Vincent Sewalt
Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.)
925 Page Mill Road
Palo Alto, CA 94304

Re: GRAS Notice No. GRN 000989

Dear Dr. Sewalt:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 000989. We received Danisco US Inc. (a wholly owned-subsidiary of International Flavors & Fragrances Inc.) (Danisco)'s GRAS notice on January 5, 2021 and filed it on June 8, 2021. Danisco submitted amendments to the notice on March 21, 2022, May 11, 2022, and May 24, 2022 that provided additional information on the identity of the enzyme, production strain, analytical methods, specifications, dietary exposure, and clarification of the notifier's name.

The subject of the notice is subtilisin enzyme preparation produced by *Bacillus subtilis* expressing a modified gene encoding a modified subtilisin[1] from *Alkalihalobacillus clausii* (subtilisin enzyme preparation) for use as an enzyme at up to 569 mg Total Organic Solids (TOS)/kg raw material to hydrolyze proteins from microbial, plant, milk, and seafood sources. The notice informs us of Danisco'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. Danisco'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 Chemical Abstracts Service number 9014-01-1 and the Enzyme Commission Number 3.4.21.62.[2] Danisco provides the amino acid sequence of subtilisin and calculates the molecular mass of the notified enzyme as 26.7 kDa based on the amino acid sequence.

[1] The modification differs from the wild-type subtilisin by three amino acid substitutions.
[2] https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC3/4/21/62.html

Danisco states that the *B. subtilis* production strain CF520B is non-pathogenic and non-toxigenic. Danisco states that the recipient strain, BG 3594-3, was used in the construction of the production strain by integration of an expression cassette carrying the gene encoding subtilisin from *A. clausii* and a chloramphenicol antibiotic resistance gene for selection.[3] Danisco states it confirmed the insertion integrity by whole genome sequencing. Danisco evaluated the stability of the integration after industrial scale fermentation by monitoring chloramphenicol resistant subtilisin production. Danisco stated that chloramphenicol resistance gene has been reported in *A. clausii* strains and no additional antibiotic resistance genes were conferred to the final production strain.

Danisco states that the subtilisin enzyme preparation is manufactured by submerged fermentation of a pure culture of the *B. subtilis* production strain under controlled conditions. The subtilisin enzyme is secreted into the medium and then recovered by centrifugation or filtration, concentrated by ultrafiltration, followed by addition of stabilizers and/or preservatives, and polish filtration. The liquid enzyme concentrate is formulated with glycerol, sodium acetate and water to produce the enzyme preparation. Danisco states that the entire process is performed using food grade raw materials and in accordance with current good manufacturing practices. Danisco states that no major allergens are present in the final enzyme preparation.

Danisco has established food grade specifications and states that the subtilisin enzyme preparation conforms to specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 12th edition, 2020), 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). Danisco provides data from analyses of three batches of subtilisin enzyme preparations to demonstrate that the manufacturing acceptance criteria have been met, including the absence of the production organism and antibacterial activity.

Danisco intends to use subtilisin enzyme preparation at a maximum use level of 569 mg TOS/kg raw material to catalyze the hydrolysis of proteins with broad specificity for peptide bonds and the hydrolysis of peptide amides. Danisco notes that the subtilisin enzyme preparation will be inactivated or removed during processing. Danisco, however, estimates a maximum dietary exposure to subtilisin enzyme preparation to be 2.73 mg TOS/kg body weight per day (mg TOS/kg bw/d) from the intended uses with the assumption that all the subtilisin enzyme preparation will remain in the final food.[4]

Danisco relies on published information to demonstrate the safety of the *B. subtilis* production organism and the safety of microbial enzyme preparations used in food processing. Danisco summarizes corroborative unpublished toxicological studies using their subtilisin liquid enzyme concentrate prior to its formulation into the enzyme preparation. A 90-day oral toxicity study in rats using the subtilisin liquid enzyme

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[3] Danisco states that transformation was performed with a plasmid carrying an expression cassette containing the *A. clausii* subtilisin gene and a chloramphenicol resistance gene from *Staphylococcus aureus* for selection.

[4] Danisco uses the Budget method to estimate dietary exposure to subtilisin enzyme preparation based on consumption of a maximum of 6 g of solid foods and 10 g of non-milk beverages/kg bw/d.

concentrate at the highest dose tested (480 mg TOS/kg bw/d) showed no treatment related effects. Based on the highest dose tested in the unpublished 90-day study and the estimated dietary exposure from the intended uses of the subtilisin enzyme preparation (2.73 mg TOS/kg bw/d), Danisco calculates the margin of exposure to be approximately 175.[5]

Danisco discusses publicly available literature, as well as the conclusions of several organizations and working groups, concerning the low risk of allergenicity posed by oral consumption of enzymes to address the potential for allergenicity to subtilisin. Based on bioinformatic analyses, Danisco reports no matches between the amino acid sequences of the subtilisin and the primary sequences of known food allergens based on the guidelines developed by Codex Alimentarius Commission (FAO, 2009). Based on the totality of the information available, Danisco concludes that it is unlikely that oral consumption of subtilisin enzyme from the intended use will result in allergenic responses.

Based on the data and information summarized above, Danisco 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 Danisco'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 Danisco provided, as well as other information available to FDA, we have no questions at this time regarding Danisco's conclusion that subtilisin enzyme preparation produced by *B. subtilis* expressing a modified gene encoding a modified subtilisin from *A. clausii* 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 modified subtilisin from *A. clausii* 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

[5] FDA notes the margin of exposure is based on unpublished safety studies and is corroborative of the published information regarding enzyme preparations used in food processing.

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 000989 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2022.06.30 17:38:20 -04'00'

Susan Carlson, Ph.D.
Director
Division of Food Ingredients
Center for Food Safety
and Applied Nutrition