BURDOCK GROUP
CONSULTANTS

DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DEACTIVATED ALKALINE SERINE PROTEASE (DASP) ENZYME AS A FOOD INGREDIENT UNDER THE CONDITIONS OF USE CITED HEREIN

July 21, 2021

FINAL

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1. EXECUTIVE SUMMARY

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, was requested by Nxt2B AB (Uppsala, Sweden) Inc. (hereinafter referred to as Nxt2B) to determine the Generally Recognized As Safe (GRAS) status, of deactivated[2] alkaline serum protease (DASP) enzyme based on scientific procedures. DASP is to be added to medical foods, such that their consumption may be up to 44.8 g/day. Nxt2B assures Burdock Group that all relevant, unpublished information in its possession related to the safety of DASP has been supplied to Burdock Group and has been summarized in this dossier. A comprehensive search of the scientific literature[3] was conducted through January 2021 for safety and toxicity information on DASP and related substances and has been summarized in this dossier as well and, along with supporting documentation, was made available to the Expert Panel as this dossier. In addition, the Expert Panel independently evaluated materials deemed appropriate and necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed that DASP is considered safe when consumed up to up to 44.8 g/day in individuals over the age of 1 (one) year, but higher levels of use have not been evaluated.

2. INTRODUCTION

Phenylketonuria (PKU) is an inborn error of metabolism (IEM) characterized by hyperphenylalaninemia (HPA) and is caused by variants in the gene coding for phenylalanine hydroxylase (PAH; EC 1.14.16.1), rendering the individual less than optimally effective in converting phenylalanine (PHE; an essential amino acid) to tyrosine (TYR) (Figure 1), resulting in formation of phenylpyruvate and urinary ketone bodies (*i.e.,* phenylketonuria) (Cederbaum, 2002; van Wegberg *et al.*, 2017). The severity of PKU depends upon the extent to which the PAH mutation reduces the activity of PAH (Blau, 2016). PHE conversion to TYR (the latter is required for production of thyroxine, catecholamines and melanin (MacLeod and Ney, 2010)) is critical for brain development; for if left untreated, severe pathologic sequalae develop (Figure 2) including, but not limited to intellectual disability, epilepsy, behavioral and social problems (Hagedorn *et al.*, 2013; van Wegberg *et al.*, 2017), or even death (Regier and Greene, 2000). It should be noted that pathological sequalae may develop even into adulthood if proper disease management is not maintained. Further, uncontrolled HPA during pregnancy is teratogenic, and produces a range of severe cognitive, neurological and physical deficits similar to fetal alcohol syndrome, mandating control of HPA in the first trimester (Hagedorn *et al.*, 2013). Children of PKU mothers have also been reported to have an abnormally small head (microcephaly) and/or congenital heart disease, and a variety of facial abnormalities (NORD, 2019); collectively, these effects are called maternal PKU (MPKU syndrome) (Vockley *et al.*, 2014). Because of the pathological sequelae resulting from uncontrolled PHE blood levels, dietary foods used to maintain low PHE blood levels were

[1] Modeled after that described in Section 201(s) of the Federal Food, Drug, and Cosmetic Act, as amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.
[2] The terms "deactivated" and "denatured" are used interchangeably in this document, when referring ASP that has been purposely converted to a form that no longer has enzymatic activity.
[3] Relevant literature cited in the electronic database search was reviewed. Literature not cited in the search or literature published subsequent to the search, may not have been included in the review.

once regarded as drugs; however, they are now considered a medical food by the Food and Drug Administration (21 CFR § 101.9(j)(8)).

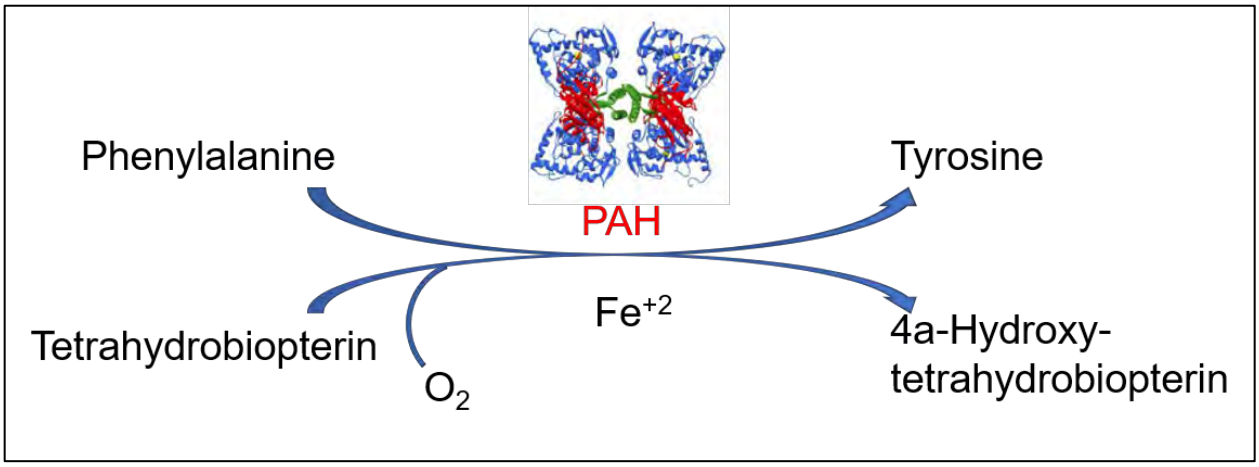

Figure 1. Metabolism of phenylalanine to tyrosine *via* phenylalanine hydroxylase (van Wegberg *et al.*, 2017). PAH=phenylalanine hydroxylase

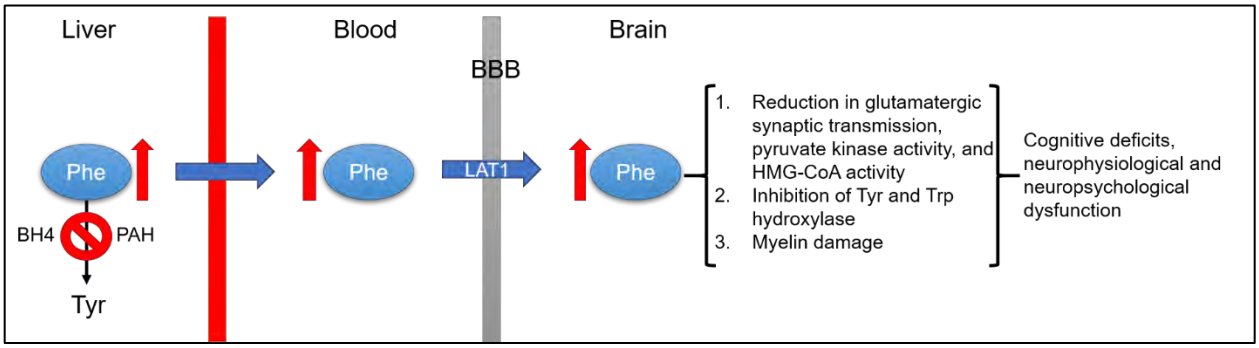

Figure 2. Pathophysiology of PKU and potential mechanisms of neurocognitive impairment by high phenylalanine concentrations (van Wegberg *et al.*, 2017). BBB=Blood brain barrier; BH4=Tetrahydrobiopterin; HMG-CoA=β-Hydroxy β-methylglutaryl-CoA; LAT1=L-type amino acid carrier; PAH=phenylalanine hydroxylase; PHE=phenylalanine; TRP=tryptophan; TYR=tyrosine

PKU can be controlled successfully through diet alone as the primary treatment method under medical supervision (Hagedorn *et al.*, 2013). Treatment consists of a near abstinence (in as much as possible) from consumption of PHE-containing foods; however, as protein is an essential part of the diet, PHE is present in significant amounts in meat and vegetables. An alternative to consuming a conventional diet is a mostly synthetic diet consisting of very low PHE. Medical foods[4] are of particular importance to dietary management of PKU, as they may be formulated to specifically exclude PHE from their composition (Camp *et al.*, 2012). Nxt2B has produced a low PHE protein source to supplement the diets of individuals with PKU. The low protein source consists of a modified alkaline serine protease (ASP) to contain a low concentration of PHE (<0.25%) using *Bacillus licheniformis* strain 2709, an organism commonly utilized for the production of enzymes for use in food (de Boer *et al.*, 1994; Olempska-Beer *et al.*, 2006). The modified alkaline serine protease (ASP) (native ASP has the CAS#9014-01-1; and is characterized as having 274 amino acids; molecular weight 27413.43; and an isoelectric point (pI) of 6.7) is

[4] Food that are specially formulated to meet the distinct nutritional requirements of a disease or other condition (21 U.S.C. 360 ee(b)(3)).

subsequently denatured with the use of heat (denatured ASP or DASP). Nxt2B plans to offer DASP as a source of low-PHE protein for incorporation into medical foods for consumption by individuals with PKU. The medical food will be nutritionally complete and deliver <25 mg PHE/serving. To evaluate the safety of DASP as an ingredient in foods, an *in vitro* bacterial reverse mutation assay, two 14-day dietary toxicity/palatability studies in rats, and a 90-day subchronic dietary toxicity study in rats were completed. This dossier is a summary of the scientific evidence that supports the general recognition that deactivated DASP is safe for human consumption as a food ingredient under the specific conditions of use.

2.1. Theory of Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 CFR § 170.36, substances evaluated by an Expert Panel to be Generally Recognized As Safe (GRAS) for intended food applications may be exempt from pre-market approval. Conventionally, a toxicological evaluation is performed utilizing the estimated daily intake (EDI) of the substance under its intended use that is then compared to an acceptable daily intake (ADI) established in a safety evaluation from data available in published literature. However, DASP is being considered for use as a low- PHE protein supplement in medical foods. As such, it may be considered a macro-additive.[5] FDA provides guidelines for the safety assessment of macro-additives in Draft Redbook II, Chapter VII (FDA, 1993):

> The common characteristic of macro-additives is that they will be consumed in large quantities compared to conventional food additives and, as a consequence, they will present testing problems that require "customized" approaches. For example, it may not be feasible to calculate safety factors in the conventional way, that is, as a fraction of the highest oral dose that has no adverse effects in animals. Other means of providing margins of safety for macro-additives will have to be used; these may include information derived from metabolic, pharmacokinetic, and human clinical studies.

DASP may be categorized as a macro-additive, thus utilizing a traditional toxicokinetic and toxicodynamic approach poses difficulties; mainly, a no observed adverse effect level (NOAEL) may not be solely utilized in the safety assessment. Instead, a weight-of-evidence approach is necessary in determining the safety of the macro-additive. The weight-of-evidence approach incorporates a holistic evaluation of data including similarities to other substances with a history of safe use in food, the toxicokinetics of the substance under its intended use, and other specification and manufacturing data in addition to traditional toxicology testing. This dossier in support of a conclusion of GRAS will incorporate all relevant data to fully assess the safety of DASP under its intended use.

Additionally, DASP is intended to be a macro-additive in medical foods to act as a PHE-free source of protein for PKU patients. Medical foods are defined in section 5(b)(3) of the Orphan Drug Act (21 U.S.C. 360 ee(b)(3)) as:

> A food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation.

[5] Macro-additives are a class of food additives that are intended to be replacements for conventional macro-nutrients such as fats, proteins, and carbohydrates and are intended for use at relatively high levels in food (FDA, 1993).

Further, FDA has established criteria that clarify the statutory definition of medical foods *per* 21 CFR § 101.9(j)(8). The criteria include:

i. special formulation and processing of the product for the partial or exclusive feeding of a patient,

ii. the product is intended for the dietary management of a patient that due to therapeutic or chronic medical needs has a limited capacity to ingest, digest, absorb, or metabolize ordinary foodstuffs or certain nutrients, or who has other medically determined nutrient requirements, the dietary management of which cannot be achieved by the modification of the normal diet alone,

iii. the product provides nutritional support specifically modified for the management of the unique nutrient needs that result from a disease or condition,

iv. the product is intended to be used under medical supervision, and

v. the product is intended only for a patient receiving active and ongoing medical supervision on a recurring basis.

FDA also references PKU as a specific IEM that may be managed with medical foods in Guidance for Industry: Frequently Asked Questions About Medical Foods (Docket Number: FDA-2013-D-0880). DASP meets the criteria set forth by FDA as a medical food ingredient as it is specially formulated and processed for exclusive use by PKU patients in their dietary management of PHE levels that are influenced by their decreased ability to metabolize PHE in normal foodstuffs. Additionally, PKU patients undergoing dietary management of their disease do so under medical supervision such that DASP would be used under medical supervision on a recurring basis.

2.2. History of use

DASP does not have a documented history of use; however, DASP is comparable to subtilisin, an ASP that is commonly used as a detergent to remove proteinaceous substances (von der Osten *et al.*, 1993; Vojcic *et al.*, 2015; Sundus *et al.*, 2016). Subtilisin may also be used as an enzyme in organic synthesis (Wong *et al.*, 1990), as a cosmetic ingredient,[6] in food processing (Bhunia *et al.*, 2012), and in contact lens cleaner (Barton *et al.*, 1988). It should be noted that DASP is a denatured ASP; therefore, it does not have the enzymatic capabilities associated with subtilisin. Active alkaline proteases have a history of use in foods, as well as food supplement intake in the US and other parts of the world. *B. licheniformis* isolated from fermented foods such as Thai fish sauce and tempeh are known to produce various proteases including subtilisin-like ASPs (Toyokawa *et al.*, 2010; Afifah *et al.*, 2017). Section 2.4 describes the regulatory status of subtilisin-like ASPs in detail; however, in short subtilisin and similarly structured proteases have been subject to several GRAS conclusions notified to FDA as food processing enzymes, see Table 2. Similarly, subtilisin produced by bacteria are approved food additives codified in the Code of Federal Regulations (21 CFR § 184.1150). Lastly, protease from *B. licheniformis* is present in FDA's Substances Added to Food list (formerly EAFUS) for use as an enzyme, flavor enhancer or agent, processing aid, and stabilizer or thickener.[7] There is significant evidence that both ASPs

[6] https://cosmetics.specialchem.com/inci/subtilisin; last accessed January 26th, 2021.
[7] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=FoodSubstances&id=PROTEASEFROMBACILLUSLICHENIFORMIS&sort=Sortterm_ID&order=ASC&startrow=1&type=basic&search=protease; last accessed January 26th, 2021.

and enzymes produced by *B. licheniformis* are overwhelmingly safe for use in food and optimizing the use of *B. licheniformis* as a production organism for ASPs continues to be an active area of research (Zhou *et al.*, 2019, 2020).

2.3. Current uses

DASP does not have any current uses. However, ASP and other proteases have a long history of use in food and continue to be utilized as food processing enzymes. Similarly, *Bacillus licheniformis* also continues to be widely utilized as a safe production organism for food grade enzymes. Table 1 describes various commercial alkaline proteases produced by *B. licheniformis* and their applications. Currently, ASPs encompass over a third of industrial enzyme sales for use in detergents, food processing, leather production, and waste processing (Bhunia *et al.*, 2012). Proteases are mainly used in food processing to improve the flavor, nutritional value, solubility, and digestibility of food proteins as well as to modify their functional properties including coagulation and emulsification (Raveendran *et al.*, 2018).

Table 1. Commercial DASP analogs in food products (adapted from Bhunia *et al.*, 2012)

Supplier	Product Trade Name	Application
Novo Nordisk	Alcalase, Savinase, Esperase, Biofeed pro, Durazym, Novozyme 243	Detergent, silk degumming, food, feed
Solvay Enzymes	Protease	Alcohol, baking, brewing, feed, food, leather, waste
Enzyme Development	Enzeco Alkaline protease	Industrial food processing
Godo Shusei	Godo-Bap	Detergent, food

2.4. Regulatory Status

DASP is not an approved food additive nor has it been subject to a conclusion of GRAS nor to our knowledge does it enjoy "prior sanction" status. A search of FDA regulatory documents did not indicate any other similarly denatured proteins were notified or approved for use in food. However, there are several DASP analogs that have been subject to regulation (Table 2). Subtilisin, an active enzyme comparable to DASP prior to denaturation, is GRAS affirmed *per* 21 CFR § 184.1150 (Bacterially-derived protease enzyme preparation). Subtilisin may only be obtained from the culture filtrate resulting from a pure culture fermentation of a nonpathogenic and nontoxigenic strain of *Bacillus subtilis* or *B. amyloliquefaciens* and may be used in foods as an enzyme to hydrolyze enzymes and polypeptides at levels not to exceed current Good Manufacturing Practice (cGMP). Similarly, protease enzyme product resulting from a pure culture fermentation of a nonpathogenic strain of *Bacillus licheniformis* is approved as a food additive *per* 21 CFR § 184.1027; it may be used as an enzyme to hydrolyze proteins in alcoholic beverages, candy, nutritive sweeteners, and protein hydrolyzates at levels not to exceed cGMP. Additionally, several GRAS conclusions for serine proteases have been notified to FDA. They include subtilisin, used as a protein hydrolysis agent in the processing of protein, produced *via* genetic engineering (GRN 714), serine protease used as a food processing (GRN 563; GRN 564), and serine endopeptidase (thermomycolin) used as a food processing enzyme (GRN 817). Notably, all GRAS notices herein discuss heat deactivation of their enzymes prior to consumption. Protease from *B. licheniformis* is included in FDA's Substances Added to Food list (formerly EAFUS) for use as an enzyme, flavor enhancer or agent, processing aid, and stabilizer or thickener, though no maximum level of use is indicated.

Table 2. Regulatory status of DASP-like analogs in food products

Substance	Production Organism	Intended Use	Regulation
Subtilisin	*B. subtilis or B. amyloliquefaciens*	Food Processing Enzyme	21 CFR § 184.1150
Subtilisin	*B. subtilis*	Food Processing Enzyme	GRN 714
Serine Protease	*B. licheniformis*	Food Processing Enzyme	GRN 564
Serine Protease	*F. venenatum*	Food Processing Enzyme	GRN 563
Serine Endopeptidase	*T. reesei*	Food Processing Enzyme	GRN 817
Protease	*B. licheniformis*	Food Processing Enzyme	21 CFR § 184.1027
Protease	*B. licheniformis*	Enzyme, flavor enhancer, flavoring agent or adjuvant, processing aid, stabilizer or thickener	FDA Substances Added to Food (formerly EAFUS)

2.5. Proposed use or uses

DASP produced by *B. licheniformis* is intended for use as a macro-additive in medical foods in the diet of individuals with PKU over the age of 1 (one) year. It will be incorporated into nutritionally complete medical foods as a source of low-PHE protein with <25 mg PHE/serving.

3. DESCRIPTION, SPECIFICATIONS AND MANUFACTURING PROCESS

3.1. Description and Specifications

DASP is an off-white powder composed of >90% protein with <0.25% of the protein composed of PHE. DASP is expressed in *B. licheniformis* strain 2709 and composed of 274 amino acids (Table 3).

3.1.1. Creation of recombinant ASP

The ASP enzyme (274 amino acids, molecular weight 27413.43 with a pI = 6.7) is an alkaline protease from *Bacillus licheniformis* strain CICC10266 (synonymous to strain 2709). This ASP gene was cloned and inserted into vectors by restriction enzyme digestion and T4 DNA ligase ligation. The PCR products were double digested with BamH I and Sma I and then ligated into pHT01, pHT43, pHT100, pHT223, pHT250, pHT431, pHT432 and pHT433 vectors (for screening purposes), which were digested with the same restriction endonuclease to form the recombinant vectors. The ligated products were first transformed into *E. coli* DH5a, a standard screening organism, and analyzed for the correct insert by DNA sequencing. They were then introduced into *B. subtilis* WB800N, a genetically engineered variant of *B. subtilis* 168, which has all extracellular proteases disrupted. Four PHE residues (F21, F50, F188, F260) in the alkaline protease gene (apr) from the *Bacillus licheniformis* CICC10266 strain were replaced with TRP and TYR by site-directed mutagenesis PCR or using a Multi-points Mutagenesis kit (TaKaRa, Japan), resulting in the final DASP sequence (10266apr-W4).

3.1.2. Integration of recombinant ASP gene into production organism

Bacillus licheniformis CICC10266 [8] (apr yhfN) was used in the final construction. CICC10266 (apr yhfN) is an alkaline protease apr and an intracellular protease yhfN genes defective derivative. Three distinct sites on the *B. licheniformis* chromosome, apr (alkaline protease locus), xyl (xylose isomerase locus), and gnt (gluconate permease locus) were used as integration sites to overproduce the DASP protein (Figure 3). For integrating the gene into three different sites, three integrative vectors containing the modified alkaline protease gene were constructed. The pEBKan194-GFP plasmid was used for construction of various knockout and knockin vectors.

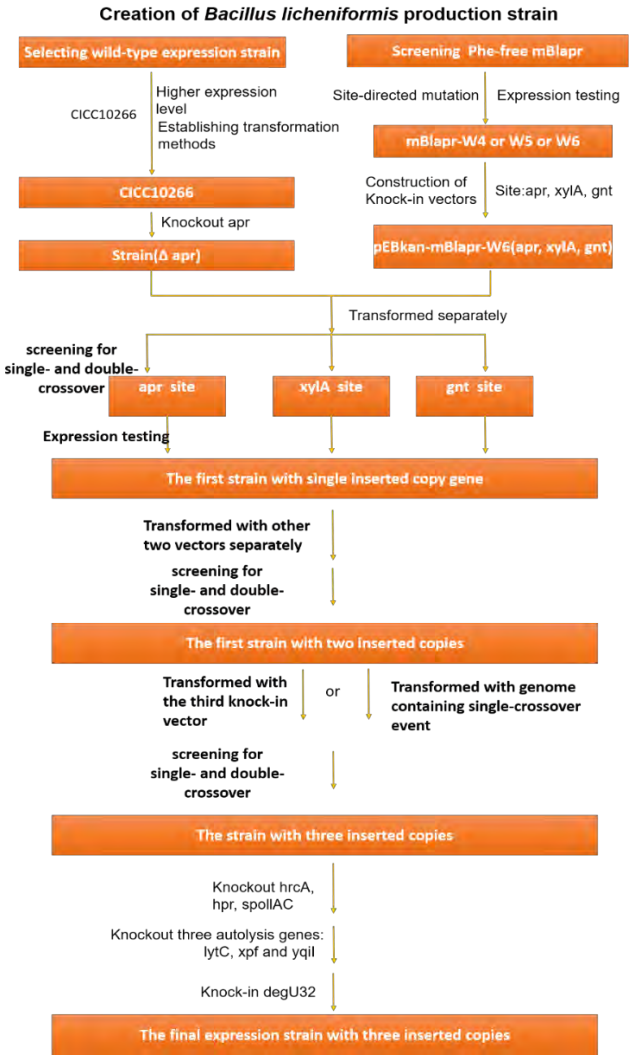

Figure 3. Recombination scheme of the *B. licheniformis* production organism

The ASP gene within the *B. licheniformis* genome of the production organism has been confirmed by Captozyme/Arranta Bio. Sequence verification of the expressed enzyme has also been performed (Captozyme Analysis, 2017).

[8] http://gcm.wfcc.info/Strain_numberToInfoServlet?strain_number=CICC%2010266; last accessed January 26th, 2021.

3.1.3. Composition and Specifications

After the gene was cloned and inserted into the genome of *B. licheniformis* strain 2709, the DASP protein was sequenced.[9] DASP is composed of >90% protein with <0.25% of the protein made up of PHE (Appendix 12.1). The amino acid composition and specifications for DASP are provided in Table 3 and Table 4, respectively.

Table 3. Amino acid composition of DASP (Burdock *et al.*, 2020)

Amino acid	Number of amino acids in DASP	Percent (%) composition	Amino acid	Number of amino acids in DASP	Percent (%) composition
Alanine	41	15.0	Leucine	16	5.8
Arginine	4	1.5	Lysine	9	3.3
Asparagine	19	6.9	Methionine	5	1.8
Aspartic Acid	9	3.3	Phenylalanine	0	0.0
Cysteine	0	0.0	Proline	9	3.3
Glutamine	7	2.6	Serine	32	11.7
Glutamic Acid	5	1.8	Threonine	19	6.9
Glycine	35	12.8	Tryptophan	4	1.5
Histidine	5	1.8	Tyrosine	14	5.1
Isoleucine	10	3.6	Valine	31	11.3

Table 4. Specifications of DASP

Analysis	Method	Specification	Batch Analysis Results (*N*=6)	
			Range	Average
Appearance	Visual inspection	Off-white to light-brown		
Enzymatic activity	SOP-125	None detected		
Moisture	AOAC 930.15	<5.0%	3.1–4.2%	3.58%
Ash	AOAC 923.03/ 32.1.05 16th Ed.	<1 g/100 g	0.2–0.6 g/100 g	0.4 g/100 g
Total organic solids	Calculated*	>93%		
Phenylalanine	AOAC 982.30	<250 mg/100 g	110–170	143
Protein	AOAC 982.30	≥90 g/100 g	92.6–94.1	93.2
Microbiological analyses				
Total aerobic microbial count	USP<61>	<10000 CFU/g	150–4200 CFU/g	1037 CFU/g
Total combined yeasts and molds count	USP<61>	<1000 CFU/g	<10 CFU/g	<10 CFU/g
Escherichia coli	USP<62>	Absent/g	ND	
Salmonella	USP<62>	Absent/10 g	ND	
Gram-negative bacteria	USP<62>	Absent/g	ND	
Heavy metals analyses				
Arsenic	AOAC 986.15 mod	<0.01 ppm	ND	MDL=0.05 ppm
Cadmium	AOAC 986.15 mod	<0.01 ppm	ND	MDL=0.05 ppm
Lead	AOAC 986.15 mod	<0.01 ppm	ND	MDL=0.05 ppm
Mercury	AOAC 986.15 mod	<0.01 ppm	ND	MDL=0.01 ppm

*%TOS=100 (A + W + D); A=ash; W=water; and D=diluents and/or other additives and ingredients.
CFU=colony forming units; MDL=method detection limit; ND=not detected; ppm=parts *per* million.

[9] Amino acid sequence:
AQTVPYGIPLIKADKVQAQGWKGANVKVAVLDTGIQASHPDLNVVGGASYVAGEAYNTDGNGHGTHVA
GTVAALDNTTGVLGVAPSVSLYAVKVLNSSGSGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAV
DNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASWSSVGAELEVMAPGAGVYST
YPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRLSSTATYLGSSWYYGKGLINVEAAAQ

Residual PHE from the production organism is present in the batch analyses of DASP with a specification of <250 mg/100 g (0.25%) (Table 4). The presence of this small amount of PHE residue is addressed in cGMP 21 CFR § 110.110(a):

> (a) Some foods, even when produced under current good manufacturing practice, contain natural or unavoidable defects that at low levels are not hazardous to health. The Food and Drug Administration establishes maximum levels for these defects in foods produced under current good manufacturing practice and uses these levels in deciding whether to recommend regulatory action.

While the FDA has not set a defect action level for PHE in food for phenylketonuria, this is a *de minimis*[10] amount and the substance is in compliance with 21 CFR § 101.100 (a)(3) (Exemptions from food labeling requirements):

> (3) Incidental additives that are present in a food at insignificant levels and do not have any technical or functional effect in that food. For the purposes of this paragraph (a)(3), incidental additives are:
>
> (i) Substances that have no technical or functional effect but are present in a food by reason of having been incorporated into the food as an ingredient of another food, in which the substance did have a functional or technical effect.
>
> (ii) processing aid(s), which are as follows:
>
> (a) Substances that are added to a food during the processing of such food but are removed in some manner from the food before it is packaged in its finished form.
>
> (b) Substances that are added to a food during processing, are converted into constituents normally present in the food, and do not significantly increase the amount of the constituents naturally found in the food.
>
> (c) Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food.

3.2. Manufacturing process

DASP is manufactured in accordance with cGMPs in four major steps: (1) submerged fermentation, (2) purification by ceramic membrane filtration and washed, (3) spray dried, then (4) packaged (Figure 4).

The genetically engineered *B. licheniformis* production strain, derived from strain 2709 is maintained in a cell bank until needed for production, at which time the cells are thawed, streaked on a lysogeny broth (LB) agar plate, and incubated overnight (37°C). Then four 60 mL flasks of LB media are inoculated with a clone and cultured for 12 hours (37°C and 220 ppm) or until the optical density (OD) at 600 nm (OD_{600}) reaches 5 – 8 to produce seed media. The seed media (50 mL) is then used to inoculate 5 L of seed media (1%) within a seed fermenter and fermented at 37°C until OD_{600} reaches 20 – 28 (*i.e.,* "first seed culture"). The 5 L from the "first seed culture" is used to inoculate 200 L of seed media (4%) in a second fermenter for 5.5 hours at 37°C to produce the "second seed culture". Finally, 140 L of the "second seed culture" inoculates 3000 L of seed media (4.6%) at 37°C for ≥16 hours (at the native pH of the seed media). After 16 hours,

[10] *De minimis* is a legal principle which allows for matters that are small scale or of insufficient importance to be exempted from a rule or requirement. It can be used by the courts as an exclusionary tool to dismiss trivial matters from litigation.

the fermentation broth is analyzed every hour for the total protein concentration. Once the total protein concentration stops increasing, the fermentation broth is discharged for purification.

During purification, the pH of the fermentation broth is adjusted to 4.5 before pumping the broth into a 200 nm ceramic membrane filter (CMF) for five minutes at 200 kPa. From the filtrate valve, the filtered fluid is collected in a storage vat and sodium chloride (1 kg NaCl/100 kg filtrate) is added. Hydrochloric acid (HCl) at a concentration of 6M is added to adjust the pH to 1.4 – 1.65, then the filtered fluid stews for 5 – 10 hours at room temperature. The supernatant is drawn away and discarded and the remaining suspension is pumped into the storage tank of the tangential flow system with 100 nm CMF. The suspension is concentrated to approximately $1/10^{th}$ of the starting volume and water is added until the pH reaches 2.6 – 3.0 with a final volume change of less than 10%. Subsequently, sodium carbonate (Na_2CO_3) is used to adjust the concentrated suspension to approximately pH 9.

To deactivate the enzyme, the concentrated suspension is heated to 70°C for 20 minutes. The concentrated suspension is then dried using a centrifugal spray dryer. The dried deactivated enzyme, which is an off-white powder, is called DASP. DASP is packaged into sterile polyurethane bags with large sealable foil pouches. Desiccant pouches are placed between the polyurethane bags and foil pouches. The packaged material is stored at room temperature.

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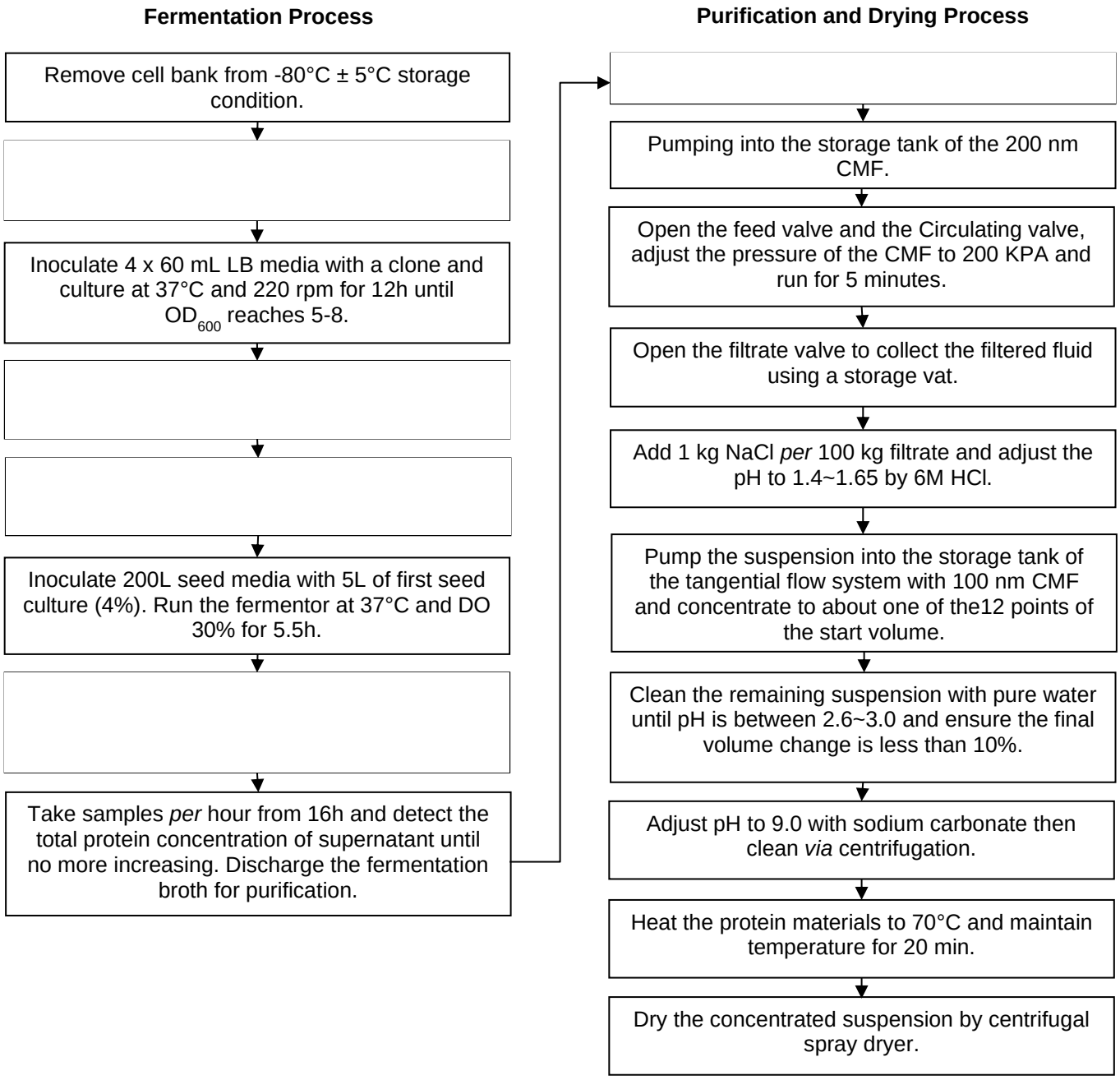

Figure 4. Deactivated ASP production scheme

3.3. Stability

DASP is a denatured enzyme intended for use as a protein source with low levels of the amino acid phenylalanine. As such, its stability over time to perform its intended function is dependent on maintaining its primary structure. Peptide bonds are notably difficult to break under normal ambient conditions; additionally, if primary structure becomes broken, this does not impact the function of the protein as a medical food. As such, DASP is very stable for up to 24 months at temperatures ranging from 5°C – 40° C with minimal degradation occurring during storage. Three lots each were analyzed at 5°C, 25°C, and 40°C for appearance, enzyme activity, protein concentration and weight, water activity and flow-ability, purity, and integrity (see Appendix 12.2) (Captozyme Stability Report, 2021). The chemical stability of DASP under these food-processing conditions demonstrates that DASP is a stable food ingredient.

4. ESTIMATED DAILY INTAKE

Table 5 describes the various phenylalanine tolerances recommended for individuals with phenylalanine-related disorders. The amount of phenylalanine that is found naturally in food is about 2 – 9% of the total protein weight (Kim and Boutin, 2015; Araújo *et al.*, 2016). As such, diets for individuals with classic PKU are recommended to contain less than 20% of protein coming from foods (approximately 5 – 10 g *per* day) (MacLeod and Ney, 2010). To maintain an adequate healthy metabolism, the diet must be supplemented with synthetic essential amino acids free of phenylalanine that would make up approximately 80% the total protein intake of individuals with PKU. For the purpose of the estimated daily intake (EDI) assessment, DASP serves as a source of amino acids free of phenylalanine that could be consumed at up to 80% the total protein intake of individuals over the age of 1 (one) year.

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Table 5. PHE tolerances for various PHE -related disorders (MacLeod and Ney, 2010; Camp *et al.*, 2014; NORD, 2019)

PHE -related disorder	Classification Schemes					
			PHE Tolerance			
	Pretreatment PHE level (traditional–modified)	PHE and PHE: TYR ratio in newborn period	Infants <1 year (mg/kg/day)	2 to 5 years old to maintain PHE at 300 µmol/L	>Age 5 years to maintain PHE 120–300 µmol/L	PAH Genotype
Tetrahydrobiopterin Deficiencies	Normal to Elevated	**PHE** 2–5 mg/dL (120–2120 µmol/L) (some normal)	Variable	Variable	Variable	N/A
PAH Deficiency Requiring Treatment						
Classical PKU	>1200 µmol/L (>20 mg/dL)	**PHE** ≥7 mg/dL (≥420 µmol/L) **PHE:** TYR >5	25–45 mg/kg/day 130–330 mg/day	<20 mg/kg/day 250–350 mg/day	<12 mg/kg/day	2 Classic mutation (often null)
Moderate PKU	900–1200 µmol/L (15–20 mg/dL)	Limited data	45–50 mg/kg/day	20–25 mg/kg/day 350–400 mg/day	12–18 mg/kg/day	Classic + moderate or 2 moderate mutations
Mild PKU	600–900 µmol/L (10–15 mg/dL)	Limited data	55 mg/kg/day	25–50 mg/kg/day 400–600 mg/day	>18 mg/kg/day	Classic, moderate or mild mutation + 1 mild HPA mutation
Mild HPA-gray zone	360–600 µmol/L (6–10 mg/dL)	Limited data	70 mg/kg/day	>50 mg/kg/day	No data	Classic, moderate or mild mutation + 1 mild HPA mutation
PAH Deficiency Not Requiring Treatment						
Mild HPA-NT	120–360 µmol/L (2–6 mg/dL)	**PHE** 151–360 µmol/L (avg 244) **PHE**: TYR 0.8–8.25 (avg 3.3)	Unrestricted diet	Unrestricted diet	Unrestricted diet	Classic, moderate or mild mutation + 1 mold HPA mutation

HPA=hyperphenylalaninemia; N/A=not applicable; PAH=phenylalanine hydroxylase deficiency.

Table 6 contains weights for various age groups and their recommended protein intake based on values published in USDA's Dietary Guidelines for Americans (2020). Conservatively assuming if 80% of all protein consumed is in the form of DASP, the estimated daily intake of DASP at various life stages in individuals with PKU would range from 460 – 912 mg/kg bw[11]/day. The highest consumption level calculated (EDI) is conventionally used as the mean for current consumption, and the 90th *percentile* current EDI of DASP may be estimated by assuming two times greater consumption than the reported mean current EDI. However, considering DASP is intended as a macro-additive for individuals with PKU that closely monitor their protein intake daily, it is unlikely that these individuals would consume protein at a much higher amount than the recommended daily intake published by USDA.

Table 6. Daily Protein Goals for Age-Sex Groups Based on Dietary Reference Intakes & Dietary Guidelines Recommendations

Age-Sex Group	Weight[a] (kg)	Protein[b] (g)	Estimated DASP intake[c] (g)	DASP Intake (mg/kg bw/day)
Child 1–3	11.4–13.8	13	10.4	754–912
Male/Female 4–8	18.6–31.8	19	15.2	478–817
Male/Female 9–13	56.8	34	27.2	479
Female 14–18	71.6	46	36.8	514
Male 14–18	71.6	52	41.6	581
Female 19–51+	80	46	36.8	460
Male 19–51+	80	56	44.8	560

[a]Based on values established by the EPA Exposure Factors Handbook (2011); [b]Values established by the USDA in 2020-2025 Dietary Guidelines; [c]DASP intake equivalent to 80% the total recommended protein intake.

5. ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION (ADME)

DASP is an off-white powder composed of 274 amino acids with a molecular weight of 27,413.43 g/mol and isoelectric point (pI) of 6.7. DASP is expressed in *B. licheniformis* strain 2709 and is composed of >90% protein with <0.25% of the protein composed of PHE. DASP is absorbed, distributed, and metabolized as any other protein. In the following toxicokinetic evaluation, DASP is similar to any other inactivated protein that leaves the stomach, *i.e.,* a chain of amino acids with a disrupted secondary and/or tertiary structure. A brief review of protein catabolism will be provided, followed by a review of amino acid metabolism.

5.1. Absorption and Bioavailability

Upon consumption, DASP is subject to the many broad acting proteases produced by the body at various stages of digestion. There is no digestion of proteins in the mouth. The first protease to act on DASP upon digestion is pepsin in the stomach. Pepsin is able to act on DASP as any secondary or tertiary structure that may have been reformed after denaturing ASP is disrupted again by HCl in the stomach, such that DASP would only maintain its primary structure (peptide bonds). As an endopeptidase, pepsin cannot break the peptide bonds of terminal amino acids – as such, pepsin only acts to break DASP into smaller amino acid chains, but not monomeric amino acids. Additional proteases in the duodenum such as trypsin and chymotrypsin further hydrolyze partially digested DASP into small peptides and amino acids (Nelson and Cox, 2005). Intestinal enterocytes then absorb amino acids and small peptides *via* several transporters, mainly sodium-dependent cotransporters. Any peptides that are not absorbed by enterocytes are subsequently digested and fermented by colonic bacteria into short-chain fatty acids, dicarboxylic

[11] BW or bw = body weight.

acids, phenols, and ammonia (Frenhani and Burini, 1999). Small peptides absorbed by enterocytes are subsequently hydrolyzed by intracellular peptidases into monomeric amino acids (Wu, 1998). Amino acids are then transported to the circulatory system for distribution throughout the body. As a result of proteolysis in the gastro-intestinal system, the bioavailability of DASP, as such in its native form, is limited.

5.2. Distribution

Upon absorption, DASP-derived amino acids can be distributed to various tissues as part of an amino acid pool that serves as a resource for various cellular demands. The body maintains a large amount of this free amino acid pool in the blood for expedient distribution. Depending on metabolic needs, amino acids may be distributed to various cells for synthesis of proteins and essential amino acid derivatives (mainly nitrogen containing compounds), as well as to the liver for gluconeogenesis and ketogenesis as needed (Nelson and Cox, 2005).

5.3. Metabolism

DASP-derived amino acids may be utilized in a variety of metabolic pathways summarized in Figure 5. The main uses for dietary amino acids are as follows: (A) use in protein synthesis, (B) precursors for nucleotides, heme, neurotransmitters, and hormones, (C) as a source of energy *via* gluconeogenesis and ketogenesis, and (D) as part of the urea cycle (Nelson and Cox, 2005). It should be noted that the preceding description and Figure 5 do not constitute an exhaustive list of the pathways by which amino acids are metabolized and that many of these reactions are reversible, rate limited, and interconnect with one another. Further discussion of amino acid metabolism is outside the scope of this evaluation.

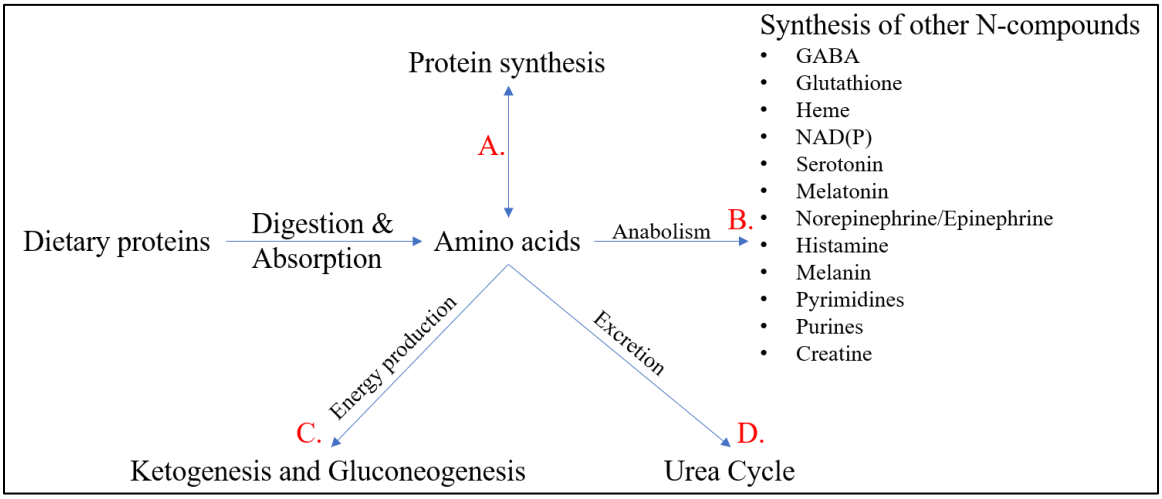

Figure 5. Various outcomes of amino acid metabolism including (A) protein synthesis, (B) synthesis of various nitrogen containing substances, (C) energy production including ketogenesis and gluconeogenesis, and (D) excretion of excess nitrogen *via* the urea cycle (Nelson and Cox, 2005).

5.4. Elimination

DASP-derived amino acid may be utilized in the biochemical pathways described previously. However, to maintain homeostatic conditions the body may excrete amino acids in the urine at values ranging from 0.26 – 0.60 mg/ml (Evered, 1956). It should also be noted that excess nitrogen from amino acid metabolism can be excreted *via* the urine in the form of metabolites including ammonium (NH_4^+), urea, uric acid, or creatinine.

6. SAFETY EVALATION

6.1. Production Strain

A concern in the safety evaluation of a protein synthesized *via* bacterial fermentation is the safety of the production strain. Certain strains may produce various protein and non-protein metabolites that induce toxicity upon consumption (Pariza and Johnson, 2001), therefore consideration of bacterial strain is critical in safety evaluation. DASP is expressed in *B. licheniformis* strain 2709, a non-toxigenic, non-pathogenic saprophytic microorganism that is widespread in nature. *B. licheniformis* has a long history of safe industrial use of producing enzymes used in human food and has been routinely used as a host for recombinant enzymes (Olempska-Beer *et al.*, 2006) and is often referenced as a well-characterized and safe production strain with a long history of safe use (IFBC, 1990). Various enzymes produced by *B. licheniformis* have been concluded as GRAS including alpha-amylase (GRN 22; GRN 24; GRN 79), pullulanase (GRN 72), xylanase (GRN 472), and glutaminase (GRN 774). Additionally, all processing-aids and raw materials used in the manufacturing of DASP are food or pharmaceutical grade quality and used in accordance with the appropriate federal regulations and/or have been previously determined to be GRAS for such uses (Table 7).

Table 7. Raw Material List

Raw Material	Process	Function
Yeast Extract	Fermentation	Nutrient
Peptone	Fermentation	Nutrient
NaCl	Fermentation	Trace Inorganic Salts
Agar	Fermentation	Nutrient
Corn Starch	Fermentation	Nutrient
Soybean powder	Fermentation	Nutrient
K_2HPO_4	Fermentation	Buffers
NaH_2PO_4	Fermentation	Buffers
Thermostable alpha-amylase	Fermentation	Other
Silicone-polyether defoamer	Fermentation	Other
$CaCl_2$	Fermentation	Trace Inorganic Salts
NaOH	Fermentation/Purification	Bases
HCl	Purification	Acids
HNO_3	Purification	Acids

6.2. Introduced Genetic Sequence

The safety of a recombinant organism is dependent on the source of the introduced genetic sequence, as well as the method of transformation and construction of the final production organism. This process is thoroughly detailed and characterized in Section 3.1. The starting ASP gene is well characterized, and all vectors and intermediate organisms are well defined and commonly utilized in protease selection, characterization, and amplification. Similarly, the methods utilized to modify the production organism are well defined and commonly used in enzyme production. Only well characterized DNA limited solely to the protease coding sequence are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

6.3. Acute Studies

No acute toxicity studies utilizing DASP have been reported in literature. As such, two 14-day toxicity studies were conducted with CRL Sprague-Dawley (SD) CD® IGS rats according to

OECD Guideline 407 (2008) and carried out according to current Good Laboratory Practice. Results are described in Sections 6.3.1 and 6.3.2 (Burdock *et al.*, 2020).

6.3.1. 14-day dietary toxicity/palatability study I

In a 14-day dose-range finding study, male and female CRL Sprague-Dawley (SD) CD® IGS rats (5 animals/sex/group) of approximately seven to eight weeks of age were treated with 0% (Group 1), 5.75% (Group 2), 11.5% (Group 3), or 23% (Group 4) DASP in the diet (Burdock *et al.*, 2020). Based on target nominal concentrations, male rat average daily intakes were 0 (control; Group 1), 5678.7 (low-dose; Group 2), 11037.2 (mid-dose; Group 3), and 11561.0 (high-dose; Group 4) mg DASP/kg bw/day, and female rat average daily intakes were 0 (control; Group 1), 4930.3 (low-dose; Group 2), 9700.7 (mid-dose; Group 3), and 12687.5 (high-dose; Group 4) mg DASP/kg bw/day. The animals in Group 2 and 3 were supplemented with the amino acids CYS and MET, while those in Group 4 were supplemented with CYS, MET, and PHE. Amino acid supplementation was administered *via* gavage (10 mL/kg bw) at levels described in Table 8. Aromatic amino acid[12] supplementation decreased from 10.2 g/kg diet to 1.9 g/kg diet once the animals were seven weeks of age, as at this age rats have either reached or are close to the maintenance stage for certain amino acids. As such, the aromatic amino acid requirement was achieved by the casein in the diet of Groups 2 and 3, and aromatic amino acid supplementation was only provided to the high-dose group. Food and water were provided *ad libitum*. Where applicable, the study was conducted according to OECD Guideline 407 (2008).

There were no treatment-related deaths. Although individual clinical observations such as thin appearances, reduced fecal volume, unkept appearance, superficial wounds, hair loss, and slight alopecia were noted in Group 3 and 4 animals, the effects were not related to treatment with DASP, and were reported as incidental. The mean body weights and mean daily body weight gains in Group 4 males and females were significantly decreased ($P<0.01$) when compared to the control. During Days 3 – 14 of treatment, male and female rats in Group 4 had significantly decreased ($P<0.01$) mean food consumption. The mean food efficiency for Group 4 males and females was also statistically significantly different ($P<0.05$) than the control. No DASP-related macroscopic findings were reported during the gross necropsy.

Table 8. 14-day palatability/toxicity study I treatment groups

Study	Treatment Group	Number of animals *per* group		Dietary protein (%) from DASP & Casein	Dietary protein (ppm) from DASP & Casein	Amino acid supplement (mg/kg bw/day)[a]		
		M	F			CYS	MET	PHE
14-day palatability /toxicity study I	1 (control)[d]	5	5	0 DASP; 23 casein	0 DASP; 230000 casein	0	0	0
	2 (low-dose)	5	5	5.75 DASP; 17.5 casein	57500 DASP; 175000 casein	14.4	28.8	0
	3 (mid-dose)	5	5	11.5 DASP; 11.5 casein	115000 DASP; 115000 casein	28.8	57.5	0
	4 (high-dose)	5	5	23 DASP; 0 casein	230000 DASP; 0 casein	57.5	115.0	79.1/ 450.0[c]

[a] Doses of amino acids in supplement as prepared for gavage in water vehicle.
[b] Concentration of amino acids in supplement as prepared for gavage in water vehicle.
[c] This group received 79.1 mg PHE/kg/day for Days 1–7 and 450 mg PHE/kg/day for Days 8–14.
[d] The control groups received only water (vehicle).
CYS=cysteine; DASP=deactivated alkaline serum protease; F=female; M=male; MET=methionine; PHE=phenylalanine; ppm=parts *per* million.

[12] Aromatic amino acids include PHE, TYR, and TRP.

6.3.2. 14-day dietary toxicity/palatability study II

In a second 14-day dose-range finding study, female CRL SD CD® IGS rats were administered DASP in the diet (5 female rats/treatment) to further assess potential toxicity and palatability of DASP (Burdock *et al.*, 2020). As shown in Table 9, Group 1 (choice diet) was provided a choice between two diets: 0% DASP (23% casein) or 23% DASP (0% casein) in the diet. Group 2 was provided a diet containing 11.5% DASP (supplemented with 11.5% casein), and Group 3 received a 23% casein diet with 0% DASP (control group). The mean daily intake for Group 2 animals was 10016.5 mg DASP/kg bw/day. The animals in Group 1 and 2 were supplemented with CYS, MET, and PHE *via* gavage (10 mL/kg bw) (Table 9). Food and water were provided to the animals *ad libitum*. There were no DASP-related deaths or abnormal clinical observations noted. Slight to moderate alopecia was noted on the forepaw of one animal and hair loss in a different animal, both in Group 3 (control). There were no statistically significant differences in mean body weight or body weight gain in Group 1 or 2 when compared to the control group (Group 3) ($P>0.05$). Group 1 (choice) food consumption of the basal diet was not significantly different from Group 3 food consumption, but there was a significant difference between the consumption of the 23% DASP by Group 1 animals when compared to Group 3 ($P<0.01$), indicating Group 1 animals preferred the basal diet over the DASP-containing diet. The food consumption of Group 2 compared to Group 3 was not significantly different ($P>0.05$). For the hematology and clinical chemistry analyses and gross necropsy, there were no DASP-related changes. Slight decreases in total serum protein (TP) ($P<0.01$), albumin (ALB) ($P<0.01$), and calcium (CA) ($P<0.05$) in Group 2 (mid-dose) females were reported, but the results were within historical controls ranges for the laboratory and unaccompanied by clinical signs or organ weight changes. When Group 2 and 3 mean absolute and relative organ weights were compared, there were no statistically significant differences ($P>0.05$).

Table 9. 14-day palatability/toxicity study II treatment groups

Study	Treatment Group	Number of animals *per* group		Dietary protein (%) from DASP & Casein	Dietary protein (ppm) from DASP & Casein	Amino acid supplement (mg/kg bw/day)[a]		
		M	F			CYS	MET	PHE
14-day palatability /toxicity study II	1 (basal/high-dose choice)	—	5	0 DASP; 23 casein	0 DASP; 230000 casein	57.5	115.0	79.1
				23 DASP; 0 casein	230000 DASP; 0 casein			
	2 (mid-dose)	—	5	11.5 DASP; 11.5 casein	115000 DASP; 115000 casein	28.8	57.5	0
	3 (control)[d]	—	5	0 DASP; 23 casein	0 DASP; 230000 casein	0	0	0

[a] Doses of amino acids in supplement as prepared for gavage in water vehicle.
[b] Concentration of amino acids in supplement as prepared for gavage in water vehicle.
[c] This group received 79.1 mg PHE/kg/day for Days 1–7 and 450 mg PHE/kg/day for Days 8–14.
[d] The control groups received only water (vehicle).
CYS=cysteine; DASP=deactivated alkaline serum protease; F=female; M=male; MET=methionine; PHE=phenylalanine; ppm=parts *per* million.

6.4. Subchronic Study

Burdock *et al.* (2020) reported a 90-day repeat-dose study in CRL SD CD® IGS rats at 0%, 2.875%, 5.75%, and 11.5% DASP (10 animals/sex/group) (Table 10). Mean daily doses were approximately equivalent to 0, 1594.3, 3100.0, or 6224.1 mg DASP/kg bw/day for male rats and 0, 1867.4, 3685.0, or 7500.9 mg DASP/kg bw/day for female rats, respectively. The animals were supplemented with CYS and MET *via* gavage (10 mL/kg bw/day). The diet and filtered tap water were provided to the animals *ad libitum*. The study was conducted according to OECD Guideline 408 (OECD, 1998) and carried out according to current Good Laboratory Practice.

Table 10. Subchronic toxicity study treatment groups (Burdock *et al.*, 2020).

Study	Treatment Group	Number of animals *per* group		Dietary protein (%) from DASP & Casein	Dietary protein (ppm) from DASP & Casein	Amino acid supplement (mg/kg bw/day)[a]	
		M	F			CYS	MET
Subchronic toxicity study	1 (control)[d]	10	10	0 DASP; 23 casein	0 DASP; 230000 casein	0	0
	2 (low-dose)	10	10	2.875 DASP; 20.125 casein	28750 DASP; 201250 casein	7.2	14.4
	3 (mid-dose)	10	10	5.75 DASP; 17.5 casein	57500 DASP; 175000 casein	14.4	28.2
	4 (high-dose)	10	10	11.5 DASP; 11.5 casein	115500 DASP; 115500 casein	28.8	57.5

[a] Doses of amino acids in supplement as prepared for gavage in water vehicle.
[b] Concentration of amino acids in supplement as prepared for gavage in water vehicle.
[c] This group received 79.1 mg PHE/kg/day for Days 1–7 and 450 mg PHE/kg/day for Days 8–14.
[d] The control groups received only water (vehicle).
CYS=cysteine; DASP=denatured alkaline serum protease; F=female; M=male; MET=methionine; PHE=phenylalanine; ppm=parts *per* million.

Two male rats (Group 1 and Group 2) were found deceased during the study, but the deaths were not considered to be DASP-related. Instead, macroscopic evaluation supported that the rats had oral gavage trauma. The ophthalmic exam results were normal for all groups, and there were no reported clinical observations in either male or female rats that were attributed to DASP consumption. Mean body weights, body weight gains, food consumption, and food efficiency of all surviving animals in the experimental groups were not significantly different that those of the control group. Similarly, there were no significant alterations in organ weights or organ-to-body/brain weight ratios between the experimental groups and control group. There were no findings attributed to treatment with DASP for hematology (Table 11 and Table 12), clinical chemistry (Table 13 and Table 14), coagulation (Table 11 and Table 12), and urinalysis (Table 13 and Table 14). Mean corpuscular hemoglobin concentration (MCHC) was significantly increased in females in the highest dose group; however, this change was reported as non-adverse considering a lack of correlation with other hematological parameters and lack of clinical or pathological signs. Several other parameters were reported as significantly different between experimental groups (including activated partial thromboplastin, prothrombin time, calcium, and globulin); these differences were reported to be within historical ranges for the laboratory. Additionally, elevated cholesterol levels in the experimental female groups were not attributed to DASP consumption as the levels were within historical ranges for the laboratory, the finding was not present in males, and high cholesterol values often induce increased bile acid synthesis, which leads to oxidative stress of the liver, causing an increase in AST and ALT and histopathologic changes in the liver – none of which were present in the females. Lastly, there were no macroscopic

or microscopic pathological findings attributed to DASP consumption. The authors reported a No Observable Adverse Effect Level (NOAEL) at 115,500 ppm DASP, the highest dose administered (approximately equivalent to 6224.1 mg DASP/kg bw/day in males and 7500.9 mg DASP/kg bw/day in females).

Table 11. Subchronic study male rats: Hematology and coagulation analyses

	0 ppm Group 1	28750 ppm Group 2	57500 ppm Group 3	115500 ppm Group 4
Hematology (Day 89)	***N*=9**	***N*=9**	***N*=10**	***N*=10**
WBC ($x10^3/\mu L$)	13.608±4.3688	11.408±1.9873	13.583±2.8179	12.230±3.0366
RBC ($x10^6/\mu L$)	9.258±0.3047	9.522±0.2283	9.231±0.2020	9.397±0.4092
HGB (g/dL)	16.09±0.428	16.23±0.328	16.16±0.310	16.41±0.4092
HCT (%)	50.50±0.912	51.54±0.866	50.14±1.132	51.01±1.524
MCV (fL)	54.61±2.007	54.17±1.105	54.33±1.342	54.30±1.371
MCH (pg)	17.37±0.658	17.07±0.545	17.49±0.453	17.48±0.577
RDW (%)	13.49±0.756	13.18±0.393	13.46±0.591	13.13±0.748
PLT ($x10^3/\mu L$)	1046.4±101.22	950.6±190.67	920.6±148.97	960.9±60.65
ANEU ($x10^3/\mu L$)	2.599±1.9075	1.958±0.5743	2.427±0.9347	2.188±1.4609
ALYM ($x10^3/\mu L$)	10.197±2.5529	8.683±1.6362	10.218±2.2304	9.184±2.3120
AMON ($x10^3/\mu L$)	0.334±0.1636	0.339±0.0929	0.389±0.1569	0.345±0.1953
AEOS ($x10^3/\mu L$)	0.223±0.0946	0.203±0.0742	0.259±0.0706	0.241±0.0736
ABAS ($x10^3/\mu L$)	0.149±0.0609	0.128±0.0758	0.175±0.1100	0.160±0.0718
ALUC ($x10^3/\mu L$)	0.098±0.0745	0.094±0.0508	0.117±0.0742	0.104±0.0885
ARET ($x10^3/\mu L$)	178.47±36.148	161.73±23.871	179.79±32.101	166.30±32.390
%RET	1.939±0.4533	1.699±0.2566	1.951±0.3719	1.780±0.3997
MCHC (g/dL)	31.81±0.575	31.49±0.523	32.23±0.340	32.19±0.428
Coagulation (Day 96)				
APTT (sec)	16.01±1.135	16.63±1.004	17.01±0.778	17.60±1.095**
PT (sec)	8.77±0.166	8.81±0.169	9.06±0.196*	9.10±0.275**

ABAS, absolute basophils; AEOS, absolute eosinophils; ALUC, absolute large unstained cells; ALYM, absolute lymphocytes; AMON, absolute monocytes; ANEU, absolute neutrophils; APTT, activated partial thromboplastin time; ARET, absolute reticulocytes; HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet count; ppm, parts *per* million; PT, prothrombin time; RBC, erythrocytes; RDW, red cell distribution width; WBC, total white blood cells; Mean ± SD shown; **Statistically significant $P \leq 0.01$; *Statistically significant $P \leq 0.05$.

Table 12. Subchronic study - female rats: Hematology and coagulation analyses

	0 ppm Group 1	28750 ppm Group 2	575000 ppm Group 3	115500 ppm Group 4
Hematology (Day 89)	***N*=10**	***N*=10**	***N*=10**	***N*=10**
WBC (x10^3/µL)	7.143±1.5248	6.815±2.1150	7.466±2.1286	8.012±2.8899
RBC (x10^6/µL)	8.821±0.5209	8.799±0.2292	8.775±0.3784	8.597±0.3175
HGB (g/dL)	15.70±0.506	15.85±0.458	15.88±0.473	15.78±0.469
HCT (%)	48.54±1.619	48.86±1.583	48.84±1.564	47.86±1.585
MCV (fL)	55.33±2.204	55.74±1.278	55.88±1.042	55.71±1.306
MCH (pg)	17.91±0.768	18.05±0.438	18.15±0.354	18.33±0.383
RDW (%)	12.09±0.412	12.00±0.350	11.68±0.355	11.97±0.386
PLT (x10^3/µL)	947.7±72.53	857.0±113.61	902.8±100.08	902.4±227.48
ANEU (x10^3/µL)	1.293±0.6470	1.082±0.6957	1.378±0.5412	1.504±1.0435
ALYM (x10^3/µL)	5.368±1.2424	5.224±1.6191	5.573±1.8929	5.863±1.8338
AMON (x10^3/µL)	0.208±0.0983	0.210±0.0831	0.233±0.0631	0.258±0.1134
AEOS (x10^3/µL)	0.142±0.0483	0.159±0.0803	0.155±0.0602	0.209±0.0681
ABAS (x10^3/µL)	0.067±0.0403	0.086±0.1197	0.085±0.0387	0.074±0.0693
ALUC (x10^3/µL)	0.068±0.0365	0.055±0.0303	0.042±0.0162	0.099±0.0677
ARET (x10^3/µL)	143.93±44.014	141.92±29.962	142.36±42.104	144.16±33.68
%RET	1.650±0.5600	1.615±0.3427	1.633±0.5212	1.684±0.4210
MCHC (g/dL)	32.36±0.310	32.42±0.539	32.45±0.443	32.94±0.347*
Coagulation (Day 97)	***N*=9-10**	***N*=9-10**	***N*=10**	***N*=10**
APTT (sec)	15.78±2.118	17.72±1.945	17.94±1.864	18.59±1.688**
PT (sec)	8.74±0.343	8.44±0.174	8.87±0.395	9.00±0.313

ABAS, absolute basophils; AEOS, absolute eosinophils; ALUC, absolute large unstained cells; ALYM, absolute lymphocytes; AMON, absolute monocytes; ANEU, absolute neutrophils; APTT, activated partial thromboplastin time; ARET, absolute reticulocytes; HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet count; ppm, parts *per* million; PT, prothrombin time; RBC, erythrocytes; RDW, red cell distribution width; WBC, total white blood cells; Mean ± SD shown; **Statistically significant $P \leq 0.01$; *Statistically significant $P \leq 0.05$.

Table 13. Subchronic study - male rats: Clinical chemistry and urinalysis parameters (Day 89)

	0 ppm Group 1	28750 ppm Group 2	575000 ppm Group 3	115500 ppm Group 4
Clinical chemistry	***N*=9**	***N*=9**	***N*=10**	***N*=10**
Sodium (mmol/L)	143.8±1.39	142.8±0.83	142.7±0.82	143.3±0.82
Potassium (mmol/L)	5.040±0.2479	4.969±0.3157	5.127±0.4692	5.177±0.3353
Chloride (mmol/L)	100.77±1.260	101.24±1.201	100.00±0.790	101.24±1.103
Albumin (g/dL)	3.98±0.186	4.09±0.105	4.04±0.227	3.93±0.206
AST (U/L)	93.7±49.10	89.4±68.79	90.4±49.12	96.1±69.35
ALT (U/L)	38.9±38.17	37.8±42.85	34.7±30.88	38.5±53.34
ALKP (U/L)	73.0±19.92	72.0±11.42	68.6±11.87	80.4±24.49
BUN (mg/dL)	12.4±1.74	12.3±1.50	11.7±1.06	11.7±0.67
Calcium (mg/dL)	10.80±0.206	10.70±0.320	10.82±0.286	10.48±0.155*
Total cholesterol (mg/dL)	87.3±26.00	83.4±23.43	83.7±14.80	79.6±22.09
Creatinine (mg/dL)	0.167±0.0218	0.153±0.0132	0.170±0.0200	0.157±0.0236
Glucose, fasting (mg/dL)	134.2±11.30	134.8±12.32	147.4±17.45	129.4±12.27
Phosphate (mg/dL)	6.89±0.389	6.61±0.369	6.84±0.737	6.97±0.548
Total protein (g/dL)	6.62±0.331	6.68±0.222	6.76±0.255	6.47±0.164
Total bilirubin (mg/dL)	0.091±0.0252	0.087±0.200	0.094±0.0284	0.100±0.0403
Triglycerides (mg/dL)	99.4±65.44	114.7±57.27	118.1±56.74	84.7±42.42
SDH (U/L)	9.23±14.802@	6.29±5.616	6.58±8.185@	8.73±16.717@
Globulin (g/dL)	2.64±0.260	2.59±0.196	2.72±0.274	2.54±0.151
Urinalysis	***N*=5-9**	***N*=7-9**	***N*=8-10**	***N*=4-10**
URO (EU/dL)	0.20±0.000	0.47±0.400	0.28±0.253	0.20±0.000
pH	6.56±0.300	6.67±0.250	6.60±0.211	7.20±0.587
Specific gravity	1.0250±0.00559	0.9687±0.17466	1.0265±0.00530	0.9146±0.21698
Urine volume (mL)	4.8±3.05	3.8±1.55	4.6±1.90	6.3±1.50

ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BILI, total bilirubin; BUN, blood urea nitrogen; ppm, parts *per* million; SDH, sorbitol dehydrogenase; UMTP, protein; URO, urobilinogen; Mean ± SD shown; *Statistically significant $P \leq 0.05$; @Number examined reduced due to excluded data

Table 14. Subchronic study - female rats: Clinical chemistry and urinalysis parameters (Day 89)

	0 ppm Group 1	28750 ppm Group 2	57500 ppm Group 3	115500 ppm Group 4
Clinical chemistry	***N*=9-10**	***N*=10**	***N*=10**	***N*=7-10**
Sodium (mmol/L)	142.2±0.79	142.6±0.70	142.2±0.92	142.0±0.94
Potassium (mmol/L)	4.448±0.3324	4.495±0.3579	4.589±0.2515	4.661±0.3347
Chloride (mmol/L)	100.57±1.568	100.56±1.375	99.88±1.769	99.55±1.210
Albumin (g/dL)	5.51±0.551	5.51±0.387	5.50±0.333	5.52±0.262
AST (U/L)	72.5±32.09	88.6±87.13	61.5±11.52	71.4±17.12
ALT (U/L)	24.2±11.32	40.7±64.92	19.6±6.15	20.0±7.41
ALKP (U/L)	35.3±9.93	33.7±4.55	39.8±12.67	37.2±10.24
BUN (mg/dL)	11.9±1.79	13.9±3.41	12.9±1.20	13.7±2.06
Calcium (mg/dL)	10.92±0.476	10.93±0.469	10.94±0.384	10.99±0.387
Total cholesterol (mg/dL)	83.6±18.52	96.1±15.52**	100.0±30.45**	109.9±21.90**
Creatinine (mg/dL)	0.255±0.0528	0.257±0.0414	0.242±0.0301	0.277±0.0435
Glucose, fasting (mg/dL)	119.0±12.77	128.5±10.91	117.1±6.49	126.1±13.32
Phosphate (mg/dL)	5.37±0.609	5.34±0.717	5.53±0.485	5.16±0.369
Total protein (g/dL)	7.68±0.537	7.88±0.371	7.74±0.353	7.98±0.349
Total bilirubin (mg/dL)	0.101±0.0307	0.108±0.0397	0.110±0.0406	0.114±0.0222
Triglycerides (mg/dL)	68.8±57.44	58.5±26.96	58.5±15.19	58.7±26.57
SDH (U/L)	4.99±2.127@	4.61±6.415	3.15±1.376	3.21±2.081@
Globulin (g/dL)	2.17±0.271	2.37±0.134	2.24±0.212	2.46±0.222*
Urinalysis	***N*=7-10**	***N*=10**	***N*=9-10**	***N*=9-10**
URO (EU/dL)	0.44±0.386	0.28±0.253	0.52±0.413	0.36±0.337
pH	6.60±0.699	6.50±0.408	6.83±0.750	6.94±0.527
Specific gravity	0.9678±0.16351	1.0215±0.00747	0.9688±0.16386	0.9151±0.21731
Urine volume (mL)	4.1±1.95	5.7±3.72	3.2±1.48	6.2±3.37

ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BILI, total bilirubin; BUN, blood urea nitrogen; ppm, parts *per* million; SDH, sorbitol dehydrogenase; UMTP, protein; URO, urobilinogen; Mean ± SD shown;**Statistically significant $P \leq 0.001$; *Statistically significant $P \leq 0.05$; @Number examined reduced due to excluded data.

6.5. Genotoxicity

Alkaline serine proteases have been evaluated for their potential genotoxicity and are considered non-mutagenic in genotoxicity assays (Basketter *et al.*, 2012). Similarly, proteins consisting of only a primary amino acid structure (denatured) are generally non-genotoxic as genotoxic proteins act by utilizing higher order structures (Thybaud *et al.*, 2016). However, to ensure non-genotoxicity of DASP, an *in vitro* bacterial reverse mutation assay was conducted in *S. typhimurium* (TA1535, TA100, TA1537, and TA98) and *E. coli* WP2 *uvr*A with (*i.e.,* S9 mix) and without metabolic activation at 0, 1.58, 5.0, 15.8, 50, 158, 500, 1580, or 5000 µg DASP/plate using sterile water for serial dilutions (control vehicle; Experiment I) (Burdock *et al.*, 2020). A pre-incubation test (Experiment II) was completed at 0, 1.58, 5.0, 15.8, 50, 158, 500, 1580, or 5000

µg DASP/plate with and without metabolic activation to serve as a confirmatory test. Each treatment was conducted in triplicate. The bacterial reverse mutation assay was conducted according to OECD Guideline 471 (1997). As shown in Table 15, the number of revertant colony counts did not increase dose-dependently and were not related to treatment with DASP. As such, the authors concluded that DASP was non-mutagenic with and without metabolic activation under the conditions of the assay (Burdock *et al.*, 2020).

Table 15. Bacterial reverse mutation assay (Ames test) with DASP

Strain	Dose (µg/plate)	Mean (SD) revertant colonies *per* plate	
		-S9	+S9
TA135	0	12(2.5)	10(2.0)
	1.58	11(4.7)	9(3.2)
	5	13(3.1)	9(2.5)
	15.8	12(2.1)	11(2.1)
	50	11(1.7)	11(1.2)
	158	11(3.1)	13(4.0)
	500	11(4.2)	14(5.0)
	1580	11(3.2)	10(1.0)
	5000*	13(6.5)	12(2.5)
	Control^	660(46.1)	295(27.1)
TA1537	0	10(3.5)	9(1.7)
	1.58	11(4.0)	9(1.5)
	5	9(2.0)	11(1.0)
	15.8	7(1.0)	7(1.0)
	50	7(2.1)	8(2.1)
	158	9(1.2)	8(2.1)
	500	8(1.0)	11(3.6)
	1580	8(3.0)	7(1.0)
	5000*	6(0.6)	9(1.5)
	Control^	2480(436.8)	401(82.7)
TA98	0	23(1.0)	30(8.5)
	1.58	19(0.6)	21(4.0)
	5	24(5.7)	21(2.1)
	15.8	23(4.2)	24(4.6)
	50	22(3.1)	23(4.9)
	158	19(7.0)	22(2.1)
	500	17(3.2)	21(3.1)
	1580	19(3.8)	24(6.8)
	5000*	25(5.0)	21(2.1)
	Control^	948(106.3)	4195(438.5)
TA100	0	94(7.0)	99(10.3)
	1.58	88(5.0)	97(4.2)
	5	94(4.4)	97(5.8)
	15.8	86(9.5)	89(24.8)+
	50	91(6.7)	91(4.0)
	158	87(5.3)	84(7.6)
	500	81(4.0)	89(9.5)
	1580	79(8.6)	90(4.7)
	5000*	86(9.9)	83(3.1)
	Control^	761(67.2)	3903(90.7)

Table 15. Bacterial reverse mutation assay (Ames test) with DASP

Strain	Dose (µg/plate)	Mean (SD) revertant colonies *per* plate	
		-S9	+S9
E. coli WP2 *uvr*A	0	45(3.1)	51(4.6)
	1.58	45(4.0)	49(8.0)
	5	45(10.1)	54(6.4)
	15.8	42(2.6)	46(2.5)
	50	43(6.7)[+]	43(7.6)
	158	47(5.0)	49(4.7)
	500	44(9.1)	55(5.6)
	1580	48(4.2)	51(7.2)
	5000[*]	41(6.0)	49(4.2)
	Control[^]	682(26.7)	129(5.3)

^Controls for –S9: strain TA1535=sodium azide, strain TA1537=ICR 191 acridine, strain TA98=daunomycin, strain TA100=sodium azide, *E. Coli* WP2 *uvr*A=MMS; controls for +S9: for all strains=2-aminoanthracene
[*]Lawn evaluation difficult due to precipitate from test substance.
[+]One replicate was contaminated; however, the contamination did not obscure revertant colony count.

6.6. Carcinogenicity

DASP is not expected to be carcinogenic as there is no indication in literature that alkaline serine proteases possess carcinogenic properties. Similarly, alkaline serine proteases, as such in their native form, are not very bioavailable as they are readily degraded by enzymes in the gastro-intestinal tract. There is no indication that carcinogenicity studies have been published regarding subtilisin, the native form of DASP (Basketter *et al.*, 2012).

6.7. Allergenicity

Alkaline serine proteases are respiratory allergens that may also cause minor dermal and ocular irritation (Matsumura, 2012; Florsheim *et al.*, 2015). Subtilisin is subject to a recommended exposure limit set by the National Institute for Occupation Safety and Health (NIOSH) and it is recommended to avoid inhalation at levels exceeding 0.00006 mg/m^3 (60 ng/m^3) over a 60-minute period.[13] There are no exposure limits regarding ingestion of subtilisin. Subtilisin has also been evaluated *via* the Human and Environmental Risk Assessment (HERA) Project for subtilisin's use in detergent products.[14] HERA evaluated subtilisin based on route of exposure and a subsequent hazard and risk assessment for each route (HERA, 2007). The respiratory and dermal/ocular exposure routes are considered in the context of allergenicity with adverse effects of exposure inducing respiratory (Type I) allergenicity and dermal/ocular irritation. However, exposure mainly occurs during dispensing detergent products or during handwashing such that exposure does not exceed 2 ng/m^3. The upper benchmark where allergic symptoms occur is 212 ng/m^3 with the lower benchmark unclear, though HERA reports that allergic symptoms can be excluded when exposure does not exceed 1 ng/m^3. Further, they report that allergic effects are not expected even at the highest exposure level (2 ng/m^3), as the threshold at which respiratory sensitization and allergies occur are likely to be distinctly higher than 1 ng/m^3. It should be noted that these exposure levels are also below the NIOSH exposure limit. As a dermal and ocular irritant, subtilisin similarly does not pose significant risk as exposure levels are small and of short duration; further, denatured subtilisin does not act as an irritant due to lack of enzymatic activity. The HERA report states that

[13] https://www.cdc.gov/niosh/npg/npgd0572.html; last accessed January 26th, 2021.
[14] HERA is a voluntary industry program to carry out Human and Environmental Risk Assessments on ingredients of household cleaning products in the EU; https://www.heraproject.com/; last accessed January 26th, 2021.

any oral exposure to subtilisin would not induce allergenicity as it is not the site of allergenic activity; additionally, subtilisin would be broken down by gastro-intestinal enzymes.

It should be noted that various proteases concluded as GRAS are not considered allergenic when consumed including serine protease (GRN 563; GRN 564), subtilisin (GRN 714), and serine endopeptidase (GRN 817).

DASP is denatured at 70°C for 20 minutes and has no enzymatic activity, as such it is not a dermal or ocular irritant. While no studies investigating allergenicity upon oral administration of DASP have been conducted, the manufacturing method and lack of enzymatic activity, along with administration route (oral), indicates that DASP should not be allergenic or irritating under its intended use. As a result, DASP should not be classified as a food allergen.

6.8. Observations in Humans

While DASP has not been subjected to clinical trials, subtilisin, the native form of DASP, has a history of use as a human food ingredient as evidenced in Section 2.2. Considering DASP is a denatured ASP, a commonly consumed food ingredient (Section 2.2), DASP is safe as a macro-additive to food.

7. EVALUATION

DASP is an off-white powder composed of 274 amino acids with a molecular weight of 27,413.43 g/mol and isoelectric point (pI) of 6.7. DASP is expressed in *B. licheniformis* strain 2709 and is composed of >90% protein with <0.25% of the protein composed of PHE. DASP is expected to be stable at ambient temperatures for up to two (2) years and DASP is stable as an added ingredient to medical foods. As a medical food ingredient, DASP meets criteria set forth by FDA for a medical food ingredient utilized in the treatment of an IEM, as DASP is specially formulated and processed for exclusive use by PKU patients. Patients utilizing DASP may facilitate the dietary management of their PHE levels that are influenced by their decreased ability to metabolize the potentially harmful amount of PHE in normal foodstuffs.

DASP is expressed in *B. licheniformis* strain 2709, a non-toxigenic, non-pathogenic saprophytic microorganism that is widespread in nature. *B. licheniformis* has a history of safe industrial use in producing enzymes used in human food and has been routinely used as a host for recombinant enzymes – it is often referenced as a well-characterized and safe production strain with a history of safe use (Section 2.2). Similarly, the method of transformation and construction of the final production organism is well detailed and characterized and the vectors and intermediate organisms utilized in characterization are commonly used in industrial enzyme production by microorganisms (Section 3; Section 6.1; Section 6.2). As such, there is no safety concern regarding the recombination and manufacturing process of DASP.

While DASP has not been subjected to human trials, subtilisin and other ASPs have a long history of use as a human food ingredient as described in Section 2.2. Considering DASP is a denatured ASP, and ASPs are commonly consumed in food, it is reasonable to assume DASP will be safe under similar use. However, DASP is intended as a macro-additive in medical foods that could replace up to 80% of protein in the diets of individuals over the age of 1 with PKU. Therefore, to evidence the safety of DASP a series of two 14-day dietary toxicity/palatability studies were conducted using various concentrations of DASP administered to rodents. In both studies, there were no DASP-related toxicological effects observed. A subsequent 90-day toxicology study was conducted in rodents at doses of 0%, 2.875%, 5.75%, and 11.5% DASP.

8. CERTIFICATION

The undersigned authors of this document—a dossier in support of GRAS status determination for use of DASP—hereby certify that, to the best of their knowledge and belief, this document is a complete and balanced representation of all available information, favorable as well as unfavorable, known by the authors to be relevant to evaluation of the substance described herein.

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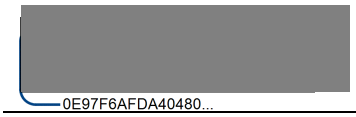
Date

9. CONCLUSION

Following a critical evaluation of the information available, the Expert Panel has determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that DASP, produced in accordance with current Good Manufacturing Practice, is safe under the intended conditions of use, and is therefore GRAS, by scientific procedures, when used as an ingredient in specified medical foods, so that total daily consumption of deactivated DASP from all sources up to 80% of total protein intake *per* day in individuals over the age of 1 (one) year, but higher levels of use have not been evaluated.

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

10. SIGNATURES



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12. APPENDIX I

12.1. Compositional Data

Results based on testing of six separate batches of DASP.

	Batch Number					
	20160416	20160422	20160503	20160508	20160514	20160520
TAMC	790	320	600	160	4200	150
TYMC	<10	<10	<10	<10	<10	<10
Bile Tolerant/g	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
E. coli/g	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
Salmonella/10g	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
P. aeruginosa/g	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
S. aureus/g	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
Pb (mg/kg)	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
Cd (mg/kg)	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
As (mg/kg)	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
Hg (mg/kg)	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
Moisture g/100g	3.9	3.1	4.2	3.3	3.8	3.2
Total Ash g/100g	0.6	0.4	0.4	0.4	0.4	0.2
Protein g/100g	93.4	94.1	92.6	93.1	93.3	92.8
Asp, g/100g	11.46	11.48	11.73	11.54	11.58	11.71
Thr, g/100g	7.00	6.80	6.99	6.98	7.00	7.05
Ser, g/100g	9.45	9.36	9.52	9.48	9.45	9.55
Glu, g/100g	6.05	6.00	6.09	6.02	6.03	6.08
Pro, g/100g	3.48	3.38	3.49	3.40	3.39	3.51
Gly, g/100g	8.41	8.13	8.45	8.46	8.48	8.54
Ala, g/100g	11.56	11.53	11.64	11.62	11.56	11.75
Val, g/100g	10.67	10.65	10.79	10.77	10.80	10.67
Met, g/100g	1.99	2.36	1.44	2.41	1.76	1.67
Ile, g/100g	3.98	3.89	3.95	3.94	3.96	3.96
Leu, g/100g	6.95	6.98	6.91	6.96	6.98	7.07
Tyr, g/100g	8.44	9.00	8.27	8.55	8.40	8.62
Phe, g/100g	0.15	0.14	0.17	0.11	0.14	0.15
Lys, g/100g	4.37	4.18	4.30	4.36	4.35	4.32
His, g/100g	2.64	2.51	2.63	2.64	2.66	2.62
Arg, g/100g	2.35	2.25	2.37	2.31	2.38	2.36

12.2. Stability Data

12.2.1. Refrigerated (+5°C ± 3°C)

As highlighted in the next three tables, the material is stable for at least 24-months when stored at (+5°C ± 3°C). There were no testing outliers or deviations during the conduct of the study.

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160416		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	93.7 g / 100 g	93.3 g / 100 g	93.7 g / 100 g	93.0 g / 100 g	93.1 g / 100 g	94.2 g / 100 g	94.7 g / 100 g	93.3 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.256	0.169	0.149	0.115	0.112	0.109	0.114	0.119
Flow-ability									
	Report	1.24	1	1.22	1.07	1.2	1.21	1.22	1.17
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Neg	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160508		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	94.1 g / 100 g	93.7 g / 100 g	94.1 g / 100 g	94.3 g / 100 g	94.7 g / 100 g	95.0 g / 100 g	95.1 g / 100 g	94.3 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.244	0.185	0.135	0.115	0.111	0.121	0.12	0.126
Flow-ability									
	Report	1.09	1	1.23	1.07	1.25	1.17	1.19	1.18
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160520		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	93.2 g / 100 g	92.2 g / 100 g	93.3 g / 100 g	93.6 g / 100 g	93.0 g / 100 g	92.6 g / 100 g	91.9 g / 100 g	92.9 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.246	0.187	0.171	0.138	0.138	0.179	0.154	0.134
Flow-ability									
	Report	1.21	1.08	1.17	1.07	1.5	1.21	1.27	1.38
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

12.2.2. Controlled Room Temperature (+25°C ± 2°C/60% RH ± 5% RH)

As highlighted in the next three tables, the material is stable for at least 24-months when stored at (+25°C ± 2°C/60% RH ± 5% RH). There were no testing outliers or deviations during the conduct of the study.

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160416		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	93.7 g / 100 g	93.2 g / 100 g	93.7 g / 100 g	93.1 g / 100 g	94.4 g / 100 g	92.7 g / 100 g	92.4 g / 100 g	93.7 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.256	0.155	0.117	0.118	0.113	0.137	0.127	0.121
Flow-ability									
	Report	1.24	1.17	1.17	1.05	1.2	1.15	1.19	1.16
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160508		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	94.1 g / 100 g	94.0 g / 100 g	93.1 g / 100 g	93.7 g / 100 g	94.6 g / 100 g	94.9 g / 100 g	92.9 g / 100 g	93.5 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.244	0.158	0.118	0.114	0.128	0.144	0.121	0.123
Flow-ability									
	Report	1.09	1.27	1.21	1.07	1.25	1.16	1.19	1.18
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months
Lot 20160520		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018	14-Mar-2019	14-Sep-2019
Appearance									
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)									
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration									
	Report	93.2 g / 100 g	93.0 g / 100 g	93.5 g / 100 g	93.7 g / 100 g	93.8 g / 100 g	94.2 g / 100 g	92.7 g / 100 g	93.4 g / 100 g
SDS-PAGE									
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)									
	Report	0.246	0.187	0.12	0.168	0.12	0.209	0.154	0.147
Flow-ability									
	Report	1.21	1.14	1.24	1.07	1.4	1.09	1.18	1.18
Purity (USP <61>)									
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass	ND	Pass
Purity (USP <62>)									
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent	ND	Absent
Integrity									
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

12.2.3. Stressed (+40°C ± 2°C/75% RH ± 5% RH)

As highlighted in the next three tables, the material is stable for at least 12-months when stored at (+40°C ± 2°C/75% RH ± 5% RH). There were no testing outliers or deviations during the conduct of the study.

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months
Lot 20160416		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018
Appearance							
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)							
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration							
	Report	93.7 g / 100 g	93.0 g / 100 g	93.7 g / 100 g	93.5 g / 100 g	94.4 g / 100 g	93.9 g / 100 g
SDS-PAGE							
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)							
	Report	0.256	0.151	0.136	0.15	0.153	0.16
Flow-ability							
	Report	1.24	1.36	1.09	1.07	1.4	1.21
Purity (USP <61>)							
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass
Purity (USP <62>)							
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent
Integrity							
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months
Lot 20160508		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018
Appearance							
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)							
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration							
	Report	94.1 g / 100 g	94.5 g / 100 g	94.9 g / 100 g	93.1 g / 100 g	93.5 g / 100 g	94.0 g / 100 g
SDS-PAGE							
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)							
	Report	0.244	0.155	0.137	0.154	0.171	0.181
Flow-ability							
	Report	1.09	1.07	1.23	1.07	1.25	1.17
Purity (USP <61>)							
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass
Purity (USP <62>)							
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent
Integrity							
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

Timepoint	Acceptance	Initial	1 Month	3 Months	6 Months	9 Months	12 Months
Lot 20160520		14-Sep-2017	14-Oct-2017	14-Dec-2017	14-Mar-2018	14-Jun-2018	14-Sep-2018
Appearance							
	Off-white free-flowing powder, free of visible contamination	Pass	Pass	Pass	Pass	Pass	Pass
Activity (Lack of Activity)							
	Report	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity	No Detectable Activity
Protein Concentration							
	Report	93.2 g / 100 g	94.2 g / 100 g	93.5 g / 100 g	93.0 g / 100 g	92.2 g / 100 g	93.2 g / 100 g
SDS-PAGE							
	Matches Reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Water Activity (aw)							
	Report	0.246	0.199	0.145	0.238	0.211	0.265
Flow-ability							
	Report	1.21	1.07	1.22	1.07	1.39	1.27
Purity (USP <61>)							
TAMC	< 10000 CFU/g	Pass	ND	ND	ND	ND	Pass
TYMC	< 1000 CFU/g	Pass	ND	ND	ND	ND	Pass
Purity (USP <62>)							
S. aureus	Absent	Absent	ND	ND	ND	ND	Absent
E. coli	Absent	Absent	ND	ND	ND	ND	Absent
Salmonella	Absent	Absent	ND	ND	ND	ND	Absent
Bile Tolerant Gram Nega	Absent	Absent	ND	ND	ND	ND	Absent
Integrity							
Visual	Report	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms

13. APPENDIX II

13.1. Regulation Cited in the Code of Federal Regulations (CFR)

21 CFR § 101.9(j)(8) Nutrition labeling of food.

(8) Medical foods as defined in section 5(b) of the Orphan Drug Act (21 U.S.C. 360ee(b)(3)). A medical food is a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. A food is subject to this exemption only if:

(i) It is a specially formulated and processed product (as opposed to a naturally occurring foodstuff used in its natural state) for the partial or exclusive feeding of a patient by means of oral intake or enteral feeding by tube;

(ii) It is intended for the dietary management of a patient who, because of therapeutic or chronic medical needs, has limited or impaired capacity to ingest, digest, absorb, or metabolize ordinary foodstuffs or certain nutrients, or who has other special medically determined nutrient requirements, the dietary management of which cannot be achieved by the modification of the normal diet alone;

(iii) It provides nutritional support specifically modified for the management of the unique nutrient needs that result from the specific disease or condition, as determined by medical evaluation;

(iv) It is intended to be used under medical supervision; and

(v) It is intended only for a patient receiving active and ongoing medical supervision wherein the patient requires medical care on a recurring basis for, among other things, instructions on the use of the medical food.

21 CFR § 101.100 Food; exemptions from labeling.

(a) The following foods are exempt from compliance with the requirements of section 403(i)(2) of the act (requiring a declaration on the label of the common or usual name of each ingredient when the food is fabricated from two or more ingredients).

(1) An assortment of different items of food, when variations in the items that make up different packages packed from such assortment normally occur in good packing practice and when such variations result in variations in the ingredients in different packages, with respect to any ingredient that is not common to all packages. Such exemption, however, shall be on the condition that the label shall bear, in conjunction with the names of such ingredients as are common to all packages, a statement (in terms that are as informative as practicable and that are not misleading) indicating by name other ingredients which may be present.

(2) A food having been received in bulk containers at a retail establishment, if displayed to the purchaser with either:

(i) The labeling of the bulk container plainly in view, provided ingredient information appears prominently and conspicuously in lettering of not less than one-fourth of an inch in height; or

(ii) A counter card, sign, or other appropriate device bearing prominently and conspicuously, but in no case with lettering of less than one-fourth of an inch in height, the information required to be stated on the label pursuant to section 403(i)(2) of the Federal Food, Drug, and Cosmetic Act (the act).

(3) Incidental additives that are present in a food at insignificant levels and do not have any technical or functional effect in that food. For the purposes of this paragraph (a)(3), incidental additives are:

(i) Substances that have no technical or functional effect but are present in a food by reason of having been incorporated into the food as an ingredient of another food, in which the substance did have a functional or technical effect.

(ii) Processing aids, which are as follows:

(*a*) Substances that are added to a food during the processing of such food but are removed in some manner from the food before it is packaged in its finished form.

(*b*) Substances that are added to a food during processing, are converted into constituents normally present in the food, and do not significantly increase the amount of the constitutents naturally found in the food.

(*c*) Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food.

(iii) Substances migrating to food from equipment or packaging or otherwise affecting food that are not food additives as defined in section 201(s) of the act; or if they are food additives as so defined, they are used in conformity with regulations established pursuant to section 409 of the act.

[Remainder of regulation omitted for brevity]

[42 FR 14308, Mar. 15, 1977, as amended at 51 FR 25017, July 9, 1986; 58 FR 2188, 2876, Jan. 6, 1993; 66 FR 17358, Mar. 30, 2001]

21 CFR § 110.110 Natural or unavoidable defects in food for human use that present no health hazard.

(a) Some foods, even when produced under current good manufacturing practice, contain natural or unavoidable defects that at low levels are not hazardous to health. The Food and Drug Administration establishes maximum levels for these defects in foods produced under current good manufacturing practice and uses these levels in deciding whether to recommend regulatory action.

(b) Defect action levels are established for foods whenever it is necessary and feasible to do so. These levels are subject to change upon the development of new technology or the availability of new information.

(c) Compliance with defect action levels does not excuse violation of the requirement in section 402(a)(4) of the act that food not be prepared, packed, or held under unsanitary conditions or the requirements in this part that food manufacturers, distributors, and holders shall observe current good manufacturing practice. Evidence indicating that such a violation exists causes the food to be adulterated within the meaning of the act, even though the amounts of natural or unavoidable defects are lower than the currently established defect action levels. The manufacturer, distributor, and holder of food shall at all times utilize quality control operations that reduce natural or unavoidable defects to the lowest level currently feasible.

(d) The mixing of a food containing defects above the current defect action level with another lot of food is not permitted and renders the final food adulterated within the meaning of the act, regardless of the defect level of the final food.

(e) A compilation of the current defect action levels for natural or unavoidable defects in food for human use that present no health hazard may be obtained upon request from the Center for Food Safety and Applied Nutrition (HFS-565), Food and Drug Administration, 5001 Campus Dr., College Park, MD 20740.

[51 FR 22475, June 19, 1986, as amended at 61 FR 14480, Apr. 2, 1996; 66 FR 56035, Nov. 6, 2001]

21 CFR § 184.1027 Mixed carbohydrase and protease enzyme product.

(a) Mixed carbohydrase and protease enzyme product is an enzyme preparation that includes carbohydrase and protease activity. It is obtained from the culture filtrate resulting from a pure culture fermentation of a nonpathogenic strain of *B. licheniformis.*

(b) The ingredient meets the specifications of the Food Chemicals Codex, 3d Ed. (1981),p. 107, which is incorporated by reference. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or available for inspection at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to:*http://www.archives.gov/federal__register/code__of__federal__regulations/ibr__locations.html.*

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

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

(2) The ingredient is used in the following foods at levels not to exceed current good manufacturing practice: alcoholic beverages, as defined in §170.3(n)(2) of this chapter, candy, nutritive sweeteners, and protein hydrolyzates.

[48 FR 240, Jan. 4, 1983]

21 CFR § 184.1150 Bacterially-derived protease enzyme preparation.

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

(b) The ingredient meets the general requirements and additional requirements in the monograph on enzyme preparations in the Food Chemicals Codex, 4th ed. (1996), pp. 128-135, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or may be examined at the Food and Drug Administration's Main Library, 10903 New Hampshire Ave., Bldg. 2, Third Floor, Silver Spring, MD 20993, 301-796-2039, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to:*http://www.archives.gov/federal__register/code__of__federal__regulations/ibr__locations.html.*

In addition, antibiotic activity is absent in the enzyme preparation when determined by an appropriate validated method such as the method "Determination of antibiotic activity" in the Compendium of Food Additive Specifications, vol. 2, Joint FAO/WHO Expert Committee on Food Additives (JECFA), Food and Agriculture Organization of the United Nations, Rome,1992. Copies are available from Bernan Associates, 4611-F Assembly Dr., Lanham, MD20706, or from The United Nations Bookshop, General Assembly Bldg., rm. 32, New York, NY 10017, or by inquiries sent to *http://www.fao.org.* Copies may be examined at the Center for Food Safety and Applied Nutrition's Library, 5001 Campus Dr., College Park, MD 20740.

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

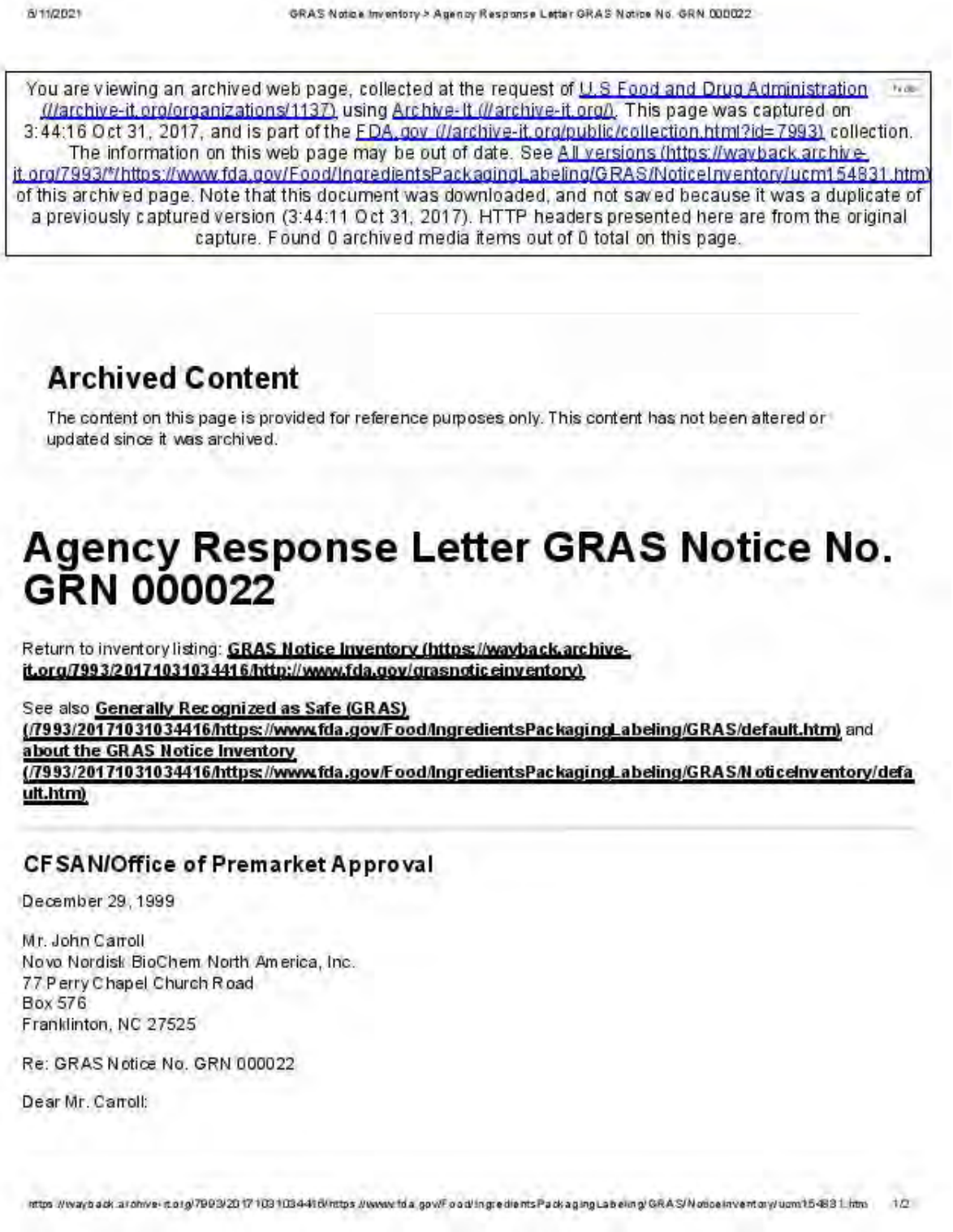
(1) The ingredient is used as an enzyme as defined in §170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

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

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

13.2. GRAS Notices and No Questions Response Letters Cited[15]

Agency Response Letter to GRAS Notice No. GRN 22.



6/11/2021 GRAS Notice Inventory > Agency Response Letter GRAS Notice No. GRN 000022

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Agency Response Letter GRAS Notice No. GRN 000022

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CFSAN/Office of Premarket Approval

December 29, 1999

Mr. John Carroll
Novo Nordisk BioChem North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000022

Dear Mr. Carroll:

https://wayback.archive-it.org/7993/20171031034416/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm154831.htm 1/2

[15] For brevity, solely FDA "no questions" response letters are included.

The Food and Drug Administration (FDA) is responding to the notice, dated May 14, 1999, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on May 24, 1999 and designated it as GRAS Notice No. GRN 000022.

The subject of your notice is alpha-amylase enzyme preparation derived from a genetically modified strain of *B. licheniformis* (which contains a recombinant gene derived from *B. licheniformis* and *B. amyloliquefaciens* encoding alpha-amylase). The notice informs FDA of the view of Novo Nordisk BioChem North America, Inc. (Novo Nordisk) that this alpha-amylase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups), and in the alcohol industry (for the thinning of starch in distilling mashes).

Your notice describes (1) published information about the host microorganism; (2) published information about microbially derived alpha-amylases, and their use in food processing; (3) unpublished studies describing the technical effect of the subject alpha-amylase enzyme preparation; (4) published information about the safety of enzymes used in food processing including enzymes derived from genetically modified microorganisms; (5) published information that discusses the safety of the genetically modified strain of B. licheniformis, including the host organism and the components of the genetic material that is introduced into the host organism; (6) information to support your conclusion that the presence of the kanamycin antibiotic resistance gene is not a concern; (7) published information about the manufacturing process, which includes methods for the fermentation, processing, and formulation of the enzyme preparation; and (8) unpublished studies to support the safety of the subject alpha-amylase enzyme preparation. In addition, your notice includes an estimate of dietary exposure to the enzyme preparation. According to your notice, the enzyme preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications for enzyme preparations provided by the Joint FAO/WHO Expert Committee on Food Additives.

Based on the information provided by Novo Nordisk, as well as other information available to FDA, the agency has no questions at this time regarding Novo Nordisk's conclusion that alpha- amylase enzyme preparation derived from this genetically modified strain of *B. licheniformis* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of alpha-amylase enzyme preparation. As always, it is your continuing responsibility to ensure that food ingredients that you market are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

More in GRAS Notice Inventory
(/7956/20171031034416/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Agency Response Letter to GRAS Notice No. GRN 24.

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Agency Response Letter GRAS Notice No. GRN 000024

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CFSAN/Office of Premarket Approval

May 15, 2000

Scott H. Shore, Ph.D.
Novo Nordisk BioChem North America, Inc.
77 Perry Chapel Church Road, Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000024

Dear Dr. Shore:

The Food and Drug Administration (FDA) is responding to the notice, dated June 4, 1999, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on June 7, 1999 and designated it as GRAS Notice No. GRN 000024.

The subject of your notice is alpha-amylase enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* expressing a gene encoding a modified alpha-amylase derived from *Bacillus stearothermophilus*. The notice informs FDA of the view of Novo Nordisk BioChem North America, Inc. (Novo Nordisk) that this alpha-amylase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for the thinning of starch in distilling mashes) at minimum levels necessary to accomplish the intended technical effect in accordance with current good manufacturing practices.

Your notice describes (1) scientific publications about the safety of the host microorganism, *B. licheniformis*; (2) scientific publications and book chapters about the structure and function of microbial derived alpha-amylases, and their use in food processing; (3) unpublished studies describing the technical effect of the enzyme preparation; (4) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing including enzymes derived from genetically modified microorganisms; (5) published scientific articles that discuss the safety of the various components of the production organism, including the host organism, and the components of the genetic material that is introduced into the host organism; (6) information to support the notifier's conclusion that the presence of the kanamycin antibiotic resistance gene is not a concern, including an FDA draft guidance document on the use of antibiotic markers in transgenic plants and a publication of the International Food Biotechnology Council; (7) chapters in several books that discuss the manufacturing process, which includes methods for the fermentation, processing, and formulation of the enzyme preparation.

Based on the information provided by Novo Nordisk, as well as other information available to FDA, the agency has no questions at this time regarding Novo Nordisk's conclusion that alpha-amylase enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* expressing a gene encoding a modified alpha-amylase derived from *Bacillus stearothermophilus* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this alpha-amylase enzyme preparation. As always, it is your continuing responsibility to ensure that food ingredients that you market are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

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Agency Response Letter to GRAS Notice No. GRN 72.

Agency Response Letter GRAS Notice No. GRN 000072

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CFSAN/Office of Premarket Approval*

June 12, 2001

Gary L. Yingling
Kirkpatrick & Lockhart, L.L.P.
1800 Massachusetts Avenue
Washington, D.C. 20036

Re: GRAS Notice No. GRN 000072

Dear Mr. Yingling:

The Food and Drug Administration (FDA) is responding to the letter, dated March 9, 2001, that you submitted on behalf of Genencor International, Inc. (Genencor). Your letter requests that FDA convert the filed GRAS affirmation petition GRP 5G0415 to a GRAS notice in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). Although GRP 5G0415 was submitted to FDA by a different firm (i.e., Solvay Enzymes, Inc.), Genencor acquired all rights and interests in GRP 5G0415 on July 1, 1996.

FDA received Genencor's conversion request on March 10, 2001 and designated it as GRAS Notice No. GRN 000072. The subject of the conversion request is pullulanase enzyme preparation obtained from a strain of *Bacillus licheniformis* that contains a gene encoding pullulanase derived from *B. deramificans*. The conversion request informs FDA of the view of Genencor that this pullulanase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid in the manufacturing of starch hydrolysates (maltodextrins, maltose, glucose, and high fructose corn syrup (HFCS)). The pullulanase enzyme preparation would be used in starch saccharification at a level of 2.3 milligrams of enzyme protein per kilogram of starch (i.e., 2.3 parts per million), in accordance with current Good Manufacturing Practices (cGMPs). Because starch hydrolysates are subjected to repeated purification steps, Genencor expects that the level of the pullulanase enzyme preparation in finished food ingredients derived from treated starch will be a small fraction of its initial concentration in liquefied starch. Genencor estimates that the enzyme residue in glucose syrup and crystalline glucose will not exceed 23 parts per billion and that the enzyme residue in HFCS will not exceed 0.23 parts per billion.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents that derive from the source organism and constituents that derive from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. GRP 5G0415 provides information about each of these components of pullulanase enzyme preparation.

The systematic name of pullulanase (EC No.3.2.1.41) is pullulan 6-glucanohydrolase. GRP 5G0415 describes published information about the technical effect of pullulanase, including the substrates for pullulanase (i.e., amylopectin and pullulan). Amylopectin is a highly branched polysaccharide that is a principal component of edible starch. Amylopectin consists of linear polymers of 1,4-alpha-linked D-glucose units joined at branch points by 1,6-alpha-glucosidic linkages. Pullulan is a linear polysaccharide composed of maltotriose units linked by alpha-D-(1,6) bonds. During the process known as "wet milling," corn starch is hydrolyzed using the enzyme glucoamylase, which catalyzes the stepwise hydrolysis of alpha-1,4-linkages in starch. Although glucoamylase also is capable of hydrolyzing the alpha-1,6- linkages, this reaction proceeds much more slowly.

Pullulanase is used in the wet milling of corn starch, during the saccharification with glucoamylase, to specifically cleave the 1,6-alpha-glucosidic linkages in amylopectin and pullulan. This use of pullulanase reduces the level of glucoamylase that is used during saccharification, increases the ultimate glucose yield, allows the saccharification process to be carried out at higher levels of dissolved solids, and shortens the saccharification time.

GRP 5G0415 describes scientific publications and recommendations, issued by FDA or by international organizations, about the safety of enzymes used in food processing, including enzymes derived from microorganisms that are modified using recombinant deoxyribonucleic acid (rDNA) technology. Consistent with those documents, GRP 5G0415 contains published information pertaining to the host organism, the various components of the plasmids that were introduced into the host organism, the production organism, and the characteristic properties of enzyme component.

GRP 5G0415 contains published information pertaining to the host organism, *B. licheniformis* strain SE2 delap1, which is used in the construction of the production strain (*B. licheniformis* strain SE2-pul-int211).[(1)] *B. licheniformis* strains are common in most soils and are listed in the Food Chemicals Codex as a source of carbohydrase and

protease enzyme preparations used in food processing. The FDA has affirmed that a mixed carbohydrase and protease enzyme product derived from *B. licheniformis* is GRAS for use in the production of certain foods (21 CFR 184.1027). GRP 5G0415 cites published reports on the cloning and expression of proteins in *B. licheniformis* for use in food products. Genencor concludes that common *B. licheniformis* strains are non-pathogenic to humans, animals, and plants.

GRP 5G0415 contains published information pertaining to the various components of the plasmids that were introduced into *B. licheniformis* strain SE2 delap1. The gene that encodes pullulanase derives from another species of *Bacillus*, *B. deramificans*. GRP 5G0415 describes the cloning process (which employed standard, well-known vectors that do not raise any safety concerns), and describes published and unpublished information pertaining to the production strain (*B. licheniformis* strain SE2-pul-int211). GRP 5G0415 includes data and information derived using the technique of Southern hybridization to demonstrate that the DNA sequence encoding pullulanase is stably integrated into the *B. licheniformis* host chromosome. GRP 5G0415 also describes an experiment analyzing the transformation potential of the recombinant production strain using competent *E. coli* cells and total DNA extracted from the production strain. Genencor concludes that the experiment shows that there is no transformation potential from the integrated vector.

GRP 5G0415 includes a published article that describes a study conducted in rats to determine the pathogenic potential of the production strain. In this study, groups of 5 male and 5 female rats received one-time intraperitoneal injections of either live or killed cells in doses of 10^6, 10^9, and 10^{11} bacteria per kilogram of body weight. No animals demonstrated signs of pathogenicity at any dose level of either live or killed cells. Genencor concludes that the production strain is, as expected, non-pathogenic in rats.

GRP 5G0415 describes the manufacturing process for pullulanase enzyme preparation. Pullulanase enzyme preparation is produced by a submerged, aerobic, and pure culture fermentation of *B. licheniformis* strain SE2-pul-int211 in accordance with cGMP. The manufacturing process consists of fermentation, recovery, and formulation. During the fermentation process, propagation is carried out on a small scale to ensure that the bacilli are viable and are a pure culture. The culture is transferred to a primary fermentor for seed fermentation, which generates an initial biomass. The biomass from the seed fermentation is then used in the main fermentation to generate large quantities of biomass. During the recovery process, the enzyme is separated from the fermentation debris and purified and concentrated by ultrafiltration, which ensures that the concentrated enzyme solution is free of the production strain and insoluble components from the fermentation medium. During the formulation process, the enzyme concentrate is stabilized with potassium sorbate and sodium benzoate and standardized at 40 percent solids with corn syrup. Pullulanase enzyme preparation complies with the general and additional requirements for enzyme preparations set forth in the Food Chemicals Codex (4th ed., 1996).

GRP 5G0415 includes data and information derived from DNA sequencing to demonstrate that the DNA sequence encoding pullulanase is the same as that derived from *B. deramificans*. GRP 5G0415 compares the pullulanase produced by the recombinant *B. licheniformis* with that obtained from *B. deramificans*. The analyses included determination of molecular weight, optimal pH and optimal temperature for pullulan hydrolysis, ion exchange chromatography profile, pattern of hydrolysis products, and amino-terminal sequencing. Genencor concludes that the pullulanase produced by the recombinant production strain and the pullulanase produced by the donor strain are essentially the same.

GRP 5G0415 describes a published article describing toxicity studies performed with the pullulanase enzyme preparation derived from the production strain. These studies include a 14-day feeding study in rats, a 28-day feeding study in rats, a bacterial reverse mutation assay in *Salmonella typhimurium* (Ames test), an *in vitro* histidine forward mutation assay in mouse lymphoma cells, *in vivo* mouse bone marrow micronucleus and chromosomal aberration assays, an acute inhalation toxicity study, and primary dermal irritation studies. The maximum concentration of the pullulanase enzyme preparation used in each of the four genetic toxicity studies showed no

indication of mutagenicity or genotoxic activity. The highest dose level used in the 14-day and the 28-day feeding studies (5 percent of the diet) revealed no signs of toxic effects. Genencor concludes that pullulanase enzyme preparation derived from the production strain is, as expected, non-mutagenic and non-toxigenic.

FDA has evaluated the information provided by Genencor, as well as the information in GRP 5G0415 and other information available to the agency. Based on this evaluation, the agency has no questions at this time regarding Genencor's conclusion that pullulanase enzyme preparation obtained from a strain of *B. licheniformis*, which contains a gene encoding pullulanase derived from *B. deramificans*, is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of pullulanase enzyme preparation. As always, it is the continuing responsibility of Genencor to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with the interim policy discussed in the GRAS proposal (62 FR 18938 at 18954), FDA has not committed any resources to review of GRP 5G0415 since March 10, 2001, the date that we received your conversion request. At this time, we request that you formally withdraw GRP 5G0415.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the Internet (at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

(1) The host organism derives from the *B. licheniformis* strain SE2. Although *B. licheniformis* strain SE2 contains a gene encoding alkaline protease, this alkaline protease gene has been deleted from *B. licheniformis* strain SE2 delap1.

* The Office of Premarket Approval became the Office of Food Additive Safety on June 18, 2001.

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Agency Response Letter to GRAS Notice No. GRN 79.

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See also **Generally Recognized as Safe (GRAS) (/7993/20171031023824/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm)** and **about the GRAS Notice Inventory (/7993/20171031023824/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**

CFSAN/Office of Food Additive Safety

December 19, 2001

John Carroll
Novozymes North America, Inc.
77 Perry Chapel Church Road
P. O. Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000079

Dear Mr. Carroll:

The Food and Drug Administration (FDA) is responding to the notice, dated June 29, 2001, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on July 3, 2001, and designated it as GRAS Notice No. GRN 000079.

The subject of the notice is alpha-amylase enzyme preparation from *Bacillus licheniformis (B. licheniformis)* carrying a gene constructed from a modified *B. licheniformis* alpha-amylase gene and a portion of the *Bacillus amyloliquefaciens (B. amyloliquefaciens)* alpha-amylase gene. The notice informs FDA of the view of Novozymes North America, Inc. (Novozymes) that this alpha-amylase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid for the liquefaction of starch in the production of syrups and for the thinning of starch in distilling mashes in the production of alcohol. The alpha-amylase enzyme preparation is used at minimum levels necessary to achieve the intended effects. The recommended use level for the alpha-amylase enzyme preparation is 0.35 kilogram per ton of starch.

The notice describes scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing, including enzymes produced from bioengineered organisms. As discussed in these documents, the safety of an enzyme preparation depends on the safety of the enzyme itself, the host organism, the inserted genetic material, the production organism, and the manufacturing process used in producing the enzyme preparation. The notice includes a safety evaluation of each of these components in support of Novozymes' GRAS determination.

In assessing the safety of the enzyme itself, the notice discusses the history of safe use of alpha-amylases in food processing. The notice cites a published article and monograph reporting the use of microbial alpha-amylases in food production since the 1950's. FDA has affirmed the GRAS status of a mixed carbohydrase (predominantly alpha-amylase) and protease enzyme product derived from *B. licheniformis* (21 CFR 184.1027). Novozymes has previously notified FDA about its determination that two other modified alpha-amylase enzyme preparations derived from *B. licheniformis* are GRAS (GRAS Notices No. GRN 000022 and GRN 000024). FDA responded to these GRAS notices with letters stating that the agency had no questions at that time regarding the GRAS determinations.

The notice includes published and unpublished structural and sequence information for several alpha-amylases, including the alpha-amylase that is the subject of GRN 000079. The alpha-amylase discussed in GRN 000079 is the same as the alpha-amylase that is the subject of GRN 000022 except that four amino acids have been changed. Both of the alpha-amylases are stable to heat, as are several other commercially available alpha-amylases. However, the alpha-amylase that is the subject of GRN 000079 can operate at lower pH and lower calcium levels than other commercially available thermostable alpha-amylases. Novozymes concludes that the alpha-amylase that is the subject of GRN 000079 is functionally equivalent to other alpha-amylases used in food production, catalyzing hydrolysis of 1,4-alpha-glucosidic linkages in amylose and amylopectin.

In assessing the safety of the host organism, *B. licheniformis* strain SJ1707, the notice describes the host strain as a sporulation deficient and alkaline protease negative derivative of the fully-characterized, well-known natural isolate of *B. licheniformis* strain ATCC 9789. Novozymes considers *B. licheniformis* to be nontoxigenic and nonpathogenic based on published criteria for the assessment of the safety of microorganisms used in the manufacture of food ingredients. Strain SJ1707 was the host strain for the production organisms described in GRAS Notices GRN 000022 and 000024. The parent of strain SJ1707 (i.e., strain ATCC 9789) has been described in the published literature and has been used for industrial production of enzymes marketed by Novozymes since 1972, including the mixed carbohydrase and protease enzyme preparation that FDA affirmed as GRAS (21 CFR 184.1027).

The notice provides information about the components of plasmid vectors introduced into the host strain SJ1707 by sequential transformation and conjugation. The notice cites published scientific articles to support Novozymes' view that all of the DNA sequences that were used in the construction of the production strain are well-known, well-characterized, and commonly used.

The final production strain, designated *B. licheniformis* strain MOL2803, complies with the Organization for Economic Co-operation and Development criteria for Good Industrial Large Scale Practice microorganisms and meets the criteria for a safe production microorganism described in scientific publications and recommendations issued by international organizations. Using the techniques of Southern hybridization, amplification by polymerase chain reaction (PCR), and DNA sequencing, Novozymes assessed the identity, location, and stability of the introduced DNA. Novozymes concludes that the DNA is stably integrated at three distinct sites on the *B. licheniformis* chromosome as expected and is not prone to genetic transfer to other organisms.

The notice describes the manufacturing process used to produce the alpha-amylase enzyme preparation. The first step is submerged fed-batch pure culture fermentation of the *B. licheniformis* production strain. Fermentation is followed by the recovery process, which includes purification and formulation. The materials used in the fermentation and recovery processes are standard materials used by the enzyme industry. Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. Novozymes provides specifications for the alpha-amylase enzyme preparation, which comply with the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications established by the Joint Food and Agriculture Organization/World Health Organization's (FAO/WHO) Expert Committee on Food Additives (Compendium of Food Additive Specifications, Volume 2, Food and Agriculture Organization of the United Nations, Rome, 1992).

Novozymes describes an unpublished 2-week oral toxicity study in rats conducted with a test batch of the alpha-amylase enzyme preparation that was manufactured from the production strain (MOL2803) using the manufacturing process described above, except that final standardization was excluded. The study did not show any treatment related toxicity. Novozymes also provides an unpublished summary of toxicology studies performed with several other test substances produced by strains derived from *B. licheniformis* strain SJ1707 (i.e., the host strain used to develop the production strain). These studies include a 4-week and a 13-week oral toxicity study in rats, an Ames mutagenicity test, chromosome aberration test, and mouse lymphoma test. These studies showed no treatment related toxicity, induction of gene mutation, or chromosomal aberrations for test articles derived from the host strain.

Conclusions

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that alpha-amylase enzyme preparation from *B. licheniformis* carrying a gene constructed from a modified *B. licheniformis* alpha-amylase gene and a portion of the *B. amyloliquefaciens* alpha-amylase gene is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this alpha-amylase enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

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

Agency Response Letter to GRAS Notice No. GRN 472.

Agency Response Letter GRAS Notice No. GRN 000472

Return to inventory listing: **GRAS Notice Inventory (https://wayback.archive-it.org/7993/20171031002401/http://www.fda.gov/grasnoticeinventory)**

See also **Generally Recognized as Safe (GRAS) (/7993/20171031002401/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm)** and **about the GRAS Notice Inventory (/7993/20171031002401/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**

CFSAN/Office of Food Additive Safety

January 10, 2014

Ms. Lori Gregg
Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000472

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated April 24, 2013, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on May 8, 2013, filed it on July 23, 2013, and designated it as GRAS Notice No. GRN 000472.

The subject of the notice is xylanase enzyme preparation derived from *Bacillus licheniformis* carrying a gene encoding a modified xylanase from *B. licheniformis* (xylanase enzyme preparation). The notice informs FDA of the view of Novozymes North America, Inc. (Novozymes) that xylanase enzyme preparation is GRAS, through scientific

procedures, for use as an enzyme in baking applications at a level of 5 milligrams (mg) of xylanase enzyme preparation per kilogram (kg) of flour. Novozymes intends to use xylanase enzyme preparation to modify arabinoxylans for improved dough handling and bread characteristics.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for the technical effect, as well as substances used as stabilizers, preservatives, or diluents. Enzyme preparations may also contain constituents derived from the production organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. Novozymes' notice provides information about each of these components of xylanase enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, xylanase is identified by the Enzyme Commission Number 3.2.1.8. The accepted name for the enzyme is xylanase and the systematic name is endo-1, 4-beta-xylanase. The CAS Registry Number for xylanase is 9025-57-4. Xylanase catalyzes the hydrolysis of the xylosidic linkages in the arabinoxylan backbone resulting in depolymerization of arabinoxylans into smaller oligosaccharides.

Novozymes states that *B. licheniformis* strain BW302 is the host strain used to develop the production strain for xylanase enzyme. The host strain is derived from *B. licheniformis* strain ATCC 9789 using a targeted recombinant DNA technique to inactivate genes encoding several proteases and peptides, as well as genes essential for sporulation. Novozymes states that these modifications improve the safety and stability of xylanase enzyme. Novozymes also cites published studies describing *B. licheniformis* as a nonpathogenic and nontoxigenic microbe with a long history of safe industrial use for the production of enzymes used in human food. In addition, Novozymes cites several GRAS notices describing the food uses of enzymes derived from *B. licheniformis*. FDA had no questions in response to these GRAS notices.

Novozymes describes the development of the production strain, *B. licheniformis* strain HyGe329. The expression plasmid pBW120 was transformed into *Bacillus subtilis* strain BW154. This *B. subtilis* strain was then used as a donor to transfer plasmid pBW120 to the host strain via conjugation. Novozymes cites published information characterizing plasmid pBW120. The plasmid contains an expression cassette that includes: 1) a chemically-synthesized gene encoding a *B. licheniformis* xylanase enzyme with a single amino acid residue difference compared to the wild type sequence. Novozymes states that the gene was synthesized based on sequence data from SWISSPROT:052730, a public database; 2) a fragment of a hybrid promoter comprised of sequences from *B. licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus thuringiensis*, and 3) a *B. licheniformis* terminator sequence. The production strain contains two copies of the expression cassette at two specific loci. According to Novozymes, Southern hybridization analyses confirmed that the expression cassette is stably integrated into the chromosome of the production strain, and that the production strain does not contain functional antibiotic resistance genes.

Novozymes states that xylanase enzyme is produced by submerged, fed-batch fermentation of a pure culture of the production strain. Each fermentation batch is initiated using a lyophilized stock culture and appropriate measures are taken to control for identity, purity, and enzyme-generating ability before use. During fermentation, xylanase enzyme is secreted to the fermentation broth. After fermentation, the enzyme is recovered using multiple filtration and concentration steps that also remove residues of the production strain. Water, glycerol, sodium benzoate, and potassium sorbate are added to preserve and stabilize the enzyme concentrate. The enzyme concentrate is then formulated with wheat flour and sodium chloride to achieve the desired xylanase enzyme activity. Novozymes states that xylanase enzyme preparation does not contain any major food allergens from the fermentation medium.(1)

Xylanase enzyme preparation is sold as a granulated product. Novozymes states that xylanase enzyme preparation is produced in accordance with current Good Manufacturing Practices and the materials used in fermentation, recovery, and formulation meet predefined quality standards and are food grade.

Novozymes states that xylanase enzyme preparation conforms to the specifications for enzyme preparations described in the Food Chemicals Codex (8th edition, 2012) 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 (2006). Novozymes provided analytical data from one batch of xylanase enzyme preparation to demonstrate compliance with these specifications.

Novozymes intends to use xylanase enzyme preparation in flour at a maximum level of 5 mg (equivalent to 4% Total Organic Solids (TOS)) of xylanase enzyme preparation per kg of flour.[2] Novozymes states that xylanase enzyme is inactivated during baking; thus, no xylanase enzyme activity is expected to remain in the final foods. However, to estimate dietary exposure to xylanase enzyme preparation, Novozymes assumes that all baked products are produced using xylanase enzyme preparation and that all of the enzyme would remain in the final foods. Based on these assumptions and the proposed maximum use level, Novozymes estimates the maximum daily intake of xylanase enzyme preparation from all the intended food applications to be 0.023 mg TOS per day, which corresponds to 0.376 x 10^{-3} mg TOS per kg body weight per day (mg TOS/kg bw/d).

In addition to published information describing the safety of the production strain and the characterization of the plasmid used in its development, Novozymes cites published information describing the long history of use of xylanases in food. Furthermore, Novozymes cites published studies demonstrating that enzymes do not generally raise safety concerns.

Novozymes summarizes unpublished toxicological studies supporting the safety of xylanase enzyme concentrate in the absence of standardization and stabilization ingredients. Tests conducted using bacterial cells showed that xylanase enzyme concentrate is not mutagenic. Tests conducted using human lymphocytes showed that xylanase enzyme concentrate is not clastogenic. The results of a 90-day oral toxicity study conducted using rats showed that consumption of xylanase enzyme concentrate did not cause adverse effects at 1020 mg TOS/kg bw/d, the highest dose tested. Novozymes designated this level as the no observed adverse effect level (NOAEL). Based on this NOAEL and the highest intended consumption level (0.376 x 10^{-3} mg/kg bw/d) for xylanase enzyme preparation, Novozymes calculates a margin of safety of 2.7 x 10^{6}.

Novozymes discusses the potential food allergenicity of xylanase enzyme. Novozymes conducted an amino acid sequence homology search for xylanase enzyme against known allergens in the Food Allergy Research and Resource Program database as well as the World Health Organization and the International Union of Immunological Societies Allergen Nomenclature Subcommittee database. No amino acid identity matches greater than 35% over 80 amino acids were found, nor were there any contiguous stretches of eight amino acids shared between the xylanase enzyme amino acid sequence and those of known allergens. Based on this information, Novozymes considers it unlikely that oral consumption of xylanase enzyme will result in allergic responses.

Based on the data and information summarized above, Novozymes concludes that xylanase enzyme preparation is GRAS for the intended uses.

Allergen Labeling

The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) amends the Federal Food, Drug, and Cosmetic Act (FD&C Act) to require that the label of a food that is or contains an ingredient that bears or contains a "major food allergen" declare the presence of the allergen (section 403(w)). FALCPA defines a "major food allergen" as one of eight foods or food groups (i.e., milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) or a food ingredient that contains protein derived from one of those foods. Xylanase enzyme preparation may require labeling under FALCPA, because it will contain protein derived from wheat. Although issues associated with labeling food are the responsibility of the Office of Nutrition, Labeling, and Dietary Supplements, issues associated with FALCPA exemptions are the responsibility of the Food Allergen Coordinator in the Office of Food Additive Safety.

Section 301(ll) of the FD&C Act

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FD&C Act to, among other things, add section 301(ll). 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 its review of Novozymes' notice that xylanase enzyme preparation is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing xylanase enzyme preparation. Accordingly, this response should not be construed to be a statement that foods that contain xylanase enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Novozymes, as well as the information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that xylanase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of xylanase enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000472, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

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

(1) Novozymes also cites publicly available information discussing the lack of a health concern posed by the used of major food allergens in fermentation media used to produce microbially-derived enzyme preparations.

(2) % TOS = 100 - A - W - D; where: A = % ash, W = % water, and D = % diluent and/or formulation ingredients.

More in GRAS Notice Inventory
(/7993/20171031002401/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Agency Response Letter to GRAS Notice No. GRN 563.

Agency Response Letter GRAS Notice No. GRN 000563

Return to inventory listing: **GRAS Notice Inventory (https://wayback.archive-it.org/7993/20171031001831/http://www.fda.gov/grasnoticeinventory)**

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CFSAN/Office of Food Additive Safety

July 23, 2015

Ms. Lori Gregg
77 Perry Chapel Church Road
P. O. Pox 576
Franklinton NC 27525

Re: GRAS Notice No. GRN 000563

Dear Ms. Gregg:

This letter corrects our letter dated July 8, 2015, sent in response to GRAS Notice No. GRN 000563. The purpose of this revised letter is to correct information regarding the margin of safety calculations reported in our previous letter.

The Food and Drug Administration (FDA) received the notice, dated December 12, 2014, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 1, 1997; Substances Generally Recognized as Safe (GRAS)), on behalf of Novozymes North America Inc. (Novozymes). FDA received this notice on December 15, 2014, filed it on December 31, 2014, and designated it as GRAS Notice No. GRN 000563.

The subject of the notice is serine protease enzyme preparation with trypsin specificity, produced by *Fusarium venenatum* carrying a serine protease gene from *F. oxysporum* (serine protease enzyme preparation). The notice informs FDA of the view of Novozymes that serine protease enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in the production of partial and extensive hydrolyzed animal and vegetable protein ingredients, up to 6.84 grams Total Organic Solids per kilogram (TOS/kg) protein raw material.

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. Novozymes' notice provides information about the components in the serine protease enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, serine protease is identified by the Enzyme Commission Number 3.4.21.4. The accepted name for the enzyme is trypsin; there is no systematic name for this serine protease. FDA notes other common names include α-trypsin, β-trypsin, cocoonase, parenzyme, parenzymol, tryptar, trypure, pseudotrypsin, tryptase, tripcellim, and sperm receptor hydrolase. The CAS Registry Number for serine protease is 9002-07-7. Serine protease catalyzes the preferential cleavage of Ser-Lys and Ser-Arg, primarily at the carboxyl side of lysine (Lys-X) or arginine (Arg-X), where X is not proline.

Novozymes describes *F. venenatum* as a nonpathogenic and nontoxigenic microbe that has been shown to be a safe host for enzyme production (GRAS Notice No. GRN 000054 and JECFA, 2003). Fusarium are saprophytes found in soil and are not considered to be human pathogens. Novozymes states that *F. venenatum* WTY842-1-11 is the host strain used to develop the production strain; it is derived from the parental strain *F. venenatum* A3/5[ATCC 20334] by transformation with a fragment containing a deleted version of *F. venenatum tri5* gene. The deletion of the *tri5* gene renders the production strain incapable of producing mycotoxin secondary metabolites in the tricothecene biosynthetic pathway. Additionally, an *amdS* gene from *A. nidulans* encoding acetamidase was inserted and is used as a selective marker during this transformation. Novozymes confirmed the transformation events in WTY-842-1-11 by demonstrating the strain's ability to grow in defined medium containing acetamide as sole nitrogen source, and via Southern blot analysis.

Novozymes describes the development of the production strain, strain WTY939-8-3. The host strain, WTY-842-1-11, was transformed with the serine protease gene from *F. oxysporum* under control of the *F. venenatum* glucoamylase promoter and of the serine protease terminator. The *bar* gene that encodes a phosphinothricin acetyltransferase (allowing growth on the antibiotic, phosphinothricin derived from *Streptomyces hygroscopicus*, is used as a selective marker. Following incubation of the plasmid containing the expression cassettes for the serine protease gene and the selective marker with protoplasts of WTY842-1-11, the high-yielding transformant, strain WTY939-8-3, was selected as the final production strain. Novozymes confirms that the transformed DNA is stably integrated and the inserted genetic material has low potential to transfer, via Southern blot analysis.

Novozymes states that the serine protease enzyme is produced by submerged fermentation of a pure culture of the production strain. Each fermentation batch is initiated from a stock culture and appropriate measures are taken to control for identity, purity, and enzyme-generating ability before use. During fermentation, serine protease enzyme is secreted to the fermentation broth. After fermentation, the enzyme is recovered from the fermentation broth using multiple filtration and concentration steps that also remove residues of the production strain. The final enzyme concentrate is spray dried to a granulated product. Novozymes states that serine protease enzyme preparation does not contain any major food allergens from the fermentation medium. Novozymes also states that serine protease enzyme preparation is produced in accordance with current good manufacturing practices. The raw materials used in fermentation, recovery, and formulation meet predefined quality standards, and are food grade.

Novozymes states that the serine protease enzyme preparation is tested to ensure compliance with established specifications prior to release, including tests to demonstrate absence of microbial pathogens, mycotoxins, and heavy metals in the final enzyme preparation. Novozymes notes that serine protease enzyme preparation conforms to the specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 2014), and to the current General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2006), by providing analytical data from non-consecutive batches of serine protease enzyme preparation.

Novozymes states that the serine protease enzyme preparation is intended for use in the manufacture of partially or extensively hydrolyzed animal and vegetable proteins such as casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, meat, and fish. Hydrolyzed proteins are used as ingredients in a variety of foods, including foods for special dietary use, medical foods, and infant formula, at up to a maximum level of 6.84 g TOS/kg of protein raw material.

To estimate dietary exposure to serine protease enzyme preparation, Novozymes assumes that the enzyme preparation will be used at the maximum intended levels, and that all of the enzyme preparation will remain in the final food. Further, Novozymes assumes that 10% of processed solid foods and 3.5% of processed liquid foods will be treated with the serine protease enzyme preparation. Based on these assumptions, Novozymes estimates a dietary intake of serine protease enzyme preparation from all intended food applications to be 14.5 mg TOS per kg body weight per day (mg TOS/kg bw/d). Novozymes estimates a dietary intake of 34.5 mg/kg bw/d of serine protease from consumption of infant formula, for infants at zero to six months, and 24.6 mg/kg bw/d for infants at seven to twelve months, respectively. Novozymes states that the reaction products resulting from serine protease enzyme activity are already part of the human diet.

Novozymes summarizes unpublished toxicological studies to support the safety of serine protease. Tests conducted using bacterial cells show that serine protease enzyme concentrate is not mutagenic. Novozymes shows that serine protease enzyme concentrate does not induce chromosomal aberrations in cultured human lymphocytes. Novozymes demonstrates with results from a 13-week oral toxicity study conducted using rats that consumption of serine protease enzyme preparation does not cause any treatment-related adverse effects in the test animals, at 581 mg TOS/kg bw/d, the highest dose tested. Novozymes also included the results from a 25-day oral toxicity study conducted using rats that consumption of serine protease enzyme preparation does not cause any treatment-related adverse effects in the test animals at 3605 mg TOS/kg bw/d, the highest dose tested. Based on the highest dose tested in the 25-day study, and the estimated daily intake from the proposed use levels of serine protease enzyme preparation, i.e. 3605 mg TOS/kg bw/d and 14.5 mg TOS/kg bw/d, respectively, Novozymes calculates a margin of safety to be 249. For use in infant formula, Novozymes calculated margins of safety of 104 and 146, for infants aged 0–6 months, and 7–12 months, respectively.

Novozymes discusses the potential food allergenicity of serine protease enzyme. Novozymes conducted an amino acid sequence homology search for serine protease enzyme against known allergens in the Food Allergy Research and Resource Program database as well as the World Health Organization and the International Union of Immunological Societies Allergen Nomenclature Subcommittee database. A search for amino acid sequence homology greater than 35% over an 80-amino acids window identified 13 mite related allergens, three insect venom related allergens, and one canine allergen. Novozymes also performed sequence identity matches across contiguous stretches of eight amino acids in the amino acid sequence of the expressed protein to known allergens, and found no matches. Based on these results Novozymes considers it unlikely that oral consumption of serine protease enzyme will result in allergic responses.

Based on the data and information summarized above, Novozymes concludes that serine protease enzyme preparation is GRAS for its intended uses.

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 its review of Novozyme's notice that serine protease enzyme preparation with trypsin specificity, produced by a genetically modified strain of F. venenatum is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing serine protease enzyme preparation with trypsin specificity, produced by a genetically modified strain of F. venenatum. Accordingly, this response should not be construed to be a statement that foods that contain serine protease enzyme preparation with trypsin specificity, produced by a genetically modified strain of F. venenatum, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that serine protease enzyme preparation is GRAS under the intended conditions. The agency has not, however, made its own determination regarding the GRAS status of the subject use of serine protease enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRAS Notice No. GRN 000563, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

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

[1] *F. venenatum* A3/5 is a natural isolate and has been deposited in the American Type Culture Collection as *F. graminearum* Schwabe ATCC 20334.

More in GRAS Notice Inventory (/7993/20171031001631/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Agency Response Letter to GRAS Notice No. GRN 564.

Agency Response Letter GRAS Notice No. GRN 000564

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CFSAN/Office of Food Additive Safety

July 7, 2015

Ms. Lori Gregg
77 Perry Chapel Church Road
P.O. Box 576
Franklinton NC 27525

Re: GRAS Notice No. GRN 000564

Dear Ms. Gregg:

The Food and Drug Administration (FDA) has received the notice, dated December 12, 2014, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 1, 1997; Substances Generally Recognized as Safe (GRAS)), on behalf of Novozymes North America Inc. (Novozymes). FDA received this notice on December 15, 2014, filed it on January 02, 2015, and designated it as GRN No. 000564.

The subject of the notice is serine protease enzyme preparation with chymotrypsin specificity, produced by a strain of *Bacillus licheniformis* expressing the gene coding for serine protease with chymotrypsin specificity from *Nocardiopsis prasina* (serine protease enzyme preparation). The notice informs FDA of the view of Novozymes that

serine protease enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in the production of partially and extensively hydrolyzed animal and vegetable protein ingredients, up to 785 milligrams Total Organic Solids per kilogram (TOS/kg) protein raw material.

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. Novozymes' notice provides information about the components in the serine protease enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, serine protease with chymotrypsin specificity is identified by the Enzyme Commission Number 3.4.21.1. The accepted name for the enzyme is chymotrypsin; there is no systematic name for serine protease. The CAS Registry Number for serine protease is 9004-07-3. FDA notes other common names include chymotrypsins A and B; α-chymar ophth; avazyme; chymar; chymotest; enzeon; quimar; quimotrase; α-chymar; α-chymotrypsin A; α-chymotrypsin. Serine protease catalyzes the hydrolysis of peptide bonds with preferential cleavage at Ser-Trp, Ser-Tyr, Ser-Phe, Ser-Leu, Ser-Met tryptophan, Ser-His and histidine. The reaction products are smaller proteins and peptides of variable lengths.

Novozymes states that *B. licheniformis* SJ6370, a variant of a natural *B. licheniformis* isolate, is the host strain used to develop the production strain *B. licheniformis* strain S10-34zEK4. *B. licheniformis* SJ6370 is constructed via classical mutagenesis to inactivate genes essential for sporulation and genes encoding several endoproteases, and an alpha-amylase. Additionally, the *amyL* locus and *xylA* locus were modified in order to facilitate integration and expression of the serine protease genes. Novozymes cites published studies describing *B. licheniformis* as a nonpathogenic and nontoxigenic microbe with a long history of safe industrial use for the production of enzymes used in human food. In addition, Novozymes cites several GRAS notices describing the food uses of enzymes derived from *B. licheniformis*. FDA had no questions in response to these GRAS notices.

Novozymes describes the development of the production strain *B. licheniformis* strain S10-34zEK4, from SJ6370 using two expression plasmids pSJ7420 and pSJ7465.[1] Novozymes states that they integrate two sets of the serine protease genes in the *B. licheniformis* genome. Specifically, two serine protease genes encoding serine protease from *N. prasina* were integrated in tandem between the hybrid *Bacillus* promoter and the *Bacillus* terminator at the modified *amyL* locus using the vector pSJ7420. Following this, two serine protease genes encoding serine protease from *N. prasina* were integrated in tandem between the hybrid *Bacillus* promoter and the *Bacillus* terminator at the modified *xylA* locus using the vector pSJ7465. The high-yielding transformant was selected as the final production strain. Novozymes confirms by Southern blot analysis that the transformed DNA is stably integrated into the production organism, that the inserted genetic material has low potential to transfer, and the absence of antibiotic resistance genes due to the genetic modifications.

Novozymes states that the serine protease enzyme is produced by submerged fed-batch fermentation of a pure culture of the production strain. Each fermentation batch is initiated from stock culture and appropriate measures are taken to confirm identity, absence of foreign microorganisms, and enzyme-generating ability before use. During fermentation, serine protease enzyme is secreted to the fermentation broth. After fermentation, the enzyme is recovered from the fermentation broth using multiple filtration and concentration steps that also remove residues of the production strain. The final enzyme concentrate is spray dried to a granulated product. Novozymes states that serine protease enzyme preparation does not contain any major food allergens from the fermentation medium. Novozymes also states that serine protease enzyme preparation is produced in accordance with current good manufacturing practices. The raw materials used in fermentation, recovery, and formulation meet predefined quality standards, and are food grade.

Novozymes states that the serine protease enzyme preparation is tested to ensure compliance with established specifications prior to release, including tests to demonstrate absence of microbial pathogens and heavy metals in the final product. Novozymes notes that serine protease enzyme preparation conforms to the specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 2014), and to the current General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2006), by providing analytical data from non-consecutive batches of serine protease enzyme preparation. Novozymes states that the reaction products resulting from serine protease enzyme activity are already part of the human diet.

Novozymes states that the intended use of serine protease enzyme preparation is in the manufacture of partially or extensively hydrolyzed animal and vegetable proteins such as casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, meat, and fish, at up to a maximum level of 785 mg TOS/kg of protein raw material. The hydrolyzed proteins are intended for use as ingredients in a variety of foods, including foods for special dietary use, medical foods, and infant formula.

To estimate dietary exposure, Novozymes assumes that all processed solid foods and beverages would contain 10% and 3.5% of protein hydrolysates, which would be manufactured using serine protease enzyme preparation at its maximum intended use levels. Novozymes also assumes that all of the serine protease enzyme preparation will remain in the final food. Based on these assumptions, Novozymes estimates a dietary intake of serine protease enzyme preparation from all intended food applications to be 1.67 mg TOS per kilogram body weight per day (mg TOS/kg bw/d). Novozymes estimates a dietary intake of serine protease enzyme preparation to be 3.96 mg/kg bw/d from consumption of infant formula, by infants at zero to six months of age, and 2.83 mg/kg bw/d by infants at seven to twelve months of age, respectively. Novozymes states that the reaction products resulting from serine protease enzyme activity are already part of the human diet.

Novozymes summarizes unpublished toxicological studies to support the safety of serine protease enzyme. Tests conducted using bacterial cells show that serine protease enzyme concentrate is not mutagenic. Novozymes shows that serine protease enzyme concentrate does not induce chromosomal aberrations in cultured human lymphocytes. Novozymes demonstrates with results from a 13-week oral toxicity study conducted using rats that consumption of serine protease enzyme concentrate does not cause any treatment-related adverse effects in the test animals, at levels up to 500.1 mg TOS/kg bw/d. Based on the highest dose tested, and the estimated daily intake from the proposed use levels of serine protease enzyme preparation, i. e. 500.1 mg TOS/kg bw/d and 3.96 mg TOS/kg bw/d, respectively, Novozymes calculates a margin of safety to be 299. For use in infant formula, Novozymes calculated margins of safety of 126 and 177, for infants aged 0–6 months, and 7–12 months, respectively.

Novozymes discusses the potential oral allergenicity of serine protease enzyme. Novozymes conducted an amino acid sequence homology search for serine protease enzyme against known allergens in the Food Allergy Research and Resource Program database as well as the World Health Organization and the International Union of Immunological Societies Allergen Nomenclature Subcommittee database. A search for amino acid sequence homology greater than 35% over an 80-amino acids window identified no matches. Novozymes also performed sequence identity matches across contiguous stretches of eight amino acids in the amino acid sequence of the expressed protein to known allergens, and did not find any matches. Based on these results Novozymes considers it unlikely that oral consumption of serine protease enzyme will result in allergic responses.

Based on the data and information summarized above, Novozymes concludes that serine protease enzyme preparation is GRAS for its intended uses.

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 its review of Novozyme's notice that serine protease enzyme preparation with chymotrypsin specificity, produced by a B. licheniformis expressing a gene coding for serine protease with chymotrypsin specificity from N. prasina is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing serine protease enzyme preparation with chymotrypsin specificity, produced by a genetically modified strain of B. licheniformis. Accordingly, this response should not be construed to be a statement that foods that contain serine protease enzyme preparation with chymotrypsin specificity, produced by a genetically modified strain of B. licheniformis, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that serine protease enzyme preparation is GRAS under the intended conditions. The agency has not, however, made its own determination regarding the GRAS status of the subject use of serine protease enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000564, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

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

[1] These vectors contain strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include i) two genes placed in tandem with slightly different DNA sequences but both encoding the same serine protease enzyme from *Nocamiopsis prasina* (NRRL 18262); ii) well-characterized non-coding regulatory sequences including a transcription terminator from *B. licheniformis*.

More in GRAS Notice Inventory
(/7993/20171031001658/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Agency Response Letter to GRAS Notice No. GRN 714.

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.

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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

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[2] DuPont states that *B. subtilis* strain BG 125 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.[3] 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

[3] 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 21CFR 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

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

Agency Response Letter to GRAS Notice No. GRN 774.

Janet Oesterling
Novozymes North America Inc.
PO BOX 576
77 Perry Chapel Church Road
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000774

Dear Ms. Oesterling:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 000774. We received Novozymes North America Inc.'s (Novozymes) notice on April 10, 2018, and filed it on May 3, 2018.

The subject of the notice is L-glutaminase enzyme preparation produced by *Bacillus licheniformis* expressing the L-glutaminase gene from *B. licheniformis* (L-glutaminase enzyme preparation) for use as an enzyme in the manufacture of wheat proteins, casein, whey protein, soy, breads, noodles, tofu, fish, cheese, and seasonings, at up to 0.17 mg TOS per g of dry protein solids. The notice informs us of Novozymes' view that these uses of L-glutaminase 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. Novozymes' notice provides information about the components in the L-glutaminase enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, L-glutaminase is identified by the Enzyme Commission Number 3.5.1.2. The accepted name for this enzyme is L-glutaminase and the systematic name is L-glutamine amidohydrolase. The enzyme is also known as glutaminase, glutaminase I, and glutamine aminohydrolase. L-glutaminase catalyzes the hydrolysis of glutamine to yield L-glutamate and ammonia. The CAS No. for L-glutaminase is 9001-47-2. Novozymes states that the primary amino acid sequence of L-glutaminase enzyme has been determined.

Novozymes describes *B. licheniformis* as a non-pathogenic, non-toxigenic, well-characterized production organism with a history of safe use in the food industry. Novozymes also states that the *B. licheniformis* production strain SJ13263 was derived from the *B. licheniformis* parental strain DSM 9552 *via* the recipient strain PP1897-3. Novozymes states that the recipient strain was modified at several chromosomal loci to improve product purity and stability. These modifications result in inactivating genes encoding proteases, eliminating the ability to sporulate, and deleting additional genes encoding unwanted proteins in the culture supernatant.

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Novozymes describes the construction of SJ13263 by targeted homologous recombination using a plasmid containing an expression cassette carrying the *ggt* gene encoding the L-glutaminase gene from *B. licheniformis*, under the control of the *B. licheniformis* promoter and a transcriptional terminator. Novozymes states that only the expression cassette with elements between the promoter and the terminator are present in the final production strain as confirmed by Southern hybridization, PCR, and DNA sequencing. Novozymes also confirmed the absence of functional antibiotic resistance genes in the final production strain by Southern hybridization.

Novozymes states that L-glutaminase enzyme is produced by submerged fed-batch fermentation of a pure culture of the production strain. Novozymes states that fermentation is carried out under controlled conditions and that the enzyme is secreted into the fermentation medium. After fermentation, the microbial biomass is removed and the supernatant containing the enzyme is clarified by adjusting pH and adding appropriate flocculants. The liquid enzyme is concentrated and filtered further to remove residual production strain. This is further used for the safety studies. The liquid enzyme concentrate is then stabilized by the addition of glycerol and preserved with potassium sorbate and sodium benzoate to obtain the L-glutaminase enzyme preparation.

Novozymes states that the entire process is performed in accordance with current good manufacturing practices. Novozymes also states that the L-glutaminase 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). Novozymes provides analytical data from three representative batches of L-glutaminase enzyme preparation to demonstrate consistency with the specifications. Novozymes states that the final L-glutaminase enzyme preparation does not contain any major food allergens from the fermentation media.

Novozymes intends to use L-glutaminase enzyme preparation during the manufacture of wheat proteins, casein, whey protein, soy, breads, noodles, tofu, fish, cheese, and seasonings, at up to 0.17 mg TOS per g of dry protein solids. Novozymes states that L-glutaminase will be used in manufacturing these foods. Novozymes states that several factors during manufacturing would render the glutaminase enzyme inactive in the final food. However, Novozymes assumes that all the L-glutaminase enzyme preparation will remain in the final food and, estimates the dietary exposure to L-glutaminase enzyme preparation to be 0.21 mg TOS/kg bodyweight per day based on the maximum intended use level.[1]

Novozymes relies on published information that discusses the safety of microbial enzyme preparations used in food processing, including the safety of the production organism. Glutaminase enzymes sourced from *Bacillus amyloliquefaciens* have a long history of use in Japan as they were first reported in the publicly available literature in 1988. Specifically, glutaminase has been used in the production of soy sauces since 1991, the production of miso since 1992 and the production of hydrolyzed vegetable protein since 2003.

Novozymes discusses potential food allergenicity of L-glutaminase enzyme. Novozymes states that naturally occurring food enzymes, if present in the final food, are unlikely to have allergenic

[1] Novozymes uses the Budget Method to calculate estimated dietary exposure to L-glutaminase enzyme preparation. Novozymes assumes consumption of a maximum of 25 g of solid foods per person per day and that 52% of these solid foods will be processed. Novozymes further assumes these foods to contain 10% of protein hydrolysates that will be treated by the L-glutaminase enzyme preparation at the maximum intended level.

potential because they are present in low concentrations, have history of safe use, or are denatured during food processing, and are susceptible to digestion in the gastrointestinal system. Novozymes further cites the conclusions of several organizations and working groups about the low risk of allergenicity posed by enzymes used as processing aids. Additionally, Novozymes states that no homology to food allergens were found when they conducted sequence homology searches using the peptide sequence of the L-glutaminase against known allergens stored in the FARRP allergen protein database as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. Novozymes also states that homology searches of the L-glutaminase sequence using UNIPROT database did not identify any significant homologies to known toxins. Based on the totality of the information available, Novozymes concludes that it is unlikely that oral consumption of L-glutaminase will result in any allergenic or toxigenic responses. In addition, Novozymes discussed the results of unpublished toxicity studies of the L-glutaminase enzyme concentrate to corroborate safety. Based on No-Observed-Adverse-Effect-Level of 702 mg TOS/kg bw/day from a 13-week oral toxicity study in rats and an estimated theoretical maximum daily intake of 0.21 mg TOS/kg bw/day, Novozymes calculated a margin of exposure of 3303. FDA notes that the margin of exposure is based on unpublished information and serves only to corroborate the published information regarding safety of enzyme preparations used in food.

Based on the data and information summarized above, Novozymes concludes that L-glutaminase 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 Novozymes's notice concluding that L-glutaminase 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 L-glutaminase enzyme preparation. Accordingly, our response should not be construed to be a statement that foods containing [notified substance], if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Novozymes provided, as well as other information available to FDA, we have no questions at this time regarding Novozymes' conclusion that L-glutaminase enzyme preparation produced by *B. licheniformis* expressing the L-glutaminase gene from *B. licheniformis* is GRAS under its intended conditions of use. This letter is not an affirmation that L-glutaminase enzyme preparation produced by *B. licheniformis* expressing the L-glutaminase gene from *B. licheniformis* 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 000774 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Dennis M. Keefe -S

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

Agency Response Letter to GRAS Notice No. GRN 817.

Candice Cryne
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
GERMANY

Re: GRAS Notice No. GRN 000817

Dear Ms. Cryne:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 000817. We received AB Enzymes GmbH (AB Enzymes)'s notice on October 5, 2018, and filed it on November 2, 2018. We received an amendment containing additional safety information on May 29, 2019.

The subject of the notice is serine endopeptidase enzyme preparation produced by *Trichoderma reesei* expressing a gene encoding serine endopeptidase from *Malbranchea cinnamomea* (serine endopeptidase enzyme preparation) for use as an enzyme at up to 10 mg Total Organic Solids (TOS)/kg of raw material in the manufacture of vegetable and animal protein hydrolysates. The notice informs us of AB Enzymes' view that these uses of serine endopeptidase enzyme preparation are 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. AB Enzymes' notice provides information about the components in the serine endopeptidase enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, serine endopeptidase is identified by the Enzyme Commission Number 3.4.21.65.[1] AB Enzymes states that the serine endopeptidase is 281 amino acids in length with a corresponding molecular weight of 28.5 kDa.

AB Enzymes states that the *T. reesei* production strain RF8963 is non-pathogenic and non-toxigenic. AB Enzymes describes the construction of the *T. reesei* production strain by targeted integration of an expression cassette carrying a gene encoding a serine endopeptidase from *M. cinnamomea* fused to a promoter and a terminator from *T.*

[1] https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC3/4/21/65.html

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reesei. AB Enzymes states that the plasmid also carries a synthetic *amdS* gene encoding acetamidase, linked to a promoter and a terminator, all from *Aspergillus nidulans* for growth and selection of the *T. reesei* transformants. AB Enzymes states that the stability of the introduced DNA has been confirmed by Southern blot analysis. AB Enzymes also states that the final production strain does not contain any functional or transferable antibiotic resistance genes.

AB Enzymes states that serine endopeptidase enzyme preparation is manufactured by submerged fed-batch fermentation of a pure culture of the production strain. AB Enzymes states that fermentation is carried out under controlled conditions and that the enzyme is secreted into the fermentation media. After fermentation, flocculants and filter aids are added to the media containing the serine endopeptidase enzyme, at controlled pH and temperature to facilitate enzyme separation. The enzyme is then recovered from the fermentation media by filtration or centrifugation and concentrated, followed by polish and germ filtration. The resulting liquid enzyme concentrate is used for the toxicological studies discussed in the notice. The liquid enzyme concentrate is formulated to a preparation with sodium benzoate, glycerol, sorbitol, and water. AB Enzymes states that the entire process is performed in accordance with current good manufacturing practices. AB Enzymes also states that the final serine endopeptidase enzyme preparation does not contain any major food allergens from the fermentation media.

AB Enzymes has established food grade specification and states that the serine endopeptidase 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). AB Enzymes provides analytical data from three batches of serine endopeptidase liquid enzyme concentrate to demonstrate that the manufacturing acceptance criteria have been met, including the absence of the production strain.

AB Enzymes intends to use serine endopeptidase enzyme preparation in the manufacture of vegetable-derived protein hydrolysates such as soy, wheat, maize, etc., and animal-derived protein hydrolysates, such as whey proteins, caseins, meat, fish, collagen, and gelatin. AB Enzymes intends to use serine endopeptidase enzyme preparation at a maximum level of 10 mg TOS/kg of protein raw material. AB Enzymes notes that the serine endopeptidase enzyme preparation will be deactivated or removed during the production of the protein hydrolysates. However, in estimating dietary exposure, AB Enzymes assumes that all of the serine endopeptidase enzyme preparation will remain in the final food. AB Enzymes estimated dietary exposure from all uses of serine endopeptidase enzyme preparation to be 0.0625 mg TOS/kg body weight per day (mg TOS/kg bw/d).[2]

AB Enzymes relies on published information that discusses the safety of microbial

[2] AB Enzymes uses the Budget method to calculate estimated dietary exposure to serine endopeptidase enzyme preparation based on consumption of a maximum of 12.5 g of solid foods and 25 g of beverages per kg body weight per day.

enzyme preparations used in food processing, including the safety of the *T. reesei* production organism. Additionally, AB Enzymes summarizes unpublished toxicological studies using serine endopeptidase enzyme liquid concentrate to corroborate safety of the intended uses of this enzyme preparation. These studies include an *in vitro* mammalian cell gene mutation assay in mouse lymphoma and *in vitro* micronucleus assay in cultured human lymphocytes with and without metabolic activation. AB Enzymes also discusses the results from an unpublished 13-week oral toxicity study in rats using the serine endopeptidase liquid enzyme concentrate that did not cause any treatment-related adverse effects up to the highest dose tested, equivalent to 1000 mg TOS/kg bw/d. AB Enzymes calculates a margin of exposure based on the No Observed Adverse Effect Level of 1000 mg TOS/kg/bw/d from this study and the estimated maximum dietary exposure from the intended uses of serine endopeptidase enzyme preparation, to be approximately 16000. FDA notes the margin of exposure is based on unpublished safety studies and is only corroborative of the published information regarding enzyme preparations used in food processing.

AB Enzymes discusses publicly available literature as well as the conclusions of several organizations and working groups about the low risk of allergenicity posed by enzymes in food to address potential allergenicity due to the proposed uses of serine endopeptidase. Further, based on bioinformatic analyses, AB Enzymes reports that the serine endopeptidase does not share any biologically meaningful sequence homology or sequence identity to potential oral allergens.[3] Based on the totality of the information available, AB Enzymes concludes that it is unlikely that oral consumption of serine endopeptidase will result in allergic responses.

Based on the data and information summarized above, AB Enzymes concludes that serine endopeptidase enzyme preparation is GRAS for its intended use.

Use in Products under USDA Jurisdiction

As provided under 21 CFR 170.270, during our evaluation of GRN 000817, we coordinated with the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture. Under the Federal Meat Inspection Act, the Poultry Products Inspection Act, and the Egg Products Inspection Act, FSIS determines the efficacy and suitability of ingredients used in meat, poultry, and egg products, and prescribes safe conditions of use. Suitability relates to the ingredient's effectiveness in performing its intended technical effect and the assurance that the ingredient's use will not result in products that are adulterated or misleading for consumers.

FSIS has completed its review and has no objection to the use of the serine endopeptidase enzyme preparation for use as an enzyme at up to 10 mg TOS/kg of protein, in meat products for the production of gelatins and animal protein hydrolysates.

[3] AB Enzymes identified sequence homology to six putative allergens (Tri m 2, Tri r 2, Penn 13, Penn 18 and Asp fl 1) during bioinformatic analyses. Based on further analyses of homology to an 8 amino acid window, potential B cell epitopes, and protease digestion predictions, AB Enzymes concluded that serine endopeptidase is not likely to cause an allergic response from its consumption.

Regarding labeling, FSIS would consider the substance a processing aid that does not require labeling under the requested conditions of use.

Any additional questions regarding regulatory guidance from FSIS should be directed to: Dr. Melanie Abley, Acting Deputy Director, Risk Management and Innovations Staff, Office of Policy and Program Development, Food Safety and Inspection Service, at (202) 690-6573 or via e-mail at Melanie.Abley@usda.gov.

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 AB Enzymes' notice concluding that serine endopeptidase 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 serine endopeptidase enzyme preparation. Accordingly, our response should not be construed to be a statement that foods containing serine endopeptidase enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that AB Enzymes provided, as well as other information available to FDA, we have no questions at this time regarding AB Enzymes' conclusion that serine endopeptidase enzyme preparation produced by *T. reesei* expressing a gene encoding serine endopeptidase from *M. cinnamomea* is GRAS under its intended conditions of use. This letter is not an affirmation that serine endopeptidase enzyme preparation produced by *T. reesei* expressing a gene encoding serine endopeptidase from *M. cinnamomea* 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 000817 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2019.08.26 16:39:27 -04'00'

Susan Carlson, Ph.D.
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