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

Re: GRAS Notice No. GRN 000714

Dear Mr. Sewalt:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 000714. We received Danisco US Inc. (operating as DuPont Industrial Sciences) (DuPont)'s GRAS notice on June 20, 2017, and filed it on August 14, 2017. We received an amendment on August 29, 2017, containing an update about the information that DuPont initially designated confidential[1] and the intended uses. We also received an amendment on December 11, 2017, containing additional safety information.

The subject of the notice is subtilisin enzyme preparation produced by *Bacillus subtilis* expressing a modified gene encoding a variant of the wild-type subtilisin from *B. amyloliquefaciens* (subtilisin enzyme preparation) for use as an enzyme to hydrolyze proteins from microbial, plant, milk, and seafood sources, at up to 369 mg Total Organic Solids (TOS)/kg substrate. The notice informs us of DuPont's view that this use of subtilisin enzyme preparation is GRAS through scientific procedures.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component that catalyzes the chemical reaction as well as substances used as stabilizers, preservatives, or diluents. Enzyme preparations may also contain components derived from the production organism and from the manufacturing process, e.g., constituents of the fermentation media or the residues of processing aids. DuPont's notice provides information about the components in the subtilisin enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, subtilisin is identified by the Enzyme Commission Number 3.4.21.62. The accepted name and systematic name for this enzyme is subtilisin. The enzyme is also known as alcalase, bacillopeptidase, alkaline proteinase, protease, thermoase, and subtilopeptidase. Subtilisin hydrolyzes native and denatured proteins, and peptide amides to release protein fragments of various lengths, peptides, and free amino acids. It has broad specificity for peptide bonds, and a

[1] GRN 000714 included information in an Appendix in Part 7 that Dupont initially designated confidential in the notice. In the August 29, 2017, amendment, Dupont confirms that the report was incorrectly marked confidential and that this information is not confidential.

preference for a large uncharged residue in P1 position. The CAS No. for subtilisin is 9014-01-1. DuPont states that the primary amino acid sequence of the expressed mature subtilisin enzyme has been determined and it consists of 275 amino acids. DuPont states that subtilisin has a molecular weight of 68.7 kDa.

DuPont states that the *B. subtilis* production strain BG3600-1425-3D was derived from the *B. subtilis* strain BG125.[2] DuPont states that this recipient strain was previously modified at several chromosomal loci to introduce mutations to enhance protease production, and to inactivate genes encoding a neutral protease and a gene necessary for sporulation. DuPont describes *B. subtilis* as a non-pathogenic, non-toxigenic, well-characterized production organism with a history of safe use in the food industry. DuPont also states that the production strain is considered suitable for Good Industrial Large Scale Practice worldwide.

DuPont describes the construction of the production strain by the targeted integration of an expression cassette carrying the modified gene encoding a variant of the wild-type subtilisin gene[3] from *B. amyloliquefaciens* and a chloramphenicol resistance gene selectable marker under control of the *B. subtilis* subtilisin promoter. DuPont verified the construction, and confirmed that the introduced DNA is stable after at least 60 generations, via Southern blot analyses. DuPont states that the final production strain does contain a chloramphenicol resistance gene,[4] but the gene product is not secreted into the culture medium.

DuPont states that subtilisin enzyme is produced by submerged fed-batch fermentation of a pure culture of the production strain. DuPont states that fermentation is carried out under controlled conditions and that the enzyme is secreted into the culture medium. The enzyme is recovered from the culture medium by filtration or centrifugation of the supernatant containing the enzyme, and concentrated by ultrafiltration. The liquid enzyme concentrate is stabilized and formulated to an enzyme preparation by the addition of sodium acetate, propylene glycol, and water. DuPont states that the entire process is performed in accordance with current good manufacturing practices. DuPont also states that the final enzyme preparation does not contain any major food allergens from the culture medium.

DuPont states that the subtilisin enzyme preparation conforms to specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 10th edition, 2016), and to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2006). DuPont provides analytical data from three batches of subtilisin enzyme concentrate to demonstrate consistency with the manufacturing

---

[2] DuPont states that *B. subtilis* strain BG125 was obtained as strain 1A10 from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio, and was derived from *B. subtilis* strain 168 via classical genetics.

[3] DuPont states that the variant subtilisin gene has a single amino acid residue difference compared to the wild-type subtilisin from *B. amyloliquefaciens*.

[4] DuPont states that the chloramphenicol resistance gene has been integrated into the *B. subtilis* production strain BG3600-1425-3D.

specifications. DuPont also confirms that a test for absence of any production organism in the final product is an established specification.

DuPont intends to use subtilisin enzyme preparation to hydrolyze protein during protein processing at a maximum level corresponding to 369 mg TOS/kg of substrate. DuPont notes that the subtilisin enzyme preparation will be inactivated or removed during processing. DuPont states that if the enzyme is present and ingested in the final food, it will be broken down by the digestive system and metabolized, and therefore poses no health risk. To estimate dietary exposure to subtilisin enzyme preparation, DuPont assumes that the enzyme preparation will be used at the maximum intended levels, and that the enzyme preparation will remain in the final food. DuPont estimated dietary exposure from all uses of subtilisin enzyme preparation to be 4.15 mg TOS/kg body weight per day (mg TOS/kg bw/d).

DuPont relies on published information that discusses the safety of microbial enzyme preparations used in food processing, including the safety of the production organism. Further, DuPont provided unpublished data from toxicological testing of the subtilisin enzyme preparation that were performed prior to the establishment of OECD guidelines. Therefore, DuPont also provided unpublished results of toxicological studies for five enzyme preparations derived from genetically engineered *B. subtilis* strains to further corroborate the safety of the subtilisin enzyme preparation. Toxicology tests included 90-day subchronic feeding studies in rats for four enzyme preparations and acute toxicity studies for two enzyme preparations. The studies also included bacterial reverse mutation assays and *in vitro* chromosomal aberration assays with human lymphocytes or Chinese hamster ovary cells. All enzyme preparations were found to be non-toxic, non-mutagenic, and not clastogenic. Based on the totality of data and information available, DuPont concludes that the *B. subtilis* is a safe strain lineage and therefore strain BG3600-1425-3D is a safe production host and the enzyme preparations resulting from it are safe for use in food.

DuPont discusses potential food allergenicity of subtilisin enzyme. DuPont states that naturally occurring food enzymes, if present in the final food, are unlikely to have allergenic potential because they are present in low concentrations and are susceptible to digestion in the gastrointestinal system. Additionally, DuPont conducted a sequence homology search with a window of 80 amino acids from the peptide sequence of the subtilisin against known allergens stored in the FARRP allergen protein database and found homology above 35% threshold to 26 allergens, of which only one was considered a food allergen.[5] Dupont states that this allergen, cucumisin (Cuc m 1), is an alkaline serine protease from muskmelon; however, neither the full length FASTA sequence analysis above 35% threshold nor eight contiguous identical amino acids search of subtilisin resulted in identification of Cuc m 1. DuPont further cites the conclusions of several organizations and working groups about the low risk of allergenicity posed by enzymes due to their low use levels and the extensive processing of enzyme-containing foods during manufacturing. Based on the totality of the information available, DuPont concludes that it is unlikely that oral consumption of subtilisin enzyme will result in any

[5] All other sequences identified were either related subtilisin genes or other serine proteases from various microorganisms, none of which are considered significant food allergens.

allergenic responses. DuPont also assessed the sequence homology of subtilisin to known toxins based on >25% homology using the UNIPROT database and did not identify any significant homology to any protein sequence identified or known to be a toxin.

Based on the data and information summarized above, DuPont concludes that subtilisin enzyme preparation is GRAS for its intended use.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FD&C Act)

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of DuPont's notice concluding that subtilisin enzyme preparation is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods containing subtilisin enzyme preparation. Accordingly, our response should not be construed to be a statement that foods containing subtilisin enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that DuPont provided, as well as other information available to FDA, we have no questions at this time regarding DuPont's conclusion that subtilisin enzyme preparation produced by *B. subtilis* expressing a modified gene encoding a variant of the wild-type subtilisin from *B. amyloliquefaciens* is GRAS under its intended conditions of use. This letter is not an affirmation that subtilisin enzyme preparation produced by *B. subtilis* expressing a modified gene encoding a variant of the wild-type subtilisin from *B. amyloliquefaciens* is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to GRN 000714 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Michael A. Adams -S

Digitally signed by Michael A. Adams -S
DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, 0.9.2342.19200300.100.1.1=1300042713, cn=Michael A. Adams -S
Date: 2018.02.06 15:01:34 -05'00'

Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition