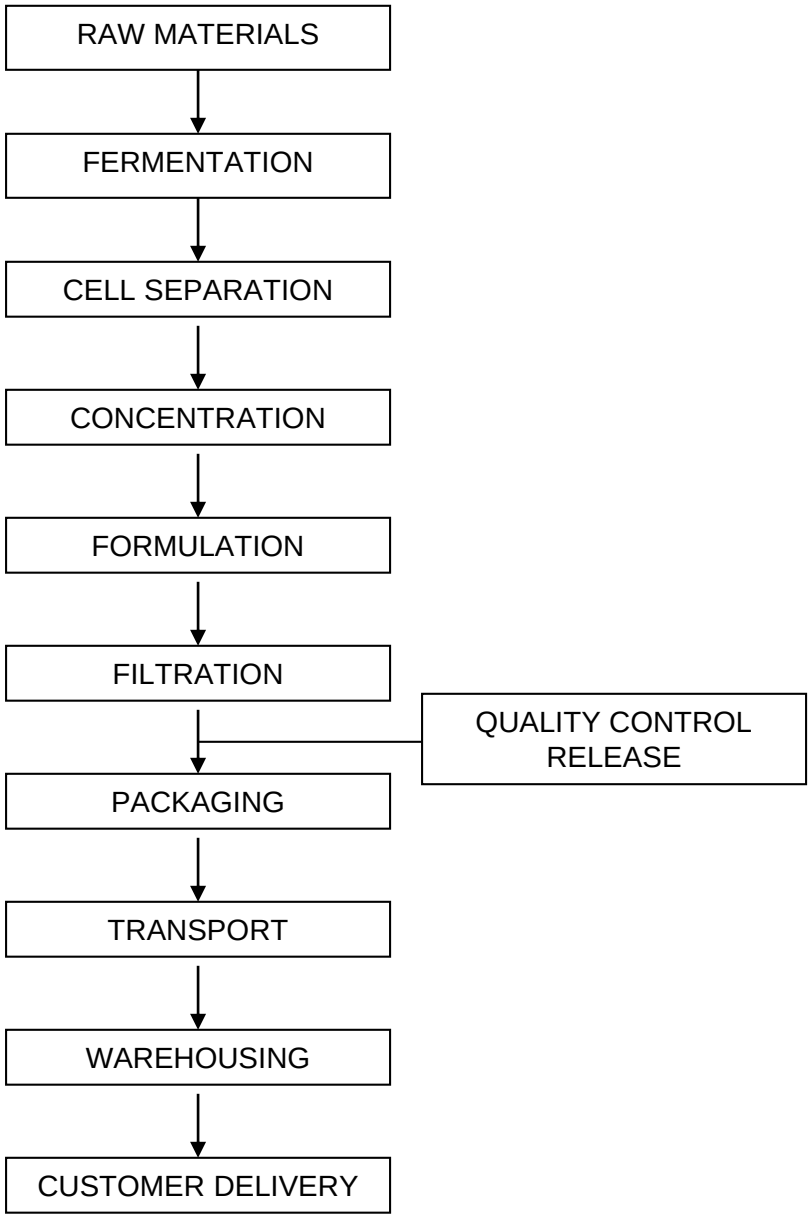
Bacillus licheniformis subtilisin (Wild-Type) 21 C.F.R. §184.1027

MMRKKSFWLGMLTAFMLVFTMAFSDSASAAQPAKNVEKDYIVGFKSGVKTASVKKDII
KESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHALAQTVPYGIPLI
KADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASFVAGEAYNTDGNGHGTHV

AGTVAALDNTTGVLGVAPSVSLYAVKVLNSSGSGTYSGIVSGIEWATTNGMDVINMSL
GGPSGSTAMKQAVDNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSN
SNRASFSSVGAELEVMAPGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLS
ASQVRNRLSSTATYLGSSFYYGKGLINVEAAAQ

Appendix 3: The Manufacturing Process

FoodPro® PXT
PROCESS FLOW DIAGRAM



Appendix 4: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663619649**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	U/g	2800-3540	3367
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Coliforms	CFU/ml	0-30	<1
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.20
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5

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.

9-Jul-2020
Date

Kelly A. Altman
QA/QC Department

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

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663620612**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	U/g	2800-3540	3245
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Coliforms	CFU/ml	0-30	<1
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.18
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5

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.

9-Jul-2020
Date

Kelly A. Altman
QA/QC Department

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

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663714790**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	U/g	2800-3540	2910
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Coliforms	CFU/ml	0-30	<1
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.18
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5

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.

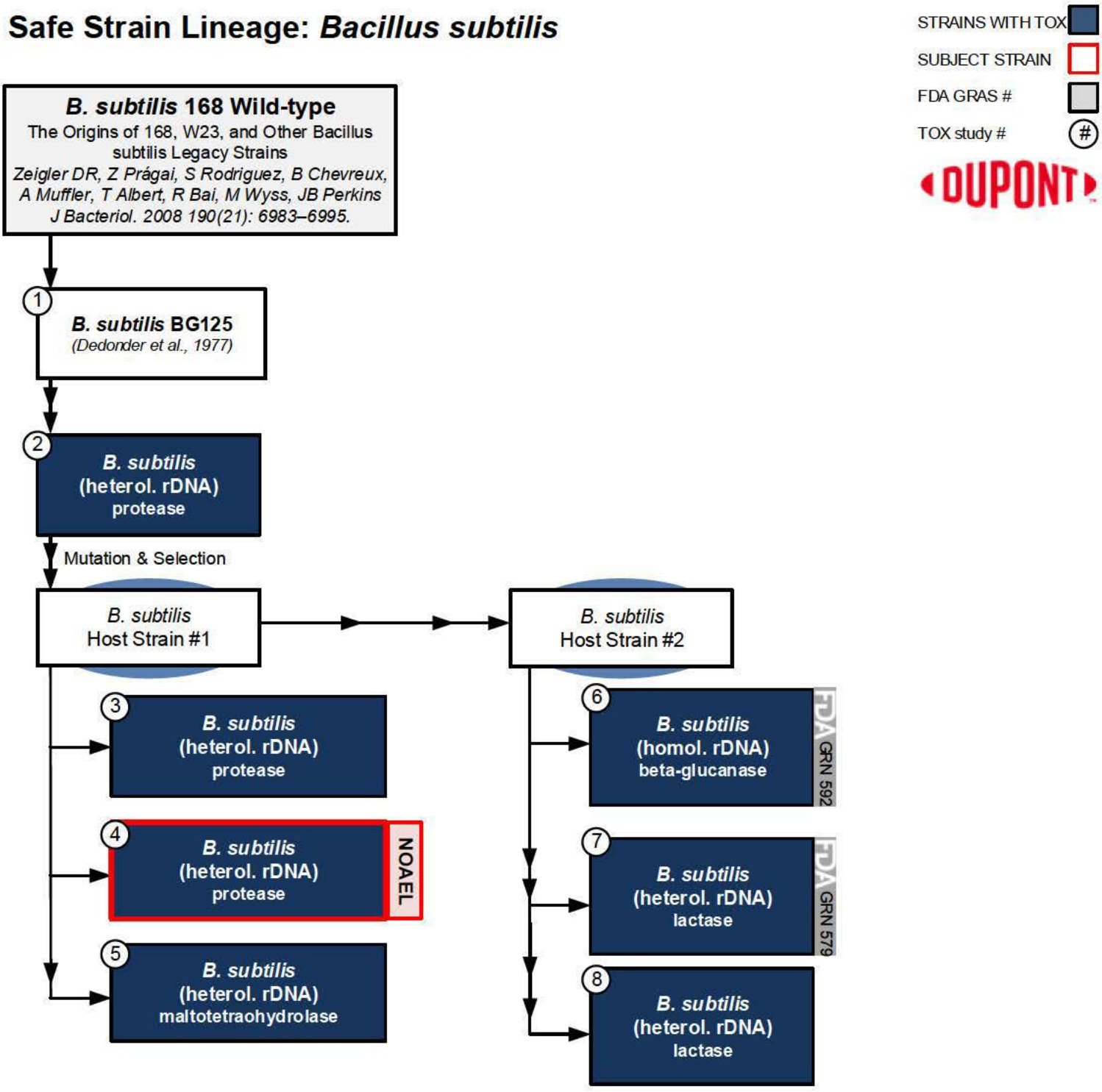
9-Jul-2020
Date

Kelly A. Altman
QA/QC Department

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

Appendix 5: *Bacillus subtilis* Strain Lineage

Safe Strain Lineage: *Bacillus subtilis*



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

Most enzymes derived from this Safe Strain Lineage were determined to be GRAS for their intended use, with GRAS Notice submitted for review by the US FDA, which resulted in a “no questions letter” for the enzyme preparations from the strains with a vertical grey FDA GRN banner indicating the GRAS Notice number..

The safety of the **protease** is fully supported by repeated testing of the protease and 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 **protease** from the production strain is used to support the safety of the **protease** in the intended use, as indicated with red flag labeled “NOAEL”.

Appendix 6: Summary of Safety Studies of Bacillus subtilis Safe Strain Lineage

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2333 CN Leiden
The Netherlands

Toxicology Test Summaries

Summary of safety studies on Bacillus subtilis derived enzymes and Bacillus subtilis strains in support of DuPont's Safe Strain Lineage

The safety of 11 DuPont *Bacillus subtilis* strains and 10 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	TOXICOLOGY TEST	RESULT
1) ***B. subtilis***	host strain	• Cytotoxicity study, Chinese hamster ovary	Non-cytotoxic
2) ***B. subtilis*** (heterol. rDNA)	protease	• Pathogenicity test	Non-toxigenic, non-pathogenic
		• 90-day subchronic study, rats	No adverse effects detected; NOAEL 50,000 ppm
3) ***B. subtilis*** (heterol. rDNA)	protease	• Cytotoxicity study, Chinese hamster ovary	Non-cytotoxic
		• 90-day subchronic study, rats	No adverse effects detected; NOAEL 50,000 ppm
4) ***B. subtilis*** (heterol. rDNA)	protease	• 90-day subchronic oral study, rats	NOAEL established at highest dose, 420 mg total protein/kg bw/day or 480.6 mg TOS/kg bw/day.
5) ***B. subtilis*** (heterol. rDNA)	maltotetrao-hydrolase	• Acute oral toxicity in rats	No signs of toxicity at 2000 mg total protein/kg bw
		• 91-day subchronic study, rats	No adverse effects detected, NOAEL = 17.5 mg enzyme protein / kg bw / day
		• Ames test	Non-mutagenic

		• *In vitro* chromosome assay, human lymphocytes	Non- clastogenic
6) ***B. subtilis*** (homol. rDNA)	glucanase	• Dermal irritation	Non-irritant
		• Eye irritation	Non-irritant
		• Ames assay	Non-mutagenic
		• Chromosomal aberration	Non-clastogenic
		• 90-day subchronic study	NOAEL 1000 mg TOS/kg bw/day
7) ***B. subtilis*** (heterol. rDNA)	lactase	• Dermal irritation	Non-irritant
		• Eye irritation	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, 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
8) ***B. subtilis*** (heterol. rDNA)	lactase	• 90-day oral study rats	NOAEL 1000 mg TOS/kg bw/day
		• *In vitro* chromosome assay, human blood lymphocytes	Non-clastogenic

		• Ames assay	Non-mutagenic
		• oral toxicity rats	Oral LD50 > 4099 mg TOS/kg (3082 mg total protein/kg)
The 90-day subchronic oral study was not conducted on the following strains, and thus they are not represented in the lineage schematic.			
9) *B. subtilis* (heterol. rDNA)	protease	• Cytotoxicity study, rats	Non-toxic, non-pathogenic
		• 4-week gavage study, rats	No adverse effects detected, NOAEL 15,000 ppm/kg/day
10) *B. subtilis* (heterol. rDNA)	protease	• Skin irritation	Mild skin irritation
		• Eye irritation	serious eye damage
		• Acute oral toxicology	No signs of toxicity at 5000 mg total protein/kg bw
		• Ames test	Non-mutagenic
11) *B. subtilis* (heterol. rDNA)	arylesterase	• Skin irritation	Non-irritant
		• Eye irritation	Mild eye irritation
		• Acute oral toxicology	No signs of toxicity at 5000 mg total protein/kg bw
		• Acute dermal toxicology	No signs of toxicity at 5000 mg total protein/kg bw
		• Local lymph node assay	not a dermal sensitizer

Appendix 7: Allergenicity Analysis on the Subject Subtilisin

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FOOD ALLERGENICITY RISK ASSESSMENT

Bacillus clausii protease FN3 (GICC03378)

By: Emily D. Melton, PhD
Regulatory Affairs Specialist
DuPont Nutrition & Biosciences

Date: June 08th, 2020

Sequence Based Analysis Risk Assessment for Potential Food Allergenicity of the *Bacillus clausii* protease expressed in *Bacillus subtilis.*

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches.

The past practice of conducting an analysis to identify short, six contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The mature *Bacillus clausii* protease sequence is given below:

```
AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSI
GVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNS
GAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQK
NPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
```

A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[1], February 10, 2020 V20, containing 2129 peer-reviewed allergen sequences listed in the database[2] (using E-value <0.1) 26 protease matches, with a peptide sequence of more than 35% identity to the mature *Bacillus clausii*

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

(alkaline) protease sequence (see Appendix A). However, none of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database[3] (see Appendix D).

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 (February 10, 2020 V20), identified 28 matches from various *Bacilli* and *Fungi*, all fungal serine proteases (see Appendix B). None of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database[3] (see Appendix D).

Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org, there is no evidence that a short identical contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (>35%). The AllergenOnline database allows isolated identity matches of eight contiguous identical amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the eight-contiguous identical amino acid search produced 28 sequence matches with known allergens, all fungal serine proteases (see Appendix C). None of these observed matches are listed as food allergens in the WHO/IUIS Allergen Nomenclature Database[3] (see Appendix D).

As noted above, none of the identified fungal serine proteases were identified as food allergens in the WHO/IUIS Allergen Nomenclature Database. Rather, the identified fungal serine proteases are known respiratory or contact allergens. Furthermore, microbial derived enzymes acting as environmental allergens have not been demonstrated to be active via the oral route. This concept was evaluated by Bindslev-Jensen *et al.* (2006) in which 19 microbial derived enzymes, including proteases, used in the food industry were tested for allergenicity in a double-blind placebo-controlled food challenge study in subjects with positive skin prick tests to inhalation allergens, food allergens, and bee and wasp allergens. None of the 19 microbial derived food enzymes were found to be food allergens and the authors concluded that ingestion of microbial derived food enzymes in general are not considered to be a concern regarding food allergy. In addition, a protease was found to be rapidly digested in an in vitro pepsin digestibility assay. Moreover, enzymes are processing aids and per definition, they are not present or active in the final food. Consequently, there is no exposure of the enzyme to the consumer. Taken together, these data indicate that the *Bacillus clausii* protease is unlikely to pose a risk of food allergenicity.

As for all enzyme products, an SDS for the protease product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

Reviewed and evaluated by:

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[3] http://www.allergen.org/index.php

This assessment is valid for 6 months from date of issue.

References

Bindslev-Jensen C, Per Stahl S, Roggen EL, Hvass P and Ditte Sidelmann B (2006) Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem Toxicol* 44:1909–1915.

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. 21-23.

Herman RA, Ping Song, and ThirumalaiswamySekhar A (2009) Value of eight-amino-acid matches in predicting the allergenicity status of proteins: an empirical bioinformatic investigation. *Clin Mol Allergy* 7: 9.

Ladics GS, Cressman RF, Herouet-Guicheney C, Herman RA, Privalle L, Song P, Ward JM and McLain S (2011) Bioinformatics and the allergy assessment of agricultural biotechnology products: Industry practices and recommendations. *Regul Toxicol Pharm* 60:46-53.

Appendix A. Allergen Online Full-Length Alignment Search Results

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v20 (February 10, 2020)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.<="" a="">

<="" a="">

<="" a="" style="color: rgb(0, 0, 0); font-family: "Times New Roman"; font-size: medium; font-style: normal; font-variant-ligatures: normal; font-variant-caps: normal; font-weight: 400; letter-spacing: normal; orphans: 2; text-align: start; text-indent: 0px; text-transform: none; white-space: normal; widows: 2; word-spacing: 0px; -webkit-text-stroke-width: 0px; text-decoration-style: initial; text-decoration-color: initial;">**fasta36.exe -q -B -m 9i -w 80 -E 0.1 -d 20 C:\Windows\Temp\allDBCF.tmp version2036 fasta**

User Query #1 >query

User Query #1
>query
AQSVPWGISR VQAPAAHNRG LTGSGVKVAV LDTGISTHPD LNIRGGASFV PGEPSTQDGN
GHGTHVAGTI AALDNSIGVL GVAPSAELYA VKVLGASGSG AISSIAQGLE WAGNNGMHVA
NLSLGSPSPS ATLEQAVNSA TSRGVLVVAA SGNSGAGSIS YPARYANAMA VGATDQNNNR
ASFSQYGAGL DIVAPGVNVQ STYPGSTYAS LNGTSMATPH VAGAAALVKQ KNPSWSNVQI
RNHLKNTATS LGSTNLYGSG LVNAEAATR
fasta36.exe -q -B -m 9i -w 80 -E 0.1 -d 20 C:\Windows\Temp\allDBCF.tmp version2036 fasta
FASTA searches a protein or DNA sequence data bank
version 36.3.8g Oct, 2018
Please cite:
W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\allDBCF.tmp
1>>>query - 269 aa
Library: version2036 fasta
517010 residues in 2171 sequences

Statistics: Altschul/Gish params: n0: 269 Lambda: 0.158 K: 0.019 H: 0.100
statistics sampled from 375 (404) to 375 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
ktup: 2, E-join: 1 (0.522), E-opt: 0.2 (0.186), width: 16
Scan time: 0.000

The best scores are:		opt	bits	E(2171)	%_id	%_sim	alen
gi|267048|gid|1174|RecName: Full=Subtilisin Savinase	(269)	1708	396.0	1e-111	0.989	1.000	269
gi|1225905|gid|1175|prepro AprM [Bacillus sp.]	(361)	1152	269.1	2e-073	0.647	0.885	269
gi|135016|gid|1171|RecName: Full=Subtilisin Carlsber	(379)	1091	255.2	3.3e-069	0.617	0.869	274
gi|11127680|gid|1171|subtilisin precursor [Bacillus	(374)	1056	247.2	8.2e-067	0.608	0.862	268
gi|6684758|gid|244|allergen Pen n 13 [Penicillium ch	(397)	480	115.9	3e-027	0.369	0.660	282
gi|4587983|gid|244|Pen c 1; alkaline serine protease	(397)	479	115.7	3.5e-027	0.365	0.663	282
gi|21069093|gid|244|alkaline serine protease [Penici	(398)	473	114.3	8.9e-027	0.379	0.660	235
gi|3549630|gid|2227|alkaline protease, partial [Aspe	(341)	438	106.4	1.9e-024	0.362	0.681	276
gi|2295|gid|2227|uncleaved alkaline protease (ALP) [	(403)	438	106.3	2.3e-024	0.362	0.681	276
gi|129235|gid|317|Oryzin precursor (Alkaline protein	(403)	424	103.2	2.1e-023	0.341	0.667	279
gi|294441150|gid|2457|extracellular alkaline serine	(403)	422	102.7	2.9e-023	0.353	0.665	272
gi|74665726|gid|317|Allergen Asp fl 1	(403)	421	102.5	3.4e-023	0.341	0.667	279
gi|54654335|gid|833|vacuolar serine protease [Rhodot	(342)	414	100.9	8.4e-023	0.390	0.676	259
gi|5813790|gid|313|Tri r 2 allergen [Trichophyton ru	(412)	390	95.4	4.6e-021	0.359	0.652	276
gi|23894244|gid|313|tri m 2 allergen [Arthroderma be	(404)	380	93.1	2.2e-020	0.377	0.646	257
gi|23894240|gid|313|tri m 2 allergen [Arthroderma be	(292)	376	92.3	2.8e-020	0.368	0.655	258

```
gi|12005501|gid|243|vacuolar serine protease [Penici ( 358)  377 92.5 3.1e-020 0.413 0.680  259
gi|74663809|gid|313|RecName: Full=Subtilisin-like pr ( 405)  371 91.1 9.1e-020 0.376 0.643  258
gi|14215732|gid|243|vacuolar serine protease [Penici ( 494)  352 86.7 2.3e-018 0.390 0.676  259
gi|7963902|gid|243|allergen Pen n 18 [Penicillium ch ( 494)  352 86.7 2.3e-018 0.390 0.676  259
gi|148361511|gid|1376|vacuolar serine protease [Clad ( 388)  322 79.9  2e-016 0.384 0.681  263
gi|60116876|gid|1227|vacuolar serine protease [David ( 518)  315 78.3 8.4e-016 0.384 0.677  263
gi|193507493|gid|2241|subtilisin-like serine proteas ( 506)  313 77.8 1.1e-015 0.388 0.669  260
gi|289172|gid|318|serine protease                    ( 533)  312 77.6 1.4e-015 0.395 0.665  263
gi|4588118|gid|243|alkaline serine protease Pen c2 [ ( 457)  306 76.2  3e-015 0.395 0.662  263
gi|739057410|gid|2857|vacuolar serine protease, part ( 386)  303 75.6  4e-015 0.369 0.665  263
gi|12005497|gid|243|vacuolar serine protease [Penici ( 503)  302 75.3 6.3e-015 0.388 0.665  263
gi|2143220|gid|318|cellular serine proteinase [Asper ( 495)  296 73.9 1.6e-014 0.395 0.658  263
gi|807698|gid|466|pre-pro-cucumisin [Cucumis melo]   ( 731)  181 47.7 1.9e-006 0.296 0.560  159
+-                                                            170 45.2 1.1e-005 0.265 0.578
185
```

>>>query, 269 aa vs version2036.fasta library

>>gi|267048|gid|1174|RecName: Full=Subtilisin Savinase; AltName: Full=Alk (269 aa)

```
 initn: 1708 init1: 1708 opt: 1708  Z-score: 2098.2  bits: 396.0 E(2171): 1e-111
Smith-Waterman score: 1708; 98.9% identity (100.0% similar) in 269 aa overlap (1-269:1-269)

               10        20        30        40        50        60        70        80
query  AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
notag| AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALNNSIGVL
               10        20        30        40        50        60        70        80

               90       100       110       120       130       140       150       160
query  GVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSIS
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
notag| GVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSIS
               90       100       110       120       130       140       150       160

              170       180       190       200       210       220       230       240
query  YPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQI
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
notag| YPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQI
              170       180       190       200       210       220       230       240

              250       260
query  RNHLKNTATSLGSTNLYGSGLVNAEAATR
       ::::::::::::::::::::::::::::::
notag| RNHLKNTATSLGSTNLYGSGLVNAEAATR
              250       260
```

>>gi|1225905|gid|1175|prepro AprM [Bacillus sp.] (361 aa)

```
 initn: 1167 init1: 1142 opt: 1152  Z-score: 1410.4  bits: 269.1 E(2171): 2e-073
Smith-Waterman score: 1152; 64.7% identity (88.5% similar) in 269 aa overlap (1-269:93-361)

                                                      10        20        30        40
query                                              AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPD
                                                   .:.:::::: ...  ::::: :.:..:::::::..:::
notag| VIHEFEEIPVIHAELTKKELKKLKKDPNVKAIEENAEVTISQTVPWGISFINTQQAHNRGIFGNGARVAVLDTGIASHPD
            60        70        80        90       100       110       120       130

               50        60        70        80        90       100       110       120
query  LNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHVA
       : : ::::::. .::: .:.:::::::::::::.:::::::::::.:::::::  .:::...:.:::.::: ::.::.
notag| LRIAGGASFISSEPSYHDNNGHGTHVAGTIAALNNSIGVLGVAPSADLYAVKVLDRNGSGSLASVAQGIEWAINNNMHII
          140       150       160       170       180       190       200       210

              130       140       150       160       170       180       190       200
query  NLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQ
       :.:::: : :.::: ::: :.. :.:.:.:.::.:  ...:::::...:::.:.:::..::::: ::  ..: ::::::.
notag| NMSLGSTSGSSTLELAVNRANNAGILLVGAAGNTGRQGVNYPARYSGVMAVAAVDQNGQRASFSTYGPEIEISAPGVNVN
          220       230       240       250       260       270       280       290

              210       220       230       240       250       260
query  STYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       ::: :. :.::.:::::::::::.::::.. ::..: :::.....::: ::: .:::.:::.:  ::.
notag| STYTGNRYVSLSGTSMATPHVAGVAALVKSRYPSYTNNQIRQRINQTATYLGSPSLYGNGLVHAGRATQ
          300       310       320       330       340       350       360
```

>>gi|135016|gid|1171|RecName: Full=Subtilisin Carlsberg; Flags: Precursor (379 aa)

```
 initn: 1083 init1: 540 opt: 1091  Z-score: 1334.8  bits: 255.2 E(2171): 3.3e-069
Smith-Waterman score: 1091; 61.7% identity (86.9% similar) in 274 aa overlap (1-269:106-379)

                                                    10        20        30
query                                             AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGI-STHP
                                                  ::.::.::  ..:  .. .:. :..:::::::::: ..::
notag| DKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHALAQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHP
         70        80        90       100       110       120       130       140

      40        50        60        70        80        90       100       110
query  DLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHV
       :::. :::::: ::  . :::::::::::::.::::::. :::::::::. ::::::::.::::. :.:..:.::: .::: :
notag| DLNVVGGASFVAGEAYNTDGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSGSGTYSGIVSGIEWATTNGMDV
         150       160       170       180       190       200       210       220

     120       130       140       150           160       170       180       190
query  ANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAG----SISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAP
        :.:::.:: :....:::..: .:::.::::.:::::.    .:.:::.: ...:::::.:.:.::::::. :: :...::
notag| INMSLGGPSGSTAMKQAVDNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMAP
         230       240       250       260       270       280       290       300

         200       210       220       230       240       250       260
query  GVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       :..: :::: ::::.::::::::.:::::::::. .:.:. :  :.::.:..::: :::.  ::.::.:.:::..
notag| GAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRLSSTATYLGSSFYYGKGLINVEAAAQ
         310       320       330       340       350       360       370

>>gi|11127680|gid|1171|subtilisin precursor [Bacillus licheniformis]       (374 aa)
 initn: 1070 init1: 526 opt: 1056  Z-score: 1291.8  bits: 247.2 E(2171): 8.2e-067
Smith-Waterman score: 1056; 60.8% identity (86.2% similar) in 268 aa overlap (1-263:106-373)

                                                    10        20        30
query                                             AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGI-STHP
                                                  ::.::.::  ..:  .. .:. :..:::::::::: ..::
notag| DKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHALAQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHP
         70        80        90       100       110       120       130       140

      40        50        60        70        80        90       100       110
query  DLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHV
       :::. :::::: ::  . :::::::::::::.::::::. :::::::::. ::::::::.::::. :.:..:.::: .::: :
notag| DLNVVGGASFVAGEAYNTDGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSGSGSYSGIVSGIEWATTNGMDV
         150       160       170       180       190       200       210       220

     120       130       140       150           160       170       180       190
query  ANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAG----SISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAP
        :.:::. : :....:::..: ..::.::::.:::::.    .:.:::.: ...:::::.:.:.::::::. :: :...::
notag| INMSLGGASGSTAMKQAVDNAYAKGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMAP
         230       240       250       260       270       280       290       300

         200       210       220       230       240       250       260
query  GVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       :..: :::: .:::.::::::::.:::::::::. .:.:. :  :.::.:..::: :::.  ::.::.:
notag| GAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRLSSTATYLGSSFYYGKGLINV
         310       320       330       340       350       360       370

>>gi|6684758|gid|244|allergen Pen n 13 [Penicillium chrysogenum]           (397 aa)
 initn: 409 init1: 275 opt: 480  Z-score: 581.7  bits: 115.9 E(2171): 3e-027
Smith-Waterman score: 484; 36.9% identity (66.0% similar) in 282 aa overlap (3-261:122-395)

                                                  10        20            30
query                                           AQSVP-WGISRVQAPAAHNRGLT-----GSGVKVAVLDTGIS
                                                  .:: ::..:...  . . . :     : ::    .::::.
notag| LKGYTASFDENTAKDIANDPAVKYIEPDMIVNATANVVQSNVPSWGLARISSKRTGTTSYTYDSTAGEGVVFYGVDTGID
              90       100       110       120       130       140       150       160

          40          50        60        70        80        90       100       110
query  -THPDLNIRG--GASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA-
        .: :.. :.  :.. : .. .  :::::::::.:.: :.     .  ::: .: : ::::::.:::. :.. .:..::
notag| ISHSDFGGRAKWGTNVVDNDNT--DGNGHGTHTASTAAG-----SKYGVAKKATLVAVKVLGADGSGTNSGVISGMDWAV
             170       180         190             200       210       220       230
```

```
                 120       130       140       150       160       170       180
query  ------GNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAG-SISYPARYANAMAVGATDQNNNRASFSQ
             : :: .: : :::.   .:. . :.: . : :... .:.:: . . : : ::  :.: ...:. .... :::..
notag| KDAKSRGANGKYVMNTSLGGEFSKAVNDAAANVVKS-GIFLSVAAGNEAENASNSSPASAAEACTIAASTSTDGSASFTN
          240       250       260       270       280       290       300       310

         190       200       210       220       230            240       250
query  YGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAA-LVKQKNPSWSN-----VQIRNHLKNTATSLGSTNLYGS
       .:. .:. ::: .. ..:::.   .:.:::::.:::::.:: :.  .. : .:     ::. .   . : :  ...:  .
notag| FGSVVDLYAPGQSITAAYPGGGSKTLSGTSMAAPHVAGVAAYLMALEGVSAGNACARIVQLATSSISRAPSGTTSKLLYN
           320       330       340       350       360       370       380       390

     260
query  GLVNAEAATR
       :.
notag| GINV
```

>>gi|4587983|gid|244|Pen c 1; alkaline serine protease [Penicillium citri (397 aa)

```
 initn: 408 init1: 274 opt: 479  Z-score: 580.5  bits: 115.7 E(2171): 3.5e-027
Smith-Waterman score: 483; 36.5% identity (66.3% similar) in 282 aa overlap (3-261:122-395)

                                                    10        20            30
query                                            AQSVP-WGISRVQAPAAHNRGLT-----GSGVKVAVLDTGIS
                                                   .:: ::..:...  . . . :     : ::    .::::.
notag| LKGYTASFDESTAKDIANDPAVKYIEPDMIVNATANVVQSNVPSWGLARISSKRTGTTSYTYDSTAGEGVVFYGVDTGID
              90       100       110       120       130       140       150       160

          40          50        60        70        80        90       100       110
query  -THPDLNIRG--GASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA-
        .: :.. :.  :.. : .. .  ::::::::.:.: :.     .  ::: .: : ::::::::.:::. :.. .:..::
notag| ISHSDFGGRAKWGTNVVDNDNT--DGNGHGTHTASTAAG-----SKYGVAKKATLVAVKVLGADGSGTNSGVISGMDWAV
            170       180         190             200       210       220       230

                 120       130       140       150       160       170       180
query  ------GNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAG-SISYPARYANAMAVGATDQNNNRASFSQ
             : :: .: :.:::.   .:. . :.: . : :... .:.:: . . : : ::  :.. ...:. .... :::..
notag| KDAKSRGANGKYVMNMSLGGEFSKAVNDAAANVVKS-GIFLSVAAGNEAENASNSSPASAAEVCTIAASTSTDGSASFTN
          240       250       260       270       280       290       300       310

         190       200       210       220       230            240       250
query  YGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAA-LVKQKNPSWSN-----VQIRNHLKNTATSLGSTNLYGS
       .:. .:. ::: .. ..:::.   .:.:::::.:::::.:: :.  .. : .:     ::. .   . : :  ...:  .
notag| FGSVVDLYAPGQSITAAYPGGGSKTLSGTSMAAPHVAGVAAYLMALEGVSAGNACARIVQLATSSISRAPSGTTSKLLYN
           320       330       340       350       360       370       380       390

     260
query  GLVNAEAATR
       :.
notag| GINV
```

>>gi|21069093|gid|244|alkaline serine protease [Penicillium chrysogenum] (398 aa)

```
 initn: 379 init1: 271 opt: 473  Z-score: 573.1  bits: 114.3 E(2171): 8.9e-027
Smith-Waterman score: 473; 37.9% identity (66.0% similar) in 235 aa overlap (6-226:126-354)

                                          10            20        30
query                                  AQSVPWGISRVQAPAAHNR-----GLTGSGVKVAVLDTGIST-HP
                                         ::.::...  .        . .: :. .  .::::.  :
notag| SASFDDRTVKDIASDPTVKYVEPDMVVNATANVVQRNAPSWGLSRISSKKSGATDYVYDSTAGEGIVIYGVDTGIDIGHA
          90       100       110       120       130       140       150       160

       40        50        60        70        80        90       100       110
query  DLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA-------
       :.. :.  .   .. .  :::::::::.:.: :.     . .::: .: . :::::::.:::. :..  :..::
notag| DFGGRAEWGTNTADNDDTDGNGHGTHTASTAAG-----SKFGVAKKASVVAVKVLGADGSGTNSQVIAGMDWAVKDSKSR
         170       180       190             200       210       220       230       240

            120       130       140       150       160       170       180       190
query  GNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGA-GSISYPARYANAMAVGATDQNNNRASFSQYGAGLD
       : .:  : :.:::.    :  . :.: . : ::.. .:.:: .  .: : ::   :. ...:. .... :::...:. .:
notag| GATGKSVMNMSLGGAYSRAMNDAAANVVRS-GVFLSVAAGNEAQDASNSSPASAPNVCTIAASTNSDGSASFTNFGSVVD
              250       260       270       280       290       300       310
```

```
            200       210       220       230       240       250       260
query  IVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       . ::: .. ..::::.   .:.::::::.:::::::
notag| LYAPGKDITAAYPGGGSKTLSGTSMAAPHVAGAAAYLMALEGVTSDKACARIVELAISSISSAPSGTTSKLLYNGINAQ
     320       330       340       350       360       370       380       390
```

>>gi|3549630|gid|2227|alkaline protease, partial [Aspergillus fumigatus] (341 aa)

```
 initn: 391 init1: 174 opt: 438  Z-score: 531.3  bits: 106.4 E(2171): 1.9e-024
Smith-Waterman score: 438; 36.2% identity (68.1% similar) in 276 aa overlap (2-259:66-332)

                                                    10           20        30
query                                            AQSVPWGISRV----QAPAAHNRGLT-GSGVKVAVLDTGIS
                                                  ...:::.. .    :: . .    . :.:. . :.:.::.
notag| DFAAYAGSFDDATIEEIRKSADVAHVEEDQIWYLDALTTQKGAPWGLGSISHKGQASTDYIYDTSAGAGTYAYVVDSGIN
         30        40        50        60        70        80        90       100

         40        50        60        70        80        90        100       110
query  T-HPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVL-GASGSGAISSIAQGLEWAGN
       . : ... :.. ..  .  :  :. ::::::::::..      . ::: ...: .:::. : :.: .:  : .:..:: :
notag| VNHVEFESRASLAYNAAGGSHVDSIGHGTHVAGTIGG-----KTYGVAKKTNLLSVKVFQGESSSTSI--ILDGFNWAVN
       110       120       130       140           150       160       170

                120       130       140       150       160       170       180
query  NGMH-------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGA-GSISYPARYANAMAVGATDQNNNRASFSQY
       . .        . :.:::. . : ....::..: ..::: :.:.:: .. .: . ::   ::..:.: ...: ::::::.:
notag| DIVSKGRTKKAAINMSLGG-GYSYAFNNAVENAFDEGVLSVVAAGNENSDASNTSPASAPNALTVAAINKSNARASFSNY
     180       190       200       210       220       230       240       250

       190       200       210         220       230       240       250       260
query  GAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAA-LVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVN
       :. .:: ::: .. :.. ::: :.  ..::::::::::.: .. :.  .: : . . .  ..:. ::.   ::. ::
notag| GSVVDIFAPGQDILSAWIGSTTATNTISGTSMATPHIVGLSVYLMGLENLS-GPAAVTARIKELATNGVVTNVKGSPNKL
       260       270       280       290       300       310       320       330


query  AEAATR

notag| AYNGN
       340
```

>>gi|2295|gid|2227|uncleaved alkaline protease (ALP) [Aspergillus fumigat (403 aa)

```
 initn: 391 init1: 174 opt: 438  Z-score: 529.8  bits: 106.3 E(2171): 2.3e-024
Smith-Waterman score: 438; 36.2% identity (68.1% similar) in 276 aa overlap (2-259:127-393)

                                                    10           20        30
query                                            AQSVPWGISRV----QAPAAHNRGLT-GSGVKVAVLDTGIS
                                                  ...:::.. .    :: . .    . :.:. . :.:.::.
notag| DFAAYAGSFDDATIEEIRKRGDVAHVEEDQIWYLDALTTQKGAPWGLGSISHKGQASTDYIYDTSAGAGTYAYVVDSGIN
         90       100       110       120       130       140       150       160

         40        50        60        70        80        90        100       110
query  T-HPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVL-GASGSGAISSIAQGLEWAGN
       . : ... :.. ..  .  :  :. ::::::::::..      . ::: ...: .:::. : :.: .:  : .:..:: :
notag| VNHVEFESRASLAYNAAGGSHVDSIGHGTHVAGTIGG-----KTYGVAKKTNLLSVKVFQGESSSTSI--ILDGFNWAVN
       170       180       190       200           210       220         230

                120       130       140       150       160       170       180
query  NGMH-------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGA-GSISYPARYANAMAVGATDQNNNRASFSQY
       . .        . :.:::. . : ....::..: ..::: :.:.:: .. .: . ::   ::..:.: ...: ::::::.:
notag| DIVSKGRTKKAAINMSLGG-GYSYAFNNAVENAFDEGVLSVVAAGNENSDASNTSPASAPNALTVAAINKSNARASFSNY
     240       250       260       270       280       290       300       310

       190       200       210         220       230       240       250       260
query  GAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAA-LVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVN
       :. .:: ::: .. :.. ::: :.  ..::::::::::.: .. :.  .: : . . .  ..:. ::.   ::. ::
notag| GSVVDIFAPGQDILSAWIGSTTATNTISGTSMATPHIVGLSVYLMGLENLS-GPAAVTARIKELATNGVVTNVKGSPNKL
      320       330       340       350       360       370       380       390


query  AEAATR

notag| AYNGNA
```

```
       400

>>gi|129235|gid|317|Oryzin precursor (Alkaline proteinase) (ALP) (Aspergi  (403 aa)
 initn: 228 init1: 164 opt: 424  Z-score: 512.6  bits: 103.2 E(2171): 2.1e-023
Smith-Waterman score: 424; 34.1% identity (66.7% similar) in 279 aa overlap (2-256:127-396)

                                                    10        20            30
query                                            AQSVPWGISRVQAPAAHNRGL-----TGSGVKVAVLDTGIS
                                                 .:.:::.. ..  . ..        .: :. . :.:.:..
notag| KFAAYAGSFDDATIEEIRKNEDVAYVEEDQIYYLDGLTTQKSAPWGLGSISHKGQQSTDYIYDTSAGEGTYAYVVDSGVN
         90       100       110       120       130       140       150       160

          40        50        60        70        80        90        100       110
query  T-HPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVL-GASGSGAISSIAQGLEWAGN
       . : ... :.. ..  .  .  :. ::::::.::::.      . :.: .: . .:::. : :.: ..  : .:..::.:
notag| VDHEEFEGRASKAYNAAGGQHVDSIGHGTHVSGTIAG-----KTYGIAKKASILSVKVFQGESSSTSV--ILDGFNWAAN
        170       180       190       200            210       220         230

                 120       130       140       150         160       170       180
query  NGMH-------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGN--SGAGSISYPARYANAMAVGATDQNNNRASFSQ
       . .        . :.::::. . : ....::..:  .::: :.:.::  : ::. : ::   .:..:.: ...:::::::.
notag| DIVSKKRTSKAAINMSLGG-GYSKAFNDAVENAFEQGVLSVVAAGNENSDAGQTS-PASAPDAITVAAIQKSNNRASFSN
     240       250       260       270       280       290       300       310

         190       200       210         220           230       240       250
query  YGAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGA----AALVKQKNPSWSNVQIRN-HLKNTATSL-GSTNLY
       .:  .:. ::: .. :.. ::. :.  ..:::::::::.:     ::: .  .:.  . .:..   :... .. :: ::
notag| FGKVVDVFAPGQDILSAWIGSSSATNTISGTSMATPHIVGLSLYLAALENLDGPAAVTKRIKELATKDVVKDVKGSPNLL
       320       330       340       350       360       370       380       390

       260
query  GSGLVNAEAATR

notag| AYNGNA
       400

>>gi|294441150|gid|2457|extracellular alkaline serine protease [Aspergill  (403 aa)
 initn: 278 init1: 167 opt: 422  Z-score: 510.1  bits: 102.7 E(2171): 2.9e-023
Smith-Waterman score: 422; 35.3% identity (66.5% similar) in 272 aa overlap (5-259:130-393)

                                                  10          20          30
query                                          AQSVPWGISRV--QAPAAHNRGL---TGSGVKVAVLDTGIST-H
                                                 :::.. .  :. :. .      .:. . . :.::::.. :
notag| AYVGSFDDATIEEIRNHKDVAHVEEDQVWYLDALTTQSDAPWGLGAISHQGDASSDYIYDTSAGADTYAYVVDTGINVDH
      90       100       110       120       130       140       150       160

       40        50        60        70        80        90        100       110
query  PDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVL-GASGSGAISSIAQGLEWAGNN--
        ... :.. ..  .  .  :. :::::::::::..      ..::. .:.: .:::. : :.: .:  : .: .::.:.
notag| SEFDGRASLAYNAAGGQHVDSVGHGTHVAGTIGG-----KTFGVSKKANLLSVKVFEGESSSTSI--ILDGYNWAANDIV
     170       180       190       200            210       220         230       240

              120       130       140       150       160       170       180
query  -----GMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGA-GSISYPARYANAMAVGATDQNNNRASFSQYGAG
           :  . :::::. . : .. .::.:: ..::: :.:.:: .. .: . ::   ::..:.:. . : :::::.::
notag| SKSRTGKSAINLSLGG-GYSYAFSNAVESAFDEGVLSVVAAGNENVDASNTSPASAPNALTVAASTERNARASFSNYGEV
            250       260       270       280       290       300       310       320

     190       200       210         220       230       240       250       260
query  LDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAA
       .:: ::: .. :.. :.. :.  ..:::::::::..: .  .   .  :  .. ...:. ::. . ... ::
notag| VDIFAPGEDILSAWIGGNSATNTISGTSMATPHIVGLSLYLIALEGLSSPGDVTSRIKELATQGALSGVSGSPNALAYNG
             330       340       350       360       370       380       390       400


query  TR

notag| AE


>>gi|74665726|gid|317|Allergen Asp fl 1                                  (403 aa)
 initn: 228 init1: 164 opt: 421  Z-score: 508.9  bits: 102.5 E(2171): 3.4e-023
Smith-Waterman score: 421; 34.1% identity (66.7% similar) in 279 aa overlap (2-256:127-396)
```

```
                                                    10        20             30
query                                            AQSVPWGISRVQAPAAHNRGL-----TGSGVKVAVLDTGIS
                                                 .:.:::.. ..  . ..        .: :. . :.:.:..
notag| KFAAYAGSFDDATIEEIRKNEDVAYVEEDQIYYLDGLTTQKSAPWGLGSISHKGQQSTDYIYDTSAGEGTYAYVVDSGVN
         90       100       110       120       130       140       150       160

          40        50        60        70        80        90        100       110
query  T-HPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVL-GASGSGAISSIAQGLEWAGN
       . : ... :.. ..  .  .  :. :::::::::::.      . :.: .: . .::::. : :.: ..  : .:..::.:
notag| VDHEEFEGRASKAYNAAGGQHVDSIGHGTHVSGTIAG-----KTYGIAKKASILSVKVFQGESSSTSV--ILDGFNWAAN
        170       180       190       200            210       220         230

                 120       130       140       150         160       170       180
query  NGMH-------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGN--SGAGSISYPARYANAMAVGATDQNNNRASFSQ
       . .        . :.:::. . : ....::..:  .::: :.:.::  : ::. : ::   .:..:.: ...:::::::.
notag| DIVSKKRTSKAAINMSLGG-GYSKAFNDAVENAFEQGVLSVVAAGNENSDAGQTS-PASAPDAITVAAIQKSNNRASFSN
     240       250       260       270       280       290       300       310

         190       200       210         220           230       240       250
query  YGAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGA----AALVKQKNPSWSNVQIRN-HLKNTATSL-GSTNLY
       .:  .:. ::: .. :.. ::. :.  ..::::::::..:     ::: .  .:.  . .:..   :... .. :: ::
notag| FGKVVDVFAPGQDILSAWIGSSSATNTISGTSMATPHIVGLSLYLAALENLDGPAAVTKRIEELATKDVVKDVKGSPNLL
       320       330       340       350       360       370       380       390

       260
query  GSGLVNAEAATR

notag| AYNGNA
       400
```

>>gi|54654335|gid|833|vacuolar serine protease [Rhodotorula mucilaginosa] (342 aa)

```
 initn: 534 init1: 176 opt: 414  Z-score: 501.8  bits: 100.9 E(2171): 8.4e-023
Smith-Waterman score: 510; 39.0% identity (67.6% similar) in 259 aa overlap (1-234:35-286)

                                                  10             20             30
query                                          AQSVPWGISRV--QAP---AAHNRGLT----GSGVKVAVLDTGIST
                                               :...:::..:.  . :   .. .. :     :.::   :.:::...
notag| TMELLEDLIEQVRQLPMVNFIEKNSLVHANEFTVAKGAPWGLARISHRDPLSLGSFDQYLYDSNGGTGVTSYVIDTGVNV
              10        20        30        40        50        60        70        80

        40           50        60        70        80        90       100       110
query  HPDLNIRGGASF---VPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA--
       : . ...: :..   .:     .::::::::: ::::..     .. ::: .::. ::::: ..:::..:.. .:.:.:
notag| HHE-QFEGRAKWGKTIPQGDEDEDGNGHGTHCAGTIGS-----NAYGVAKNAEIVAVKVLRSNGSGSMSDVIKGVEFAVK
               90       100       110            120       130       140       150

                   120       130       140       150         160       170       180
query  --------GNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI-SYPARYANAMAVGATDQNNNRASF
               :.:.. .::.:::. . : .:. :::.:.. :.  ..:.:: .  .  . ::   ::..:::.  .. :: :
notag| SHQDSVKKGKNSFSTANMSLGG-GKSPALDLAVNAAVKAGLHFAVAAGNENQDACNTSPASAENAITVGASTISDARAYF
          160       170       180       190       200       210       220       230

           190       200       210         220       230       240       250       260
query  SQYGAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGL
       :.::  .:: :::.:. ::: ::  :.  :.:::::.::.::  .   . .::
notag| SNYGKCVDIFAPGLNILSTYIGSDAATAYLSGTSMASPHIAGLLTYYLSLQPSSDSEFFIGAEGITPAQLKKNLIAFGTP
           240       250       260       270       280       290       300       310


query  VNAEAATR

notag| DVLADIPADTPNILAFNGAGQNLTKFWGH
           320       330       340
```

>>gi|5813790|gid|313|Tri r 2 allergen [Trichophyton rubrum] (412 aa)

```
 initn: 441 init1: 228 opt: 390  Z-score: 470.5  bits: 95.4 E(2171): 4.6e-021
Smith-Waterman score: 474; 35.9% identity (65.2% similar) in 276 aa overlap (3-252:132-398)

                                                    10          20           30
query                                            AQSVP-WGISRV--QAPAAHNRGL---TGSGVKVAVLDTGIS
                                                   .:: ::..::  . :.. .      .:.:: . ..::::.
notag| SGEFDDAMIKDISSHDDVDFIEPDFVVRTTTNGTNLTHQDNVPSWGLARVGSKKPGGTTYYYDPSAGKGVTAYIIDTGID
```

```
            100       110       120       130       140       150       160       170

          40          50        60        70        80        90       100       110
query  T-HPDLNIRG--GASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA-
         : :.. :.  : .::  . .. : ::::::::::...        :.: .. : :::::  .:::. :.. .:.:::
notag| IDHEDFQGRAKWGENFV--DQQNTDCNGHGTHVAGTVGGTK-----YGLAKGVSLVAVKVLDCDGSGSNSGVIKGMEWAM
            180         190       200       210            220       230       240

                      120       130       140       150       160       170       180
query  -----GNNGM------HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI-SYPARYANAMAVGATDQNNNR
           :.::        : :.::::.:   :. .::... .. :.....:.:: .  .  : ::   .. .:.:. .....
notag| RQASGGGNGTAKAAGKSVMNMSLGGPRSEAS-NQAAKAISDAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTKDDGK
          250       260       270       280       290       300       310       320

              190       200       210       220           230       240       250
query  ASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAA----LVKQKNPSWSNVQIRNHLKNTATSLGSTNL
       :.::.::: .:. ::: .. :  ::..  .:.::::::.::: : .:    : :: .:.  .. :..  ...  : :
notag| ADFSNYGAVVDVYAPGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDT-IKKMANDVIQSPGEGTT
           330       340       350       360       370       380       390       400

       260
query  YGSGLVNAEAATR

notag| GKLIYNGSGK
            410
```

>>gi|23894244|gid|313|tri m 2 allergen [Arthroderma benhamiae] (404 aa)

```
 initn: 455 init1: 228 opt: 380  Z-score: 458.4  bits: 93.1 E(2171): 2.2e-020
Smith-Waterman score: 477; 37.7% identity (64.6% similar) in 257 aa overlap (2-233:129-377)

                                                    10            20        30
query                                            AQSVP-WGISRVQAPAA-----HNRGLTGSGVKVAVLDTGI
                                                  ..:: ::..:: .  :     .  . .:.:: . :.::::
notag| YSGEFDDAMIKDISNHDDVDYIEPDFVVRTSTNGTNLTRQENVPSWGLARVGSKQAGGTTYYYDSSAGKGVTAYVIDTGI
       90       100       110       120       130       140       150       160

           40          50        60        70        80        90       100       110
query  ST-HPDLNIRG--GASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA
       .  : :.. :.  : .::  .   .: ::::::::::...        :.: :. : :::::  .:::. :.. .:.:::
notag| DIEHEDFGGRAKWGKNFV--DQRDEDCNGHGTHVAGTVGGTK-----YGLAKSVSLVAVKVLDCDGSGSNSGVIRGMEWA
      170       180         190       200           210       220       230       240

                      120       130       140       150       160       170       180
query  -----GNNGM------HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI-SYPARYANAMAVGATDQNNNR
           :.::        : :.::::.:  .:. ..:. . .  :.....:.:: .  .  : ::   .. .:.:. .....
notag| MREASGGNGTAKAAGKSVMNMSLGGPRSQAS-NDAARAISEAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTEDDGK
            250       260       270       280       290       300       310       320

              190       200       210       220           230       240       250
query  ASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAA----LVKQKNPSWSNVQIRNHLKNTATSLGSTNL
       : ::.::: .:. ::: .. :  ::..  .:.::::::.::: : .:    : :: .:
notag| AEFSNYGAVVDVYAPGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDTIKQMANEAIQRPGEGTTG
            330       340       350       360       370       380       390       400

       260
query  YGSGLVNAEAATR

notag| KLIY
```

>>gi|23894240|gid|313|tri m 2 allergen [Arthroderma benhamiae] (292 aa)

```
 initn: 443 init1: 228 opt: 376  Z-score: 456.4  bits: 92.3 E(2171): 2.8e-020
Smith-Waterman score: 471; 36.8% identity (65.5% similar) in 258 aa overlap (2-233:27-276)

                                     10            20        30        40
query                             AQSVP-WGISRVQAPAA-----HNRGLTGSGVKVAVLDTGIS-THPDLNIRG--G
                                  ..:: ::..:: .  :     .  . .:.:: . ..:::. .: :.. :.  :
notag| NHDDVDYIEPDFVVRTSTNGTNLTRQENVPSWGLARVGSKKAGGTTYYYDSSAGKGVTAYIIDTGIDINHEDFGGRAKWG
              10        20        30        40        50        60        70        80

         50        60        70        80        90       100       110
query  ASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA------GNNGMH--
        .::  .   .: ::::::::::...        :.: .. : :::::  .:::. :.. .:.:::      :.::
```

```
notag| KNFV--DKMDEDCNGHGTHVAGTVGGTK-----YGLAKGVTLVAVKVLDCDGSGSNSGVIEGMEWAMREASGGGNGTAKA
                90       100            110       120       130       140       150

          120       130       140       150       160       170       180       190
query  ----VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI-SYPARYANAMAVGATDQNNNRASFSQYGAGLDIV
           : :.::::.:  .:. ..:... .. :.....:.:: .  .  : ::   .. .:.:. .....: ::.::: .:.
notag| AGKAVMNMSLGGPRSQAS-NDAAKAISDAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTEDDGKAEFSNYGAVVDVY
           160       170       180       190       200       210       220       230

           200       210       220           230       240       250       260
query  APGVNVQSTYPGSTYASLNGTSMATPHVAGAAA----LVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       ::: .. :  ::..  .:.::::::::: : .:    : :: .:
notag| APGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDTIKEMANEAIQR
            240       250       260       270       280       290
```

>>gi|12005501|gid|243|vacuolar serine protease [Penicillium citrinum] (358 aa)

```
 initn: 394 init1: 179 opt: 377  Z-score: 455.8  bits: 92.5 E(2171): 3.1e-020
Smith-Waterman score: 536; 41.3% identity (68.0% similar) in 259 aa overlap (2-234:7-259)

                  10            20            30        40        50        60
query       AQSVPWGISRVQ-----APAAHNRGLT----GSGVKVAVLDTGIST-HPDLNIRGG-ASFVPGEPSTQDGNGHGT
             ...::::.:..     . .. :. :     : :: . :.::: .: : :.. :.. .. .:      :::::::
notag| DSPSVEKNAPWGLARISHRDSLSFGTFNKYLYAEDGGEGVDAYVIDTGTNTDHVDFEGRANWGKTIPEGDEDVDGNGHGT
              10        20        30        40        50        60        70        80

           70        80        90       100       110                  120       130
query  HVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA----------GNNGMH--VANLSLGSPSPSAT
       : .:::::        ::: .:..:::::: ..::::..:...:.:::          :..:..  :::.:::. : : :
notag| HCSGTIAGKK-----YGVAKKANVYAVKVLRSNGSGTMSDVVKGVEWAAEAHIKKAKAGKKGFKGSVANMSLGGGS-SRT
              90            100       110       120       130       140       150

            140       150       160       170       180       190       200
query  LEQAVNSATSRGVLVVAASGNSGAGSISY-PARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYA--
       :. :::.:.. :.  ..:.::..: . .: ::   ::..:::.   ..:: ::.::   :: :::.:. ::. :: ::
notag| LDLAVNAAVDAGIHFAVAAGNDNADACNYSPAAAENAVTVGASTLADERAYFSNYGKCTDIFAPGLNILSTWIGSKYAVN
           160       170       180       190       200       210       220       230

     210       220       230       240       250       260
query  SLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
       ...::::::.::.::  :   . .::
notag| TISGTSMASPHIAGLLAYYVSLQPSDDSAFAVEKITPKKLKEALITVATSGALTDIPSDTPNLLAWNGGGSSNYTDIVAQ
           240       250       260       270       280       290       300       310
```

>>gi|74663809|gid|313|RecName: Full=Subtilisin-like protease 6; AltName: (405 aa)

```
 initn: 448 init1: 228 opt: 371  Z-score: 447.2  bits: 91.1 E(2171): 9.1e-020
Smith-Waterman score: 475; 37.6% identity (64.3% similar) in 258 aa overlap (2-233:129-378)

                                                  10            20        30
query                                          AQSVP-WGISRVQAPAA-----HNRGLTGSGVKVAVLDTGI
                                                ..:: ::..:: .  :     .  . .:.:: . :.:::::
notag| YSGEFDDAMIKDISNHDDVDYIEPDFVVRTSTNGTNLTRQENVPSWGLARVGSKQAGGTTYYYDSSAGKGVTAYVIDTGI
       90       100       110       120       130       140       150       160

           40          50        60        70        80        90       100       110
query  ST-HPDLNIRG--GASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWA
       .  : :.. :.  : .::  .   .: ::::::::::...        :.: :. : :::::  .:::. :.. .:.:::
notag| DIEHEDFGGRAKWGKNFV--DQRDEDCNGHGTHVAGTVGGTK-----YGLAKSVSLVAVKVLDCDGSGSNSGVIRGMEWA
      170       180         190       200            210       220       230       240

                       120       130       140       150       160       170
query  ------GNNGM------HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI-SYPARYANAMAVGATDQNNN
             :.::        : :.::::.:  .:. ..:. . .  :.....:.:: .  .  : ::   .. .:.:. ....
notag| MREASGGGNGTAKAAGKSVMNMSLGGPRSQAS-NDAARAISEAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTEDDG
             250       260       270       280       290       300       310       320

     180       190       200       210       220           230       240       250
query  RASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAA----LVKQKNPSWSNVQIRNHLKNTATSLGSTN
       .: ::.::: .:. ::: .. :  ::..  .:.:::::.::: : .:    : :: .:
notag| KAEFSNYGAVVDVYAPGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDTIKQMANEAIQRPGEGTT
              330       340       350       360       370       380       390       400

        260
query  LYGSGLVNAEAATR
```

```
notag| GKLIY
```

>>gi|14215732|gid|243|vacuolar serine protease [Penicillium chrysogenum] (494 aa)

```
 initn: 534 init1: 169 opt: 352  Z-score: 422.1  bits: 86.7 E(2171): 2.3e-018
Smith-Waterman score: 516; 39.0% identity (67.6% similar) in 259 aa overlap (2-234:143-395)

                                                  10            20            30
query                                          AQSVPWGISRVQ-----APAAHNRGLT----GSGVKVAVLD
                                                ...::::.:..     . .  :. :     : :: . :.:
notag| LLGYAGHFHEDVIEQIRRHPDVDYIEKDSEVRTMSEGSVEKNAPWGLARISHRESLSFGNFNKYLYAEEGGEGVDAYVID
            110       120       130       140       150       160       170       180

               40         50        60        70        80        90       100       110
query  TGIST-HPDLNIRGG-ASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLE
       :: .. : :.. :.. .. .:   . .::::::::: .::::.       .::: .:..:::::: ..::::.:....:.:
notag| TGANVKHVDFEGRANWGKTIPQGDADEDGNGHGTHCSGTIAGKK-----FGVAKKANVYAVKVLRSNGSGTMSDVVKGVE
            190       200       210       220            230       240       250

                        120       130       140       150       160       170
query  WAGNNGMH------------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISY-PARYANAMAVGATDQN
       ::..  ..             :::.:::. : : ::. :::.:.. :.  ..:.::..: . .: ::   .:..:::.
notag| WAAEAHIKKSKKGDKKFKGSVANMSLGGGS-SRTLDLAVNAAVDAGIHFAVAAGNDNADACNYSPAAAEKAITVGASTLA
       260       270       280       290       300       310       320       330

       180       190       200       210         220       230       240       250
query  NNRASFSQYGAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTN
       ..:: ::.::   :: :::.:. ::. :: .:.  ..::::::.::.::  :   .  :.
notag| DERAYFSNYGKCTDIFAPGLNILSTWVGSDHATNTISGTSMASPHIAGLLAYYVSLAPAKDSAYAVADVTPKQLKAALIS
        340       350       360       370       380       390       400       410

        260
query  LYGSGLVNAEAATR

notag| VATEGTLTDIPSDTPNLLAWNGGGSANYTKILADGGYKAHNAETTVEDRIGGIIDSAEKAFHKELGAIYSEIKDAVSA
        420       430       440       450       460       470       480       490
```

>>gi|7963902|gid|243|allergen Pen n 18 [Penicillium chrysogenum] (494 aa)

```
 initn: 534 init1: 169 opt: 352  Z-score: 422.1  bits: 86.7 E(2171): 2.3e-018
Smith-Waterman score: 516; 39.0% identity (67.6% similar) in 259 aa overlap (2-234:143-395)

                                                  10            20            30
query                                          AQSVPWGISRVQ-----APAAHNRGLT----GSGVKVAVLD
                                                ...::::.:..     . .  :. :     : :: . :.:
notag| LLGYAGHFHEDVIEQIRRHPDVDYIEKDSEVRTMSEGSVEKNAPWGLARISHRESLSFGNFNKYLYAEEGGEGVDAYVID
            110       120       130       140       150       160       170       180

               40         50        60        70        80        90       100       110
query  TGIST-HPDLNIRGG-ASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLE
       :: .. : :.. :.. .. .:   . .::::::::: .::::.       .::: .:..:::::: ..::::.:....:.:
notag| TGANVKHVDFEGRANWGKTIPQGDADEDGNGHGTHCSGTIAGKK-----FGVAKKANVYAVKVLRSNGSGTMSDVVKGVE
            190       200       210       220            230       240       250

                        120       130       140       150       160       170
query  WAGNNGMH------------VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISY-PARYANAMAVGATDQN
       ::..  ..             :::.:::. : : ::. :::.:.. :.  ..:.::..: . .: ::   .:..:::.
notag| WAAEAHIKKSKKGDKKFKGSVANMSLGGGS-SRTLDLAVNAAVDAGIHFAVAAGNDNADACNYSPAAAEKAITVGASTLA
       260       270       280       290       300       310       320       330

       180       190       200       210         220       230       240       250
query  NNRASFSQYGAGLDIVAPGVNVQSTYPGSTYAS--LNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTN
       ..:: ::.::   :: :::.:. ::. :: .:.  ..::::::.::.::  :   .  :.
notag| DERAYFSNYGKCTDIFAPGLNILSTWVGSDHATNTISGTSMASPHIAGLLAYYVSLAPAKDSAYAVADVTPKQLKAALIS
        340       350       360       370       380       390       400       410

        260
query  LYGSGLVNAEAATR

notag| VATEGTLTDIPSDTPNLLAWNGGGSANYTKILADGGYKAHNAETTVEDRIGIIIDSAEKAFHKELGAIYSEIKDAVSV
        420       430       440       450       460       470       480       490
```

>>>///

```
269 residues in 1 query   sequences
517010 residues in 2171 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Fri Jun 05 02:47:29 2020 done: Fri Jun 05 02:47:30 2020
 Scan time:  0.000 Display time:  1.000

Function used was FASTA [36.3.8g Oct, 2018]
```

Appendix B. Allergen Online 80mmer Sliding Window Search Results

80mer Sliding Window Search Results

Database	AllergenOnline Database v20 (February 10, 2020)
Input Query	>query AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGN GHGTHVAGTIAALDNSIGVLGVAPSAELYAVKVLGASGSGAISSIAQGLEWAGNNGMHVA NLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNR ASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQI RNHLKNTATSLGSTNLYGSGLVNAEAATR
Length	269
Number of 80 mers	190
Number of Sequences with hits	28

Hit #	Defline	Species	Best %ID	# Hits > 35%	Full Alignment			Links	
					E-val	%ID	length	NCBI	Details
1	gi|267048|gid|1174|RecName: Full=Subtilisin Savinase; Alt	Bacillus lentus	100.00%	190of190	1e-111	98.90%	269	gi|267048	GO!
2	gi|1225905|gid|1175|prepro AprM [Bacillus sp.]	Bacillus sp.	77.52%	190of190	2e-073	64.70%	269	gi|1225905	GO!
3	gi|135016|gid|1171|RecName: Full=Subtilisin Carlsberg; Fl	Bacillus licheniformis	75.30%	190of190	3.3e-069	61.70%	274	gi|135016	GO!
4	gi|11127680|gid|1171|subtilisin precursor [Bacillus liche	Bacillus licheniformis	75.30%	190of190	8.2e-067	60.80%	268	gi|11127680	GO!
5	gi|148361511|gid|1376|vacuolar serine protease [Cladospor	Cladosporium cladosporioides	53.80%	150of190	2e-016	38.40%	263	gi|148361511	GO!
6	gi|60116876|gid|1227|vacuolar serine protease [Davidiella	Davidiella tassiana	53.80%	148of190	8.4e-016	38.40%	263	gi|60116876	GO!
7	gi|193507493|gid|2241|subtilisin-like serine protease [Cu	Cochliobolus lunatus	50.00%	174of190	1.1e-015	38.80%	260	gi|193507493	GO!
8	gi|2295|gid|2227|uncleaved alkaline protease (ALP) [Asper	Aspergillus fumigatus	50.00%	140of190	2.3e-024	36.20%	276	gi|2295	GO!
9	gi|12005501|gid|243|vacuolar serine protease [Penicillium	Penicillium citrinum	50.00%	180of190	3.1e-020	41.30%	259	gi|12005501	GO!
10	gi|3549630|gid|2227|alkaline protease, partial [Aspergill	Aspergillus fumigatus	50.00%	138of190	1.9e-024	36.20%	276	gi|3549630	GO!
11	gi|54654335|gid|833|vacuolar serine protease [Rhodotorula	Rhodotorula mucilaginosa	49.97%	173of190	8.4e-023	39.00%	259	gi|54654335	GO!
12	gi|74665726|gid|317|Allergen Asp fl 1	Aspergillus flavus	48.80%	106of190	3.4e-023	34.10%	279	gi|74665726	GO!
13	gi|129235|gid|317|Oryzin precursor (Alkaline proteinase)	Aspergillus oryzae	48.80%	114of190	2.1e-023	34.10%	279	gi|129235	GO!
14	gi|289172|gid|318|serine protease	Aspergillus niger	48.70%	171of190	1.4e-015	39.50%	263	gi|289172	GO!
15	gi|4588118|gid|243|alkaline serine protease Pen c2 [Penic	Penicillium citrinum	48.70%	176of190	3e-015	39.50%	263	gi|4588118	GO!
16	gi|739057410|gid|2857|vacuolar serine protease, partial [	Fusarium proliferatum	48.70%	148of190	4e-015	36.90%	263	gi|739057410	GO!
17	gi|12005497|gid|243|vacuolar serine protease [Penicillium	Penicillium oxalicum	48.70%	173of190	6.3e-015	38.80%	263	gi|12005497	GO!
18	gi|2143220|gid|318|cellular serine proteinase [Aspergillu	Aspergillus fumigatus	47.60%	172of190	1.6e-014	39.50%	263	gi|2143220	GO!
19	gi|4587983|gid|244|Pen c 1; alkaline serine protease [Pen	Penicillium citrinum	47.53%	142of190	3.5e-027	36.50%	282	gi|4587983	GO!
20	gi|6684758|gid|244|allergen Pen n 13 [Penicillium chrysog	Penicillium chrysogenum	47.53%	145of190	3e-027	36.90%	282	gi|6684758	GO!
21	gi|7963902|gid|243|allergen Pen n 18 [Penicillium chrysog	Penicillium chrysogenum	47.50%	174of190	2.3e-018	39.00%	259	gi|7963902	GO!
22	gi|14215732|gid|243|vacuolar serine protease [Penicillium	Penicillium chrysogenum	47.50%	174of190	2.3e-018	39.00%	259	gi|14215732	GO!
23	gi|294441150|gid|2457|extracellular alkaline serine prote	Aspergillus versicolor	47.50%	160of190	2.9e-023	35.30%	272	gi|294441150	GO!
24	gi|23894244|gid|313|tri m 2 allergen [Arthroderma benhami	Arthroderma benhamiae	45.70%	131of190	2.2e-020	37.70%	257	gi|23894244	GO!
25	gi|74663809|gid|313|RecName: Full=Subtilisin-like proteas	Trichophyton schoenleinii	45.70%	130of190	9.1e-020	37.60%	258	gi|74663809	GO!
26	gi|21069093|gid|244|alkaline serine protease [Penicillium	Penicillium chrysogenum	45.00%	106of190	8.9e-027	37.90%	235	gi|21069093	GO!
27	gi|5813790|gid|313|Tri r 2 allergen [Trichophyton rubrum]	Trichophyton rubrum	43.90%	132of190	4.6e-021	35.90%	276	gi|5813790	GO!
28	gi|23894240|gid|313|tri m 2 allergen [Arthroderma benhami	Arthroderma benhamiae	43.40%	120of190	2.8e-020	36.80%	258	gi|23894240	GO!

AllergenOnline Database v20 (February 10, 2020)

Appendix C. Allergen Online 8 Amino Acid Exact Match Search Results

>query

number of 8mer = 262

Number of Sequences with at least one 8mer match = 28

GI: **267048** Hits: **245** -- Def: **RecName: Full=Subtilisin Savinase; AltName: Full=Alkaline protease**

GI: **135016** Hits: **11** -- Def: **RecName: Full=Subtilisin Carlsberg; Flags: Precursor**

GI: **11127680** Hits: **11** -- Def: **subtilisin precursor [Bacillus licheniformis]**

GI: **1225905** Hits: **17** -- Def: **prepro AprM [Bacillus sp.]**

GI: **4588118** Hits: **1** -- Def: **alkaline serine protease Pen c2 [Penicillium citrinum]**

- 210 - DGNGHGTH

GI: **7963902** Hits: **1** -- Def: **allergen Pen n 18 [Penicillium chrysogenum]**

GI: **12005497** Hits: **1** -- Def: **vacuolar serine protease [Penicillium oxalicum]**

GI: **12005501** Hits: **1** -- Def: **vacuolar serine protease [Penicillium citrinum]**

GI: **14215732** Hits: **1** -- Def: **vacuolar serine protease [Penicillium chrysogenum]**

GI: **4587983** Hits: **1** -- Def: **Pen c 1; alkaline serine protease [Penicillium citrinum]**

GI: **6684758** Hits: **1** -- Def: **allergen Pen n 13 [Penicillium chrysogenum]**

GI: **21069093** Hits: **2** -- Def: **alkaline serine protease [Penicillium chrysogenum]**

GI: **289172** Hits: **1** -- Def: **serine protease**

GI: **2143220** Hits: **1** -- Def: **cellular serine proteinase [Aspergillus fumigatus]**

GI: **54654335** Hits: **1** -- Def: **vacuolar serine protease [Rhodotorula mucilaginosa]**

GI: **60116876** Hits: **1** -- Def: **vacuolar serine protease [Davidiella tassiana]**

GI: **148361511** Hits: **1** -- Def: **vacuolar serine protease [Cladosporium cladosporioides]**

GI: **193507493** Hits: **1** -- Def: **subtilisin-like serine protease [Curvularia lunata]**

GI: **739057410** Hits: **1** -- Def: **vacuolar serine protease, partial [Fusarium proliferatum]**

GI: **5813790** Hits: **3** -- Def: **Tri r 2 allergen [Trichophyton rubrum]**

GI: **23894240** Hits: **3** -- Def: **tri m 2 allergen [Arthroderma benhamiae]**

GI: **23894244** Hits: **3** -- Def: **tri m 2 allergen [Arthroderma benhamiae]**

GI: **74663809** Hits: **3** -- Def: **RecName: Full=Subtilisin-like protease 6; AltName: Allergen=Tri m 2; Flags: Precursor**

GI: **2295** Hits: **4** -- Def: **uncleaved alkaline protease (ALP) [Aspergillus fumigatus]**

GI: **3549630** Hits: **4** -- Def: **alkaline protease, partial [Aspergillus fumigatus]**

GI: **294441150** Hits: **4** -- Def: **extracellular alkaline serine protease [Aspergillus versicolor]**

GI: **129235** Hits: **1** -- Def: **Oryzin precursor (Alkaline proteinase) (ALP) (Aspergillus proteinase B) (Aspergillopeptidase B)**

GI: **74665726** Hits: **1** -- Def: **Allergen Asp fl 1**

Appendix D. Matches from the AllergenOnline Database in the WHO/IUIS Database

GI	GenBank	Full name	E-score	%ID	% similarity	WHO/IUIS search results[1]
267048	P29600	Full=Subtilisin Savinase; AltName: Full=Alkaline protease	1e-100	0.989	1.000	0
1225905	BAA05540	prepro AprM [Bacillus sp.]	2.9e-066	0.647	0.885	0
135016	P00780	Full=Subtilisin Carlsberg; Flags: Precursor	1.8e-062	0.617	0.869	0
11127680	AAG31026	subtilisin precursor, partial [Bacillus licheniformis]	2.6e-060	0.608	0.862	0
6684758	AAF23726	allergen Pen n 13 [Penicillium chrysogenum]	1.1e-024	0.369	0.660	Not listed as a Food allergen
4587983	AAD25926	Pen c 1 [Penicillium citrinum]	1.2e-024	0.365	0.663	0
21069093	AAM33821	alkaline serine protease [Penicillium chrysogenum]	2.9e-024	0.379	0.660	0
3549630	CAA75805	alkaline protease, partial [Aspergillus fumigatus]	3.8e-022	0.362	0.681	0
2295	CAA77666	uncleaved alkaline protease (ALP) [Aspergillus fumigatus]	4.3e-022	0.362	0.681	Not listed as a Food allergen
294441150	ADE74975	extracellular alkaline serine protease [Aspergillus versicolor]	4.2e-021	0.353	0.665	Not listed as a Food allergen
54654335	AAT37679	vacuolar serine protease, partial [Rhodotorula mucilaginosa]	1.1e-020	0.390	0.676	Not listed as a Food allergen
5813790	AAD52013	Tri r 2 allergen [Trichophyton rubrum]	4e-019	0.359	0.652	Not listed as a Food allergen
23894244	CAD23614	tri m 2 allergen, partial [Trichophyton benhamiae]	1.6e-018	0.377	0.646	0
23894240	CAD23613	tri m 2 allergen, partial [Trichophyton benhamiae]	2.2e-018	0.368	0.655	0
12005501	AAG44480	vacuolar serine protease, partial [Penicillium citrinum]	2.3e-018	0.413	0.680	0
74663809	Q8J077	RecName: Full=Subtilisin-like protease 6; AltName: Allergen=Tri m 2; Flags: Precursor	5.9e-018	0.376	0.643	0
7963902	AAF71379	allergen Pen n 18 [Penicillium chrysogenum]	1e-016	0.390	0.676	Not listed as a Food allergen

14215732	AAG44693	vacuolar serine protease [Penicillium chrysogenum]	1e-016	0.390	0.676	0
148361511	ABQ59329	vacuolar serine protease, partial [Cladosporium cladosporioides]	6.1e-015	0.384	0.681	Not listed as a Food allergen
60116876	AAX14379	vacuolar serine protease [*Cladosporium herbarum*]	2.1e-014	0.384	0.677	Not listed as a Food allergen
193507493	ACF19589	subtilisin-like serine protease [Curvularia lunata]	2.7e-014	0.388	0.669	Not listed as a Food allergen
289172	AAA32702	serine protease [Aspergillus niger]	3.3e-014	0.395	0.665	Not listed as a Food allergen
4588118	AAD25995	alkaline serine protease Pen c2 [*Penicillium citrinum*]	6.8e-014	0.395	0.662	0
739057410	AJA79001	vacuolar serine protease, partial [Fusarium proliferatum]	9.2e-014	0.369	0.665	Not listed as a Food allergen
12005497	AAG44478	vacuolar serine protease [Penicillium oxalicum]	1.3e-013	0.388	0.665	Not listed as a Food allergen
2143220	CAA73782	cellular serine proteinase [Aspergillus fumigatus]	3e-013	0.395	0.658	Not listed as a Food allergen
129235	P12547	RecName: Full=Alkaline protease 1; Short=ALP; AltName: Full=Aspergillopeptidase B; AltName: Full=Aspergillus proteinase B; AltName: Full=Elastase; AltName: Full=Elastinolytic serine proteinase; AltName: Full=Oryzin; Flags: Precursor	NA	48.80 %	NA	Not listed as a Food allergen
74665726	Q9UVU3	Allergen Asp fl 1	NA	48.80 %	NA	Not listed as a Food allergen

[1.] A screenshot from the search result is provided below when the result is not listed as a Food allergen. If the enzyme did not return any results, it was not listed in the WHO/IUIS Allergen nomenclature database.

allergen Pen n 13 [*Penicillium chrysogenum*], GenBank AAF23726
http://www.allergen.org/viewallergen.php?aid=484

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Fungi Ascomycota › Eurotiales › *Penicillium chrysogenum* › Pen ch 13

Allergen Details:

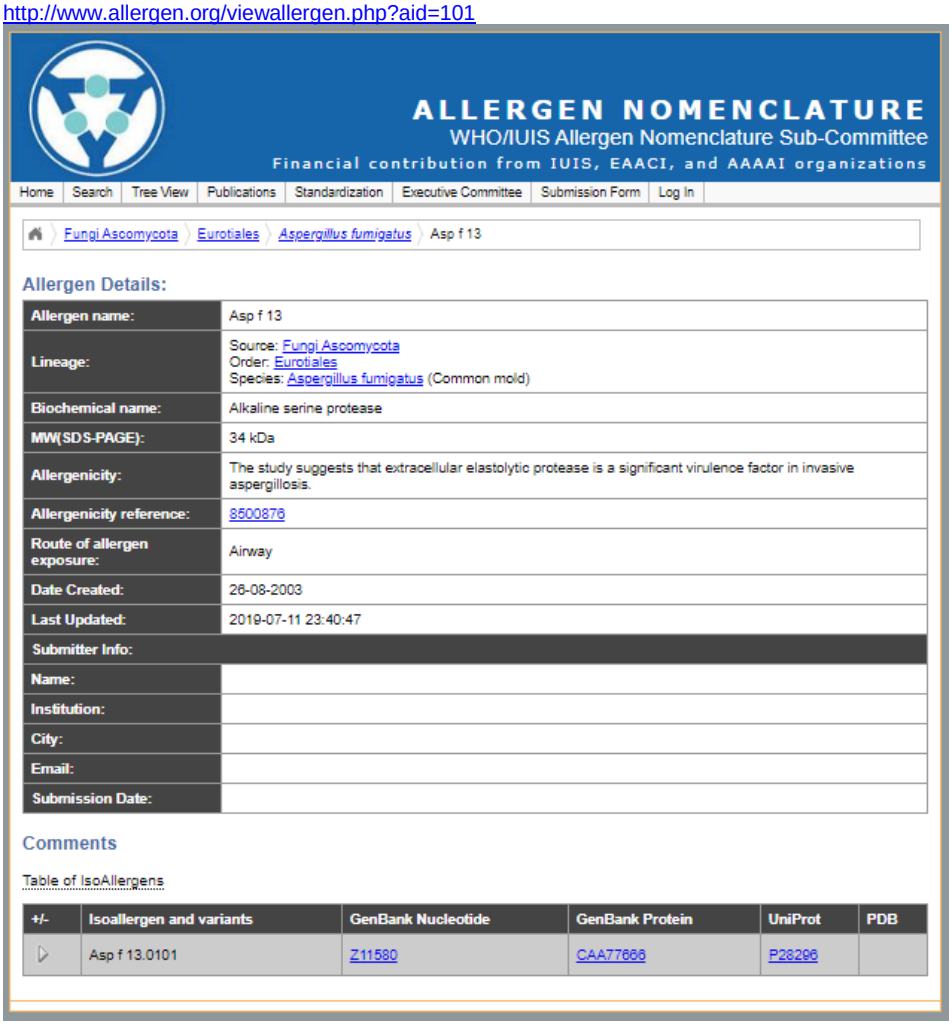
Allergen name:	Pen ch 13
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Penicillium chrysogenum
Biochemical name:	Alkaline serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	Among 70 sera from asthmatics patients, 17 had IgE reactivity towards P.chrysogenum on immunoblot. 15 patient sera (88%) showed IgE binding to 34 kD Pen ch 13 (identified by N-terminal sequence analysis) on immunoblot. - Basophils from 5 patients with a positive immunoblot for Pen ch 13, showed histamine release in response to Pen ch 13: medline 21890913
Allergenicity reference:	10231324
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-09-05 01:44:43
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Pen ch 13.0101	AF193420	AAF23726	Q9URR2	

Uncleaved alkaline protease (ALP) [*Aspergillus fumigatus*] GenBank: CAA77666
http://www.allergen.org/viewallergen.php?aid=101



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Fungi Ascomycota › Eurotiales › *Aspergillus fumigatus* › Asp f 13

Allergen Details:

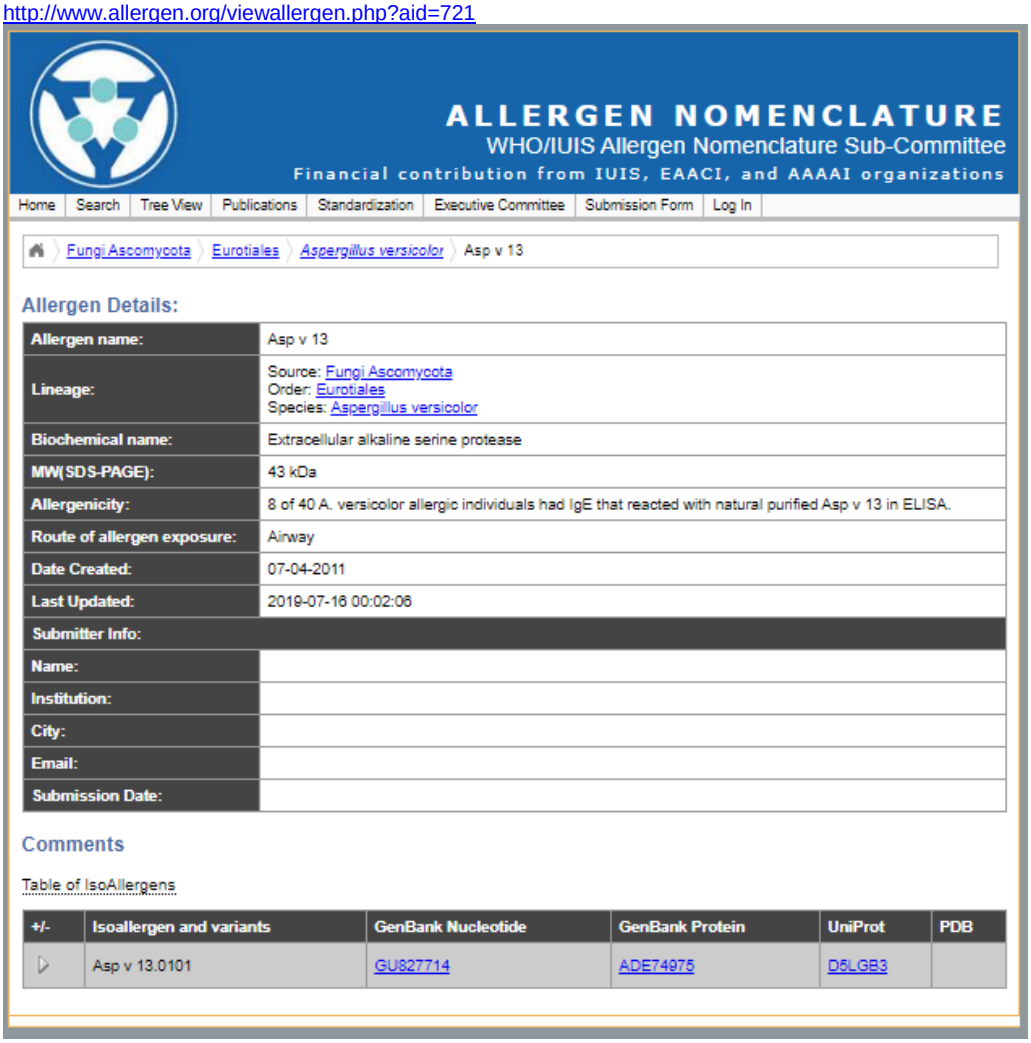
Allergen name:	Asp f 13
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus fumigatus (Common mold)
Biochemical name:	Alkaline serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	The study suggests that extracellular elastolytic protease is a significant virulence factor in invasive aspergillosis.
Allergenicity reference:	8500876
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-07-11 23:40:47
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp f 13.0101	Z11580	CAA77666	P28296	

Extracellular alkaline serine protease [*Aspergillus versicolor*], GenBank ADE74975
http://www.allergen.org/viewallergen.php?aid=721



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Fungi Ascomycota › Eurotiales › *Aspergillus versicolor* › Asp v 13

Allergen Details:

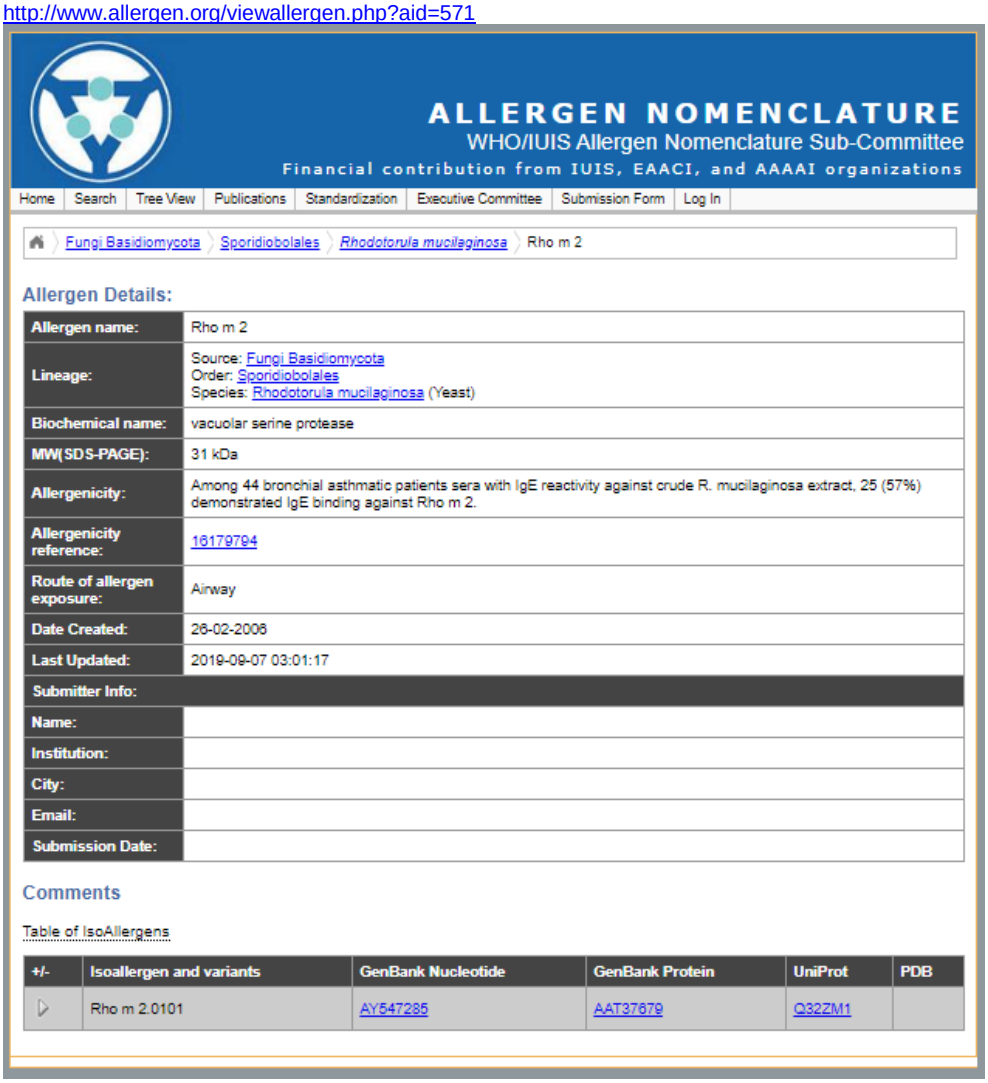
Allergen name:	Asp v 13
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus versicolor
Biochemical name:	Extracellular alkaline serine protease
MW(SDS-PAGE):	43 kDa
Allergenicity:	8 of 40 A. versicolor allergic individuals had IgE that reacted with natural purified Asp v 13 in ELISA.
Route of allergen exposure:	Airway
Date Created:	07-04-2011
Last Updated:	2019-07-16 00:02:06
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp v 13.0101	GU827714	ADE74975	D5LGB3	

vacuolar serine protease, partial [*Rhodotorula mucilaginosa*], GenBank AAT37679
http://www.allergen.org/viewallergen.php?aid=571



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Fungi Basidiomycota › Sporidiobolales › *Rhodotorula mucilaginosa* › Rho m 2

Allergen Details:

Allergen name:	Rho m 2
Lineage:	Source: Fungi Basidiomycota Order: Sporidiobolales Species: Rhodotorula mucilaginosa (Yeast)
Biochemical name:	vacuolar serine protease
MW(SDS-PAGE):	31 kDa
Allergenicity:	Among 44 bronchial asthmatic patients sera with IgE reactivity against crude R. mucilaginosa extract, 25 (57%) demonstrated IgE binding against Rho m 2.
Allergenicity reference:	16179794
Route of allergen exposure:	Airway
Date Created:	26-02-2006
Last Updated:	2019-09-07 03:01:17
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Rho m 2.0101	AY547285	AAT37679	Q32ZM1	

Tri r 2 allergen [*Trichophyton rubrum*], GenBank AAD52013
http://www.allergen.org/viewallergen.php?aid=627

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Fungi Ascomycota › Onygenales › *Trichophyton rubrum* › Tri r 2

Allergen Details:

Allergen name:	Tri r 2
Lineage:	Source: Fungi Ascomycota Order: Onygenales Species: Trichophyton rubrum (Athlete's foot fungus)
Biochemical name:	Putative secreted alkaline protease Alp1
MW(SDS-PAGE):	29 kDa
Allergenicity:	Specific IgE Ab were measured in 73 sera: the prevalence of IgE Ab was significantly higher among subjects with IH skin test reactions (43%) compared with those with DTH or negative skin test reactions (12%). Five of nine individuals with delayed reactions to the Trichophyton mixture showed a positive delayed type hypersensitivity reaction in response to rTri r 2 maximal at 24 h
Allergenicity reference:	9792655 15653817
Route of allergen exposure:	Contact
Date Created:	31-08-2003
Last Updated:	2019-09-27 08:34:32
Submitter Info:	
Name:	Judith A Woodfolk
Institution:	University of Virginia
City:	Charlottesville, VA
Email:	jaw4m@virginia.edu
Submission Date:	0000-00-00

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Tri r 2.0101	AF082515	AAD52013	Q9UW97	

Allergen Pen n 18 [*Penicillium chrysogenum*], GenBank: AAF71379
http://www.allergen.org/viewallergen.php?aid=485

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Fungi Ascomycota › Eurotiales › *Penicillium chrysogenum* › Pen ch 18

Allergen Details:

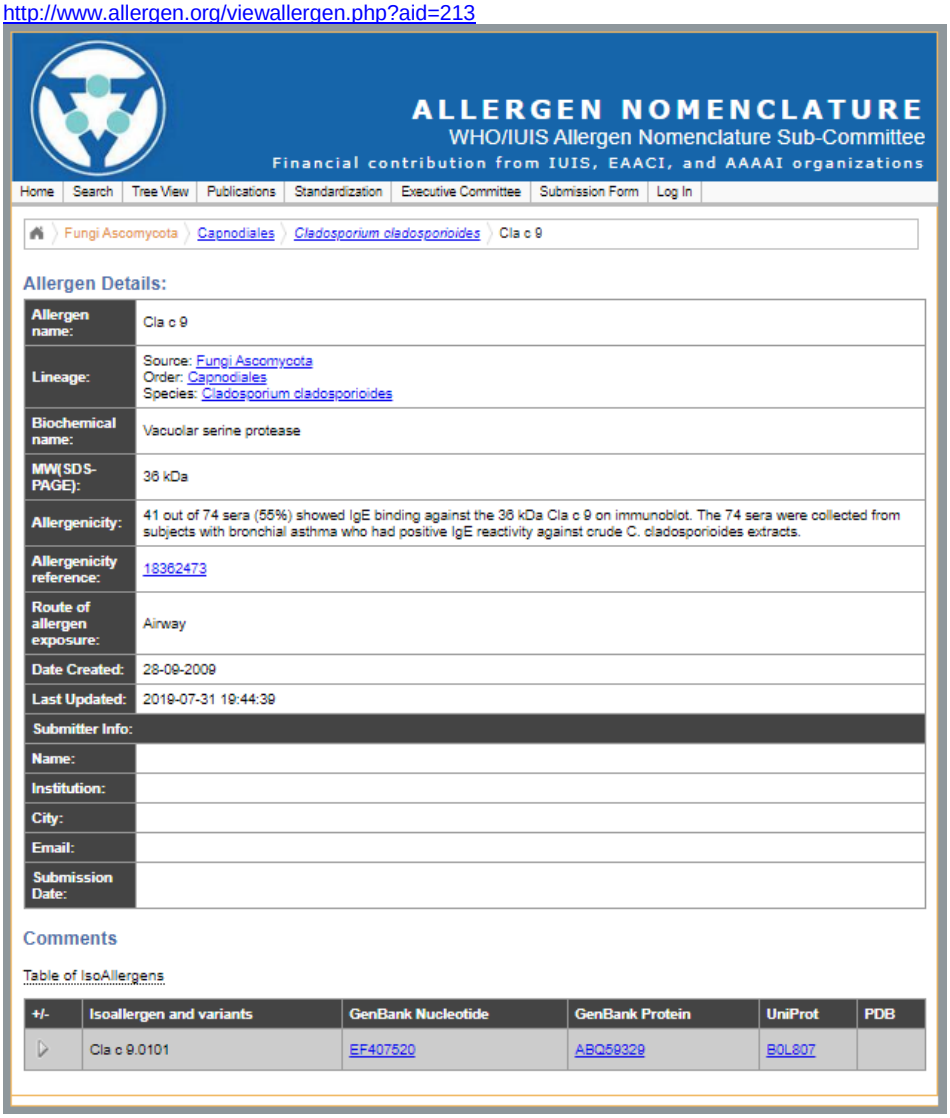
Allergen name:	Pen ch 18
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Penicillium chrysogenum
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	32 kDa
Allergenicity:	Among 17 P.chrysogenum-sensitized patients, 14 (82%) showed IgE binding to Pen ch 18 (identified by N-terminal sequence analysis) on immunoblot. - IgE epitope analysis: medline 21961432 (peptide C12, located in the N-terminal region of the molecule, was recognized by serum IgE in 75% of the patients tested)
Allergenicity reference:	10231324
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-09-05 01:45:16
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Pen ch 18.0101	AF264027	AAF71379	Q9P8G3	

Vacuolar serine protease, partial [*Cladosporium cladosporioides*], GenBank ABQ59329
http://www.allergen.org/viewallergen.php?aid=213



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Fungi Ascomycota > Capnodiales > *Cladosporium cladosporioides* > Cla c 9

Allergen Details:

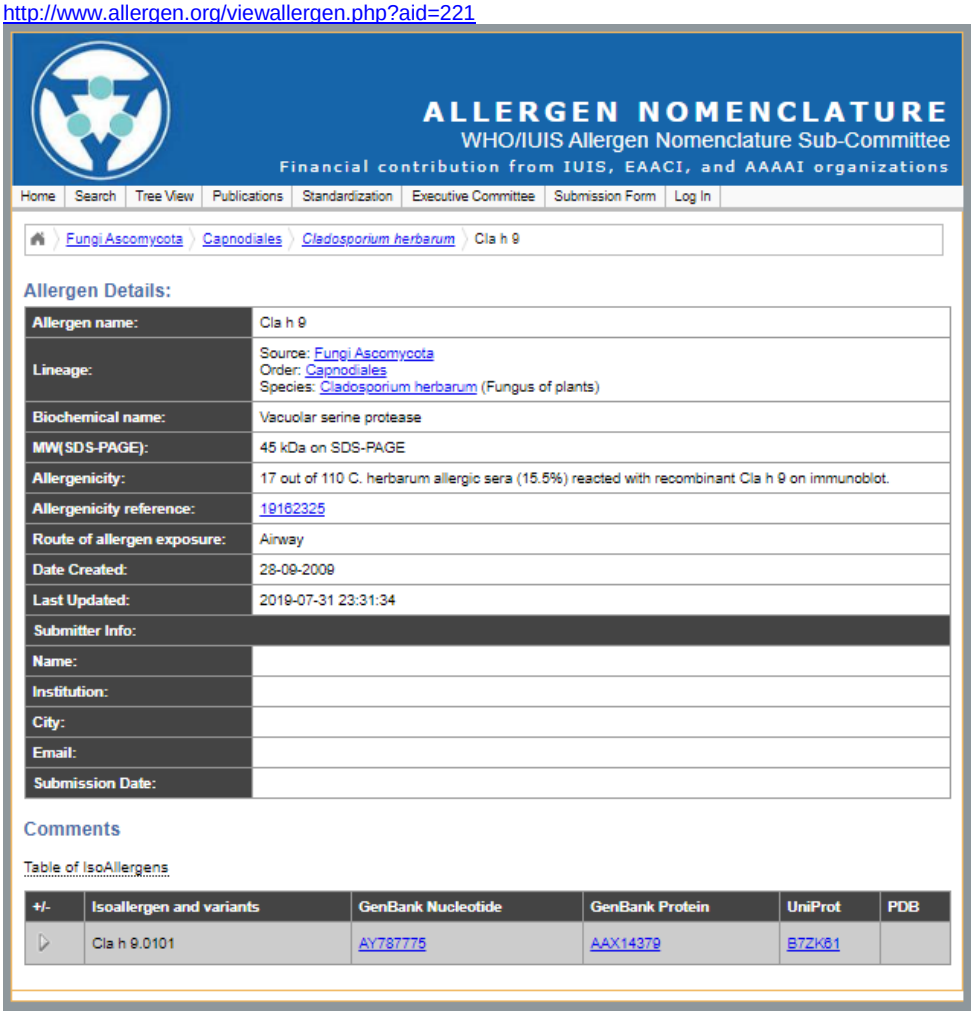
Allergen name:	Cla c 9
Lineage:	Source: Fungi Ascomycota Order: Capnodiales Species: Cladosporium cladosporioides
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	36 kDa
Allergenicity:	41 out of 74 sera (55%) showed IgE binding against the 36 kDa Cla c 9 on immunoblot. The 74 sera were collected from subjects with bronchial asthma who had positive IgE reactivity against crude C. cladosporioides extracts.
Allergenicity reference:	18362473
Route of allergen exposure:	Airway
Date Created:	28-09-2009
Last Updated:	2019-07-31 19:44:39
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Cla c 9.0101	EF407520	ABQ59329	B0L807	

Vacuolar serine protease [*Cladosporium herbarum*], GenBank AAX14379
http://www.allergen.org/viewallergen.php?aid=221



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Fungi Ascomycota › Capnodiales › *Cladosporium herbarum* › Cla h 9

Allergen Details:

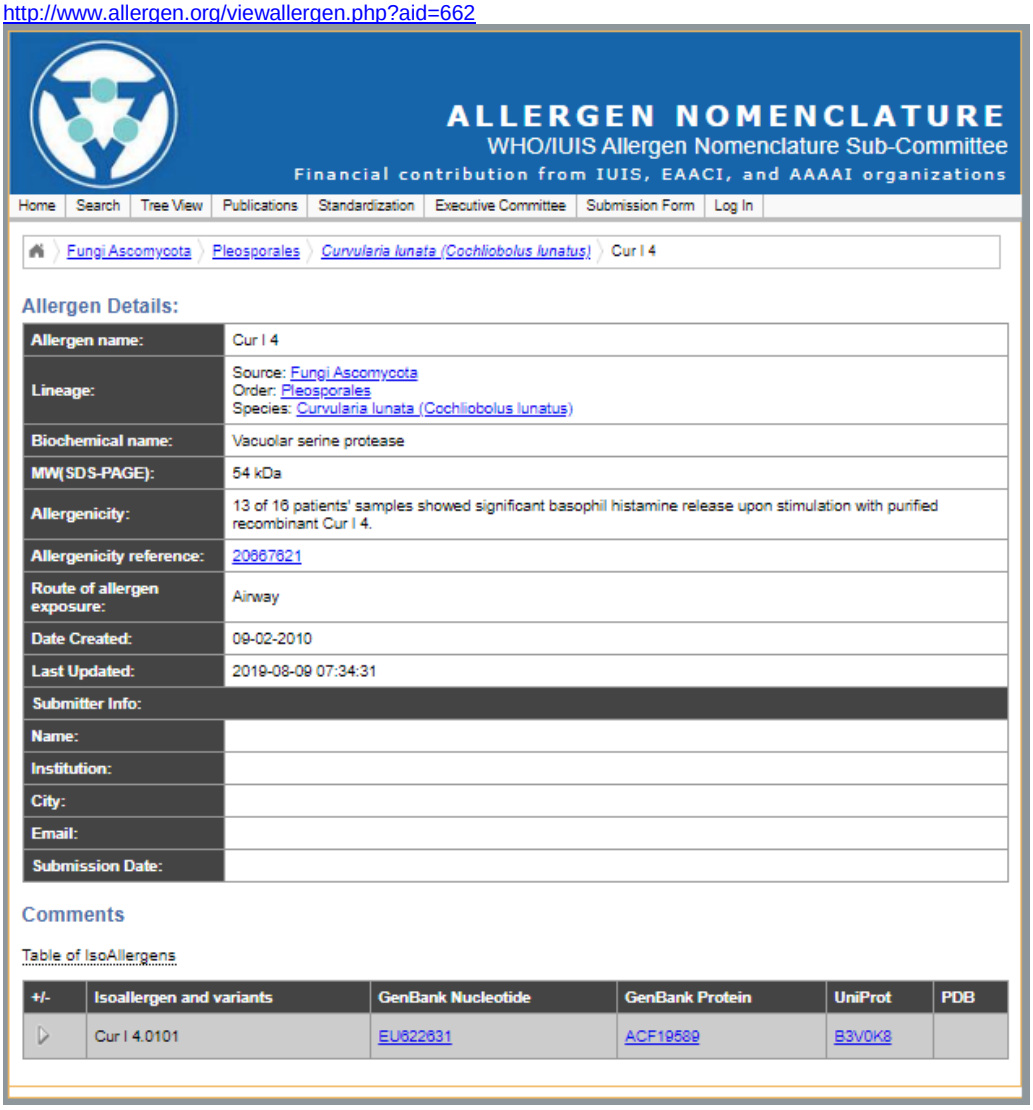
Allergen name:	Cla h 9
Lineage:	Source: Fungi Ascomycota Order: Capnodiales Species: Cladosporium herbarum (Fungus of plants)
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	45 kDa on SDS-PAGE
Allergenicity:	17 out of 110 C. herbarum allergic sera (15.5%) reacted with recombinant Cla h 9 on immunoblot.
Allergenicity reference:	19162325
Route of allergen exposure:	Airway
Date Created:	28-09-2009
Last Updated:	2019-07-31 23:31:34
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Cla h 9.0101	AY787775	AAX14379	B7ZK61	

Subtilisin-like serine protease [*Curvularia lunata*], GenBank ACF19589
http://www.allergen.org/viewallergen.php?aid=662



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Fungi Ascomycota › Pleosporales › *Curvularia lunata (Cochliobolus lunatus)* › Cur l 4

Allergen Details:

Allergen name:	Cur l 4
Lineage:	Source: Fungi Ascomycota Order: Pleosporales Species: Curvularia lunata (Cochliobolus lunatus)
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	54 kDa
Allergenicity:	13 of 16 patients' samples showed significant basophil histamine release upon stimulation with purified recombinant Cur l 4.
Allergenicity reference:	20667621
Route of allergen exposure:	Airway
Date Created:	09-02-2010
Last Updated:	2019-08-09 07:34:31
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Cur l 4.0101	EU622631	ACF19589	B3V0K8	

Serine protease [*Aspergillus niger*], GenBank: AAA32702
http://www.allergen.org/viewallergen.php?aid=122

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Fungi Ascomycota › Eurotiales › *Aspergillus niger* › Asp n 18

Allergen Details:

Allergen name:	Asp n 18
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus niger (Black mold)
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	34 kDa
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-07-15 23:27:36
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp n 18.0101				

Vacuolar serine protease, partial [*Fusarium proliferatum*], GenBank AJA79001
http://www.allergen.org/viewallergen.php?aid=799

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Fungi Ascomycota › Hypocreales › *Fusarium proliferatum* › Fus p 9

Allergen Details:

Allergen name:	Fus p 9
Lineage:	Source: Fungi Ascomycota Order: Hypocreales Species: Fusarium proliferatum
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	36.5 kDa
Allergenicity:	Nine of seventeen respiratory atopic patients from Taipei who had IgE binding to proteins of Fusarium proliferatum, had IgE binding to the Vacuolar serine protease. The crude extract used for testing was from a combination of hyphae and spores. Specific IgE binding was tested with recombinant protein produced by a cDNA clone.
Allergenicity reference:	27334782
Route of allergen exposure:	Airway
Date Created:	24-07-2014
Last Updated:	2019-08-17 03:01:32
Submitter Info:	
Name:	Horng-Der Shen
Institution:	Taipei Veterans General Hospital, Dept. of Medical Research and Education
City:	Taipei, Taiwan
Email:	hdshen@vghtpe.gov.tw
Submission Date:	2014-07-04

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Fus p 9.0101	KJ462778	AJA79001	A0A0U1Y1N5	

Vacuolar serine protease [*Penicillium oxalicum*], GenBank AAG44478
http://www.allergen.org/viewallergen.php?aid=492

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Fungi Ascomycota › Eurotiales › *Penicillium oxalicum* › Pen o 18

Allergen Details:

Allergen name:	Pen o 18
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Penicillium oxalicum
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	Among 70 asthmatic sera tested, 18 (26%) had IgE immunoblot reactivity towards components of P. oxalicum. Of these 18 sera, > 80% showed IgE-binding to a 34 kD protein, identified as a vacuolar serine proteinase by N-terminal sequence analysis.
Allergenicity reference:	10231324
Route of allergen exposure:	Airway
Date Created:	31-08-2003
Last Updated:	2019-09-05 21:57:35
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Pen o 18.0101	AF243425	AAG44478	Q9HF12	

Cellular serine proteinase [*Aspergillus fumigatus*], GenBank CAA73782
http://www.allergen.org/viewallergen.php?aid=105

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Fungi Ascomycota › Eurotiales › *Aspergillus fumigatus* › Asp f 18

Allergen Details:

Allergen name:	Asp f 18
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus fumigatus (Common mold)
Biochemical name:	Vacuolar serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	Of 19 asthmatic, A.fumigatus-sensitized patients, 15 (79%) showed serum IgE binding to 34 kD Asp f 18 (identified by N-terminal amino acid sequence analysis) on immunoblot. Of another 14 patients, 11 (79%) showed IgE binding to Asp f 18.
Allergenicity reference:	11251631
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-07-11 23:44:13
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

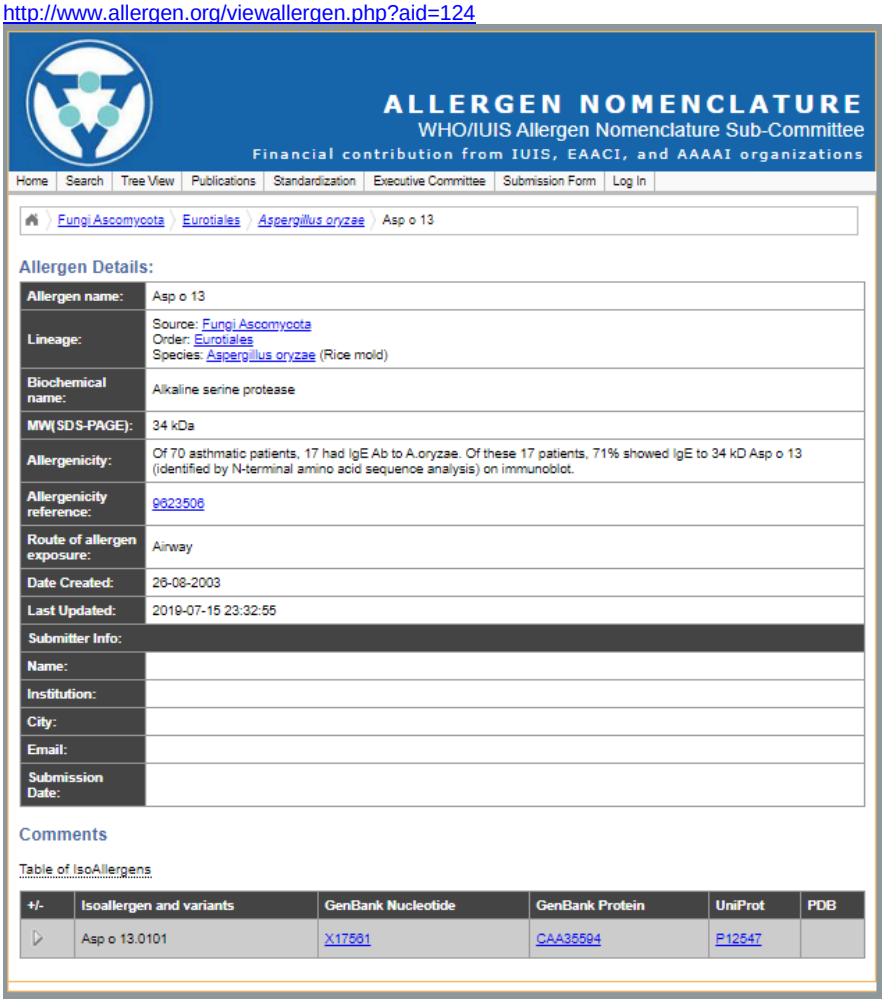
Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp f 18.0101	Y13338	CAA73782	P87184	

***Aspergillus proteinase* B, UniProt code P12547**

http://www.allergen.org/viewallergen.php?aid=124



ALLERGEN NOMENCLATURE

WHO/IUIS Allergen Nomenclature Sub-Committee

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Fungi Ascomycota › Eurotiales › *Aspergillus oryzae* › Asp o 13

Allergen Details:

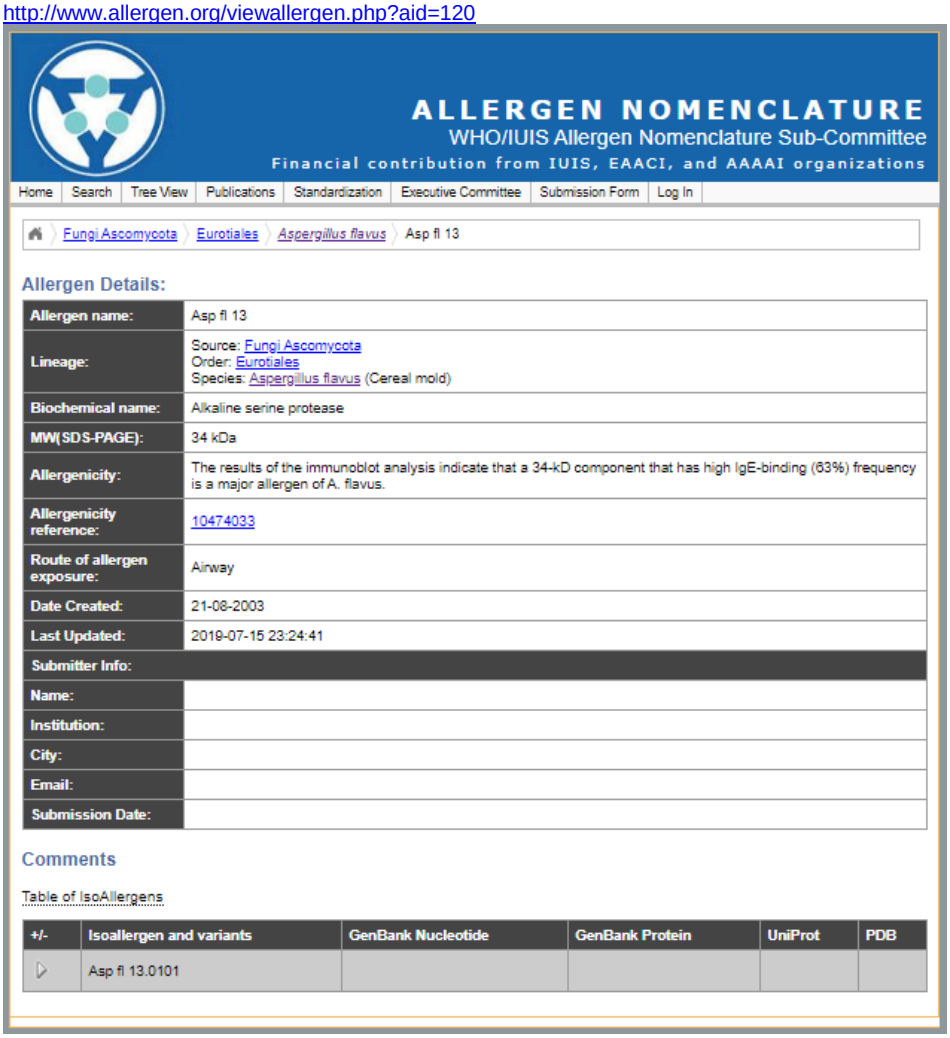
Allergen name:	Asp o 13
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus oryzae (Rice mold)
Biochemical name:	Alkaline serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	Of 70 asthmatic patients, 17 had IgE Ab to A.oryzae. Of these 17 patients, 71% showed IgE to 34 kD Asp o 13 (identified by N-terminal amino acid sequence analysis) on immunoblot.
Allergenicity reference:	9623506
Route of allergen exposure:	Airway
Date Created:	26-08-2003
Last Updated:	2019-07-15 23:32:55
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp o 13.0101	X17561	CAA35594	P12547	

Allergen Asp fl 1 (*Aspergillus flavus*), GenBank Q9UVU3
http://www.allergen.org/viewallergen.php?aid=120



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Fungi Ascomycota › Eurotiales › *Aspergillus flavus* › Asp fl 13

Allergen Details:

Allergen name:	Asp fl 13
Lineage:	Source: Fungi Ascomycota Order: Eurotiales Species: Aspergillus flavus (Cereal mold)
Biochemical name:	Alkaline serine protease
MW(SDS-PAGE):	34 kDa
Allergenicity:	The results of the immunoblot analysis indicate that a 34-kD component that has high IgE-binding (63%) frequency is a major allergen of A. flavus.
Allergenicity reference:	10474033
Route of allergen exposure:	Airway
Date Created:	21-08-2003
Last Updated:	2019-07-15 23:24:41
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Submission Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Asp fl 13.0101				

Appendix 8: External Expert Opinion Letter from Dr. Michael Pariza

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

April 29, 2016

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
DuPont Industrial Biosciences
Genencor / Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS Opinion on the intended uses of DuPont's FN3 alkaline protease enzyme preparation that is produced by *Bacillus subtilis* CF520B (GICC3378)

Dear Dr. Sewalt,

I have reviewed the information that you provided on the intended uses of DuPont's FN3 alkaline protease enzyme preparation that is produced by *Bacillus subtilis* CF520B (GICC3378). FN3 is a protein-engineered variant of the alkaline protease enzyme, commonly referred to as 'subtilisin', which is derived from *Bacillus lentus*. The proposed use of FN3 is as a processing aid in a variety of applications that include plant protein processing, fish and seafood protein processing, yeast processing, animal protein processing, xanthan gum processing, and microalgae processing, with the resulting protein hydrolysates intended for human consumption, or for use in animal feed and pet food, where the enzyme will not be present in the final food/feed, or present in negligible amounts with no function in the final food/feed.

In evaluating FN3 I considered the biology of *Bacillus subtilis*, the biology of *Bacillus lentus* which was the source of the wild-type alkaline protease gene sequence that was subsequently modified (engineered) via three amino acid substitutions to produce FN3, relevant information available in the peer-reviewed scientific literature, information that you provided regarding the cloning methodology that was utilized, information pertaining to the safe strain lineage within which *B. subtilis* CF520B (GICC3378) was developed, and results of a 90-day oral (gavage) study in SPF Sprague Dawley rats that was conducted on behalf of DuPont.

By way of background, *B. subtilis* is a ubiquitous gram positive spore-forming bacterium that is rarely associated with opportunistic infections or food poisoning outbreaks. Many non-toxic

strains of this species are utilized by enzyme manufacturers worldwide to produce enzymes and other products for industrial applications, including human food and animal feed uses. Carbohydrase and protease enzyme preparations derived from *B. subtilis* have been affirmed as GRAS by the U.S. FDA per 21 CFR 184.1148 and 184.1150, respectively.

DuPont's safe lineage of *B. subtilis* production strains, including *B. subtilis* CF520B (GICC3378), was developed from the wild-type *B. subtilis* 168 via a series of modifications that included classical mutagenesis, as well as rDNA and protein engineering utilizing techniques and reagents that are appropriate for the development of a safe lineage of food ingredient production microorganisms. The safety of the enzymes from these production strains have been evaluated with various *in vitro* genetic toxicity tests, as well as oral toxicity tests in rats (90-day, 28-day, or acute oral toxicity.) Strains within this safe lineage are used to manufacture many food and feed enzymes, including proteases, arylesterase, maltotetraohydrolase, xylanase, cellulase, and β-glucanase. Published literature, government laws and regulations, and DuPont's unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the development and manufacture of food-grade and feed-grade enzymes. Expert opinion letters supporting GRAS status for the proposed uses were received for Multifect P300 protease for food and feed (Dr. Pariza to G. Mercer 18 May 1994, and to A. Caddow 1 October 1994), maltotetrahydrolases (SAS 1, 2, & 3 amylase; 27 May 2004, 17 October 2005, & 29 August 2006, respectively from Drs. Pariza, Borzelleca, and Blumenthal) from three strains for baking, a xylanase for baking (Dr. Pariza to A. Caddow, 28 September 2006), a lactase for dairy (Dr. Pariza to Sewalt, 13 June 2014) and a β-glucanase for brewing (Dr. Pariza to Sewalt, 13 June 2014). GRAS notices for lactase and β-glucanase were submitted to FDA, which responded with 'no questions' letters (GRN 579 and GRN 592, respectively).

FN3 is a variant of the wild-type alkaline protease (subtilisin) gene from *B. lentus* ATCC21536. This species is not associated with illness in humans or animals. It is listed as a Class 1 Agent per NIH guidelines, and complies with the Good Industrial Large Scale Practice (GILSP) criteria suggested by OECD. The wild-type alkaline protease gene from *B. lentus* ATCC21536 was modified via protein engineering to generate FN3, which differs from the wild-type subtilisin by three amino acid substitutions. FN3 hydrolyzes a wide variety of protein substrates including slaughter waste proteins such as feathers and hair.

The safety of FN3 was evaluated by repeated daily oral administration to SPF Sprague Dawley rats of both sexes for 90 consecutive days. No treatment related adverse effects were observed, and the NOAEL was determined to be the highest dose tested, 420 mg total protein/kg BW/day. This is approximately 230 times the maximum estimated consumption of FN3 for humans, and from 117-311 times the maximum estimated consumption of FN3 for companion animals (dogs and cats) and production animals (cattle, pigs, poultry, fish and shrimp).

The proposed raw materials, product formulation, and specifications for FN3 meet Food Chemicals Codex and FAO/WHO JEFCA requirements. The proposed use of FN3 is as a processing aid in a variety of applications that include plant protein processing, fish and seafood protein processing, yeast processing, animal protein processing, xanthan gum processing, and microalgae processing, with the resulting protein hydrolysates intended for human consumption, or for use in animal feed and pet food, where the enzyme will not be present in the final food/feed, or present in negligible

amounts with no function in the final food/feed.

The safety of FN3 was formally evaluated using the Pariza-Johnson decision tree. The conclusion of this analysis was that the test article (FN3) was accepted. The chloramphenicol resistance marker gene (CAT) is recognized to be in the *Bacillus* gene pool. This marker is excised during strain construction, but is later integrated into the genome of the final construct, where it is no longer transferable to other species.

Based on the foregoing, I concur with the evaluation made by DuPont that the *B. subtilis* CF520B (GICC3378) production strain is safe to use for the manufacture of FN3, to be used as a processing aid in a variety of applications that include plant protein processing, fish and seafood protein processing, yeast processing, animal protein processing, xanthan gum processing, and microalgae processing, with the resulting protein hydrolysates intended for human consumption, or for use in animal feed and pet food, where the enzyme will not be present in the final food/feed, or present in negligible amounts with no function in the final food/feed.

I further conclude that FN3, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in a variety of applications that include plant protein processing, fish and seafood protein processing, yeast processing, animal protein processing, xanthan gum processing, and microalgae processing, with the resulting protein hydrolysates intended for human consumption, or for use in animal feed and pet food, where the enzyme will not be present in the final food/feed, or present in negligible amounts with no function in the final food/feed.

It is my professional opinion that other qualified experts would also concur in this conclusion.

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 the subject product by you or others.

Sincerely,

Michael W. Pariza, Ph.D.
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

Viebrock, Lauren

From: Vincent Sewalt <Vincent.Sewalt@iff.com>
Sent: Monday, March 21, 2022 7:49 PM
To: Viebrock, Lauren
Cc: Annie Han
Subject: [EXTERNAL] FW: GRN 000989 Questions
Attachments: Attachment 1 Molecular Weight of FN3.pdf; Attachment 2 Updated Certificate of Analysis.pdf; Response letter to FDA 21mar2021.pdf

Dear Lauren,

Hereby our response to CFSAN's questions to GRN 000989, in the form of a response letter and 2 attachments.

Best regards,

VINCENT SEWALT, Ph.D.
Head of Regulatory Science & Advocacy, IFF
Danisco US Inc.
vincent.sewalt@iff.com
M +1 650 799 0871
iff.com
LinkedIn | Twitter | Facebook | YouTube | Instagram

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, March 8, 2022 12:08 PM
To: Sewalt, Vincent <Vincent.Sewalt@dupont.com>; Vincent Sewalt <Vincent.Sewalt@iff.com>
Subject: GRN 000989 Questions

Dear Mr. Sewalt,

During our review of GRAS Notice No. 000989, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

Date: March 21, 2022

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To: Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

From: Vince Sewalt, Ph.D.
Head of Regulatory Sciences and Advocacy
Danisco US Inc.
vincent.sewalt@iff.com

RE: GRAS Notice GRN000989

Dear Dr VieBrook,

Thank you for your review of our submission. We are providing this letter in response to FDA's questions that was sent via email on March 8, 2022 regarding our GRAS Notice GRN00989 on the subtilisin enzyme preparation produced with genetically engineered *Bacillus subtilis*. We have included your questions with our responses for your reference:

1. The subtilisin enzyme sequence is modified from wild type. Please provide additional information on the nature of the modification(s).

   The subtilisin enzyme sequence was modified from the wild type subtilisin from *Bacillus clausii* strain ATCC21536,[1] which is a 3 amino acid variant (N76D, S103A, and V104I) of the wild type.

   In addition, the taxonomic classification of the donor wild type genus has been updated from *Bacillus clausii* to *Alkalihalobacillus clausii* (Nielsen *et al*., 1995; Patel & Gupta, 2020).

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[1] https://www.atcc.org/products/21536

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2. Please provide the molecular weight of the enzyme.

   The molecular weight is 26.698 kDa (Attachment 1).

3. Please state whether the enzyme is secreted into the culture medium.

   The enzyme is secreted into the culture medium.

4. Please provide the production strain identification. The GRAS opinion letter provided by Dr. Pariza refers to *Bacillus subtilis* CF520B (GICC3378), which is not mentioned in the GRAS notice. Please confirm that the opinion letter was not based on information not provided in the GRAS notice submitted to our office.

   We confirm the GRAS opinion letter from Dr. Pariza to *Bacillus subtilis* CF520B (GICC03378) was based on the same information provided in the GRAS Notice GRN000989 submitted to FDA. *Bacillus subtilis* CF520B is the internal strain designation and the GICC03378 is the Danisco culture collection number of this strain.

5. The specifications for total viable cell counts, coliforms, and toxic elements are given as a range (e.g., the lead specification provided is 0-5 mg/kg). Please revise the specifications as "< " the value.

   Upon your request, we updated the Certificate of Analysis in Attachment 2.

6. Please specify the method used for the analyses shown on the certificates of analysis on pages 49-51. Please provide the values for the results that were found in the batch analysis supporting the specifications.

   The methods used for the analyses on the Certificate of Analysis and the values for the results for each batch can be found in updated Certificate of Analysis (Attachment 2).

7. The dietary exposure using the Budget method was calculated based on the assumptions of 25% solid food and 10% beverage. Please provide reasoning for the deviation from the typical 50% of solid and 25% of beverages assumptions.

   IFF believes the following data support the use of the revised, yet still conservative Budget Method assumptions for high protein containing products such as sport drinks and protein bars.

1) The Budget method enables a calculation of the Theoretical Maximum Daily Intake (TMDI) based on very conservative assumptions regarding physiological requirements for energy from food and the energy density of

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food rather than on food consumption survey data. The regular assumption is for all processed food to be 50% of total solid food and for all soft drinks to be 25% of total beverages. As the application of the enzyme is focused on high-protein food (such as protein bars) & drinks (such as sport drinks), the following reduced yet still conservative assumptions for intake were used: high-protein processed food: 25% of total solid food (kg); protein-containing soft drinks: 10% of total beverages (L). IFF believes that for high-protein containing products, the revised assumptions, while still conservative, are more in line with consumer exposure. To demonstrate the relevance of the revised Budget Method assumptions to consumer exposure, IFF utilized the USDA Food Consumption and Nutrient Intake Table data for Protein Foods[2] and data for consumption of diet soft drinks/other drinks and regular soft/other drinks.[3] For both Protein Foods and diet soft/other drinks and regular soft/other drinks, a worst-case approach was assumed in which all Protein Foods and Protein drinks contained protein processed with the subtilisin enzyme. For Protein Foods, adults 20-64 had the highest daily consumption at 370.25 grams/day. For beverages, males 20 years and over had the highest daily consumption at 615g.

Protein Foods: 0.370 kg/day x 170.7 mg TOS/kg food = 63.2 mg TOS/day/60 kg = 1.05 mg TOS/kg bw/day

Beverages: 0.615 kg/day x 170.7 mg TOS/kg food = 104.98 mg TOS/day/60 kg = 1.75 mg TOS/kg bw/day

Protein Foods (1.05 mg TOS/kg bw/day) + Beverages (1.75 mg TOS/kg bw/day) = 2.8 mg TOS/kg bw/day

Margin Of Exposure (MOA) = 480.6 mg TOS/kg bw/day/2.8 = 171.6

The value calculated in the GRAS document using the revised Budget Method calculations = 2.73 mg TOS/kg bw/day

MOA = 480.6 mg TOS/kg bw/day/2.73 = 176,

in line with the calculation based on USDA intake data for protein foods.

I addition to the above MOA calculations, IFF would like to share the following considerations:

The conduct of repeated dose toxicological testing with enzymes derived from the same strain lineage (*e.g., Trichoderma reesei; Bacillus licheniformis,*

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[2] https://www.ers.usda.gov/data-products/food-consumption-and-nutrient-intakes/food-consumption-and-nutrient-intakes/#Food%20Consumption%20Estimates
[3] https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/dbrief/6_beverage_choices_adults_0708.pdf

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Bacillus subtilis) with no adverse effects allows for the establishment of a Safe Strain Lineage (SSL) (Pariza and Johnson, 2001; Sewalt et al., 2016). Demonstrating the safety of the production strain is the most critical factor in evaluating the safety of an enzyme preparation. A Safe Strain Lineage can be established by repeated assessment of members of the lineage, which involves addressing the potential for antimicrobial activity and toxic microbial metabolites. This generally also includes a repeat-dose oral toxicity study to produce a No Observed Adverse Effect Level (NOAEL) to determine the margin of exposure in the intended use. Once the SSL has been established, the decision tree allows for any strains within the lineage that are upstream from the production strains already evaluated toxicologically to be supported as safe production hosts for other enzymes, and any new enzyme preparations from the new strains in the lineage would also be considered safe, based upon the decision tree requirements of the use of safe and well-documented transformation techniques, thorough characterization of the introduced DNA, and the expressed enzyme activity having a history of safe use (Pariza and Johnson, 2001). For the subtilisin protease produced by strain GICC3378, the NOAEL was 480.6 mg/kg bw/day, the highest dose tested. Produced by another member (GICC00896) of the same *Bacillus subtilis* SSL, another subtilisin BG2843 evaluated in a 28-day oral gavage Toxicology study had a NOAEL of 1500 mg TOS/kg bw/day, the highest dose tested. The two strains are very close relatives within the same SLL, as both are constructed by integration of an expression cassette in host BG3594. The expression cassette is different, but in both cases expressing the same subtilisin enzyme. For the purpose of calculating a margin of exposure, the NOEAL derived from the notified subtilisin produced in GICC3378 itself was used (as is customary), but since the top dose in either toxicology study showed no adverse effects, the 3-fold higher top dose with no adverse effects from the second study conducted with subtilisin produced in parallel strain GICC00896 is actually a better quantitative indicator of the lack of toxigenic potential of the production strain.

Finally, microbially-produced food enzymes in general don't produce acute toxicity, genotoxicity, or repeated dose oral toxicity. As reviewed in (Ladics and Sewalt, 2018)[4], several hundred mutagenicity studies have been conducted on bacterial and mammalian cells for a variety of enzymes. No positive findings were observed. Over 225 90-day studies have been performed and submitted to EFSA (as part of EFSA's review program under the FIAP regulation) with no adverse findings, including in the bone marrow. The data showing no adverse effects for enzyme preparations also confirms that any residual microbial metabolites and fermentation materials, to the extent present, lack toxicity as well.

[4] https://doi.org/10.1016/j.yrtph.2018.07.016

Taken together, these data provide ample weight-of-evidence demonstrating the safety of microbial-derived enzymes in general, and the notified subtilisin in particular, when the notified subtilisin is used at the Maximum Use Rate in protein processing applications for food and beverages.

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References

Ladics G.S. and Sewalt V. (2018).[5] Industrial microbial enzymes safety: What does the weight-of-evidence indicate? *Regulatory Toxicology and Pharmacology* 98:151-154

Nielsen P., Fritze D., and Priest F.G. (1995). Phenetic diversity of alkaliphilic Bacillus strains: proposal for nine new species. *Microbiology* 141: 1745-1761.

Pariza M.W. and Johnson E.A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regulatory Toxicology and Pharmacology* 33: 173-186.

Patel S. and Gupta R.S. (2020). A phylogenomic and comparative genomic framework for resolving the polyphyly of the genus Bacillus: Proposal for six new genera of Bacillus species, Peribacillus gen. nov., Cytobacillus gen. nov., Mesobacillus gen. nov., Neobacillus gen. nov., Metabacillus gen. nov. and Alkalihalobacillus gen. nov. *International Journal of Systematic and Evolutionary Microbiology* 70: 406-438.

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: 295-302.

Attachments

Attachment 1	Molecular Weight
Attachment 2	Certificate of Analysis (3 lots)

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[5] https://doi.org/10.1016/j.yrtph.2018.07.016

Compute pI/Mw

Compute pI/Mw

Theoretical pI/Mw (average) for the user-entered sequence:

```
        10         20         30         40         50         60
AQSVPWGISR VQAPAAHNRG LTGSGVKVAV LDTGISTHPD LNIRGGASFV PGEPSTQDGN

        70         80         90        100        110        120
GHGTHVAGTI AALDNSIGVL GVAPSAELYA VKVLGASGSG AISSIAQGLE WAGNNGMHVA

       130        140        150        160        170        180
NLSLGSPSPS ATLEQAVNSA TSRGVLVVAA SGNSGAGSIS YPARYANAMA VGATDQNNNR

       190        200        210        220        230        240
ASFSQYGAGL DIVAPGVNVQ STYPGSTYAS LNGTSMATPH VAGAAALVKQ KNPSWSNVQI

       250        260
RNHLKNTATS LGSTNLYGSG LVNAEAATR
```

Theoretical pI/Mw: 8.98 / 26697.51

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Attachment 2: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663619649**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	3367	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7521 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Salmonella	/25ml	Negative by test	Negative	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Production Strain	/ml	Negative by test	Negative	IFF H&B method C892
Antibacterial activity	/ml	Negative by test	Negative	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218; Appendix A
PHYSICAL PROPERTIES				
Specific gravity		Report	1.20	IFF H&B method C711
OTHER ASSAYS				
Lead	mg/kg	≤5	0.01	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.
Arsenic	mg/kg	≤3	<0.02	Same as Lead
Cadmium	mg/kg	≤0.5	ND	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision
Mercury	mg/kg	≤0.5	<0.01	Same as Lead

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.

9-Mar-2022
Date

Kelly A. Altman
QA/QC Department

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CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663620612**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	3245	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7521 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Salmonella	/25ml	Negative by test	Negative	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Production Strain	/ml	Negative by test	Negative	IFF H&B method C892
Antibacterial activity	/ml	Negative by test	Negative	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218; Appendix A
PHYSICAL PROPERTIES				
Specific gravity		Report	1.18	IFF H&B method C711
OTHER ASSAYS				
Lead	mg/kg	≤5	0.01	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.
Arsenic	mg/kg	≤3	0.02	Same as Lead
Cadmium	mg/kg	≤0.5	ND	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision
Mercury	mg/kg	≤0.5	<0.01	Same as Lead

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.

9-Mar-2022
Date

Kelly A. Altman
QA/QC Department

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

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CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663714790**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	2910	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7521 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Salmonella	/25ml	Negative by test	Negative	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Production Strain	/ml	Negative by test	Negative	IFF H&B method C892
Antibacterial activity	/ml	Negative by test	Negative	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218; Appendix A
PHYSICAL PROPERTIES				
Specific gravity		Report	1.18	IFF H&B method C711
OTHER ASSAYS				
Lead	mg/kg	≤5	0.02	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.
Arsenic	mg/kg	≤3	0.02	Same as Lead
Cadmium	mg/kg	≤0.5	ND	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision
Mercury	mg/kg	≤0.5	<0.01	Same as Lead

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.

9-Mar-2022
Date

Kelly A. Altman
QA/QC Department

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

Viebrock, Lauren

From: Vincent Sewalt <Vincent.Sewalt@iff.com>
Sent: Wednesday, May 11, 2022 11:26 AM
To: Viebrock, Lauren
Cc: Annie Han
Subject: RE: [EXTERNAL] FW: GRN 000989 Questions

Dear Lauren,

Apologies for the delay in responding.

Yes, “Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.)” is preferred.

Best regards,
Vince

VINCENT SEWALT
Head of Regulatory Science & Advocacy, Global Regulatory Affairs, IFF
Danisco US Inc.
vincent.sewalt@iff.com
M +1 650 799 0871
iff.com
LinkedIn | Twitter | Facebook | YouTube | Instagram

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, April 14, 2022 9:38 AM
To: Vincent Sewalt <Vincent.Sewalt@iff.com>
Cc: Annie Han <Annie.Han@iff.com>
Subject: RE: [EXTERNAL] FW: GRN 000989 Questions

Dear Vince,

Thank you for the response and additional information regarding GRN 989.

Can you please clarify how the name of the company should be written in our correspondences? Is “Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.)” or “Danisco US Inc.” preferred? Thank you.

Best,
Lauren

From: Vincent Sewalt <Vincent.Sewalt@iff.com>
Sent: Monday, March 21, 2022 7:49 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Cc: Annie Han <Annie.Han@iff.com>
Subject: [EXTERNAL] FW: GRN 000989 Questions

Dear Lauren,

Hereby our response to CFSAN's questions to GRN 000989, in the form of a response letter and 2 attachments.

Best regards,

VINCENT SEWALT, Ph.D.
Head of Regulatory Science & Advocacy, IFF
Danisco US Inc.
vincent.sewalt@iff.com
M +1 650 799 0871
iff.com
LinkedIn | Twitter | Facebook | YouTube | Instagram

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, March 8, 2022 12:08 PM
To: Sewalt, Vincent <Vincent.Sewalt@dupont.com>; Vincent Sewalt <Vincent.Sewalt@iff.com>
Subject: GRN 000989 Questions

Dear Mr. Sewalt,

During our review of GRAS Notice No. 000989, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety

U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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Viebrock, Lauren

From: Vincent Sewalt <Vincent.Sewalt@iff.com>
Sent: Tuesday, May 24, 2022 6:28 PM
To: Viebrock, Lauren
Cc: Annie Han
Subject: FW: [EXTERNAL] FW: GRN 000989 Questions
Attachments: A01053_GRAS_1663714790_19may2022.pdf; A01053_GRAS_1663620612_19may2022.pdf; A01053_GRAS_1663619649_19may2022.pdf

Public

Dear Lauren,

With regard to your remaining questions, we provide responses below:

1. Please confirm that in attachment 2 of March 21, 2022, that the ISO method for *E. coli* detection is supposed to be written as ISO 7251.

Response: we confirm that the ISO method is ISO 7251, and have corrected this typo on the attached certificates of analysis.

2. FDA noted that specifications for arsenic, cadmium, mercury and lead are < 5, < 3, < 0.5, and < 0.5. As the batch analyses results support lower specification, please consider reducing the specification to reflect the batch analyses.

Response: indeed the batch analytical results are well within the stated limit as provided on the certificates of analysis. We'll take FDA's suggestion of lowering the specification under consideration once we complete a thorough study of analytical results on our entire food-grade enzyme portfolio as produced at various manufacturing sites.

Best regards,

Vincent Sewalt, PhD
Head of Regulatory Science & Advocacy, Global Regulatory Affairs, IFF
Danisco US Inc.
vincent.sewalt@iff.com
M +1 650 799 0871
iff.com
LinkedIn | Twitter | Facebook | YouTube | Instagram

Public

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, May 17, 2022 11:03 AM

To: Vincent Sewalt <Vincent.Sewalt@iff.com>
Cc: Annie Han <Annie.Han@iff.com>
Subject: RE: [EXTERNAL] FW: GRN 000989 Questions

External Warning: This email is from Lauren.Viebrock@fda.hhs.gov - if this email address is unfamiliar, do not click links and forward as an attachment to SuspiciousEmail@iff.com

Dear Mr. Sewalt,

Thank you for the information on the company name. We have two additional points to be addressed regarding GRN 000989, which are provided below:

1. Please confirm that in attachment 2 of March 21, 2022, that the ISO method for *E. coli* detection is supposed to be written as ISO 7251.

2. FDA noted that specifications for arsenic, cadmium, mercury and lead are < 5, < 3, < 0.5, and < 0.5. As the batch analyses results support lower specification, please consider reducing the specification to reflect the batch analyses.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

From: Vincent Sewalt <Vincent.Sewalt@iff.com>
Sent: Wednesday, May 11, 2022 11:26 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Cc: Annie Han <Annie.Han@iff.com>
Subject: RE: [EXTERNAL] FW: GRN 000989 Questions

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Lauren,

Apologies for the delay in responding.

Yes, “Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.)” is preferred.

Best regards,
Vince

VINCENT SEWALT
Head of Regulatory Science & Advocacy, Global Regulatory Affairs, IFF
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Subject: RE: [EXTERNAL] FW: GRN 000989 Questions

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Dear Vince,

Thank you for the response and additional information regarding GRN 989.

Can you please clarify how the name of the company should be written in our correspondences? Is "Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.)" or "Danisco US Inc." preferred? Thank you.

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Sent: Monday, March 21, 2022 7:49 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Cc: Annie Han <Annie.Han@iff.com>
Subject: [EXTERNAL] FW: GRN 000989 Questions

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Dear Lauren,

Hereby our response to CFSAN's questions to GRN 000989, in the form of a response letter and 2 attachments.

Best regards,

VINCENT SEWALT, Ph.D.
Head of Regulatory Science & Advocacy, IFF
Danisco US Inc.
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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, March 8, 2022 12:08 PM

To: Sewalt, Vincent <Vincent.Sewalt@dupont.com>; Vincent Sewalt <Vincent.Sewalt@iff.com>
Subject: GRN 000989 Questions

Dear Mr. Sewalt,

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We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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suppliers, service providers and other business partners, please refer to the Privacy Notice for EEA Customers and Vendors.

Where science
& creativity meet

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663619649**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	3367	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7251 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Salmonella	/25ml	Negative by test	Negative	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Production Strain	/ml	Negative by test	Negative	IFF H&B method C892
Antibacterial activity	/ml	Negative by test	Negative	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218; Appendix A
PHYSICAL PROPERTIES				
Specific gravity		Report	1.20	IFF H&B method C711
OTHER ASSAYS				
Lead	mg/kg	≤5	0.01	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.
Arsenic	mg/kg	≤3	<0.02	Same as Lead
Cadmium	mg/kg	≤0.5	ND	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision
Mercury	mg/kg	≤0.5	<0.01	Same as Lead

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.

19-May-2022
Date

Kelly A. Altman
QA/QC Department

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

Where science
& creativity meet

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663620612**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	3245	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7251 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Salmonella	/25ml	Negative by test	Negative	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Production Strain	/ml	Negative by test	Negative	IFF H&B method C892
Antibacterial activity	/ml	Negative by test	Negative	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218; Appendix A
PHYSICAL PROPERTIES				
Specific gravity		Report	1.18	IFF H&B method C711
OTHER ASSAYS				
Lead	mg/kg	≤5	0.01	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.
Arsenic	mg/kg	≤3	0.02	Same as Lead
Cadmium	mg/kg	≤0.5	ND	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision
Mercury	mg/kg	≤0.5	<0.01	Same as Lead

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.

19-May-2022
Date

Kelly A. Altman
QA/QC Department

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Where science
& creativity meet

CERTIFICATE OF ANALYSIS

PRODUCT: **FoodPro PXT**
LOT NUMBER: **1663714790**

ASSAY	UNIT	SPECIFICATION	FOUND	METHOD
ENZYME ACTIVITY				
Protease	U/g	2800-3540	2910	IFF H&B method C310L
MICROBIOLOGICAL ANALYSIS				
Total Viable Count	CFU/ml	0-50000	<1000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
Coliforms	CFU/ml	0-30	<1	ISO 4832 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
E. coli	/25ml	Negative by test	Negative	ISO 7251 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International
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PHYSICAL PROPERTIES				
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19-May-2022
Date

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