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OFFICE OF FOOD ADDITIVE SAFETY

19 June 2020

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Exemption Claim for Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285 [Generally Recognized as Safe (GRAS) Notice] as published in the *Federal Register* [81 FR 54960 (17 August 2016)], Shin Nihon Chemical Co., Ltd. (Shin Nihon), as the notifier [Shin Nihon Chemical Co., Ltd., 19-10 Showa-cho, Anjo, Aichi 446-0063, Japan], is submitting one hard copy and one electronic copy (on CD), of a Notice of the evaluation that arabinase enzyme preparation from *Aspergillus tubingensis* GPA41 is GRAS on the basis of scientific procedures in accordance with 21 CFR §170.30(a) and (b), for use in conventional food and beverage products across multiple categories as defined in the enclosed documents. Based on the conclusion of GRAS status, use of arabinase from *A. tubingensis* GPA41 under the intended conditions of use is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act*. Information setting forth the basis for Shin Nihon's conclusions regarding the determination of arabinase from *A. tubingensis* GPA41 as GRAS, as well as a consensus opinion of an independent panel of experts, are enclosed for review by the agency.

The enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Nobuo Okado
Shin Nihon Chemical Co., Ltd.
19-10 Showa-cho, Anjo
Aichi 446-0063
Japan

Email: nokado@snc-enzymes.co.jp
Tel: +81-566-75-5555

Enclosures:

- One hard copy of the GRAS Notice
- One electronic copy of the GRAS Notice (on CD)

GRAS NOTICE FOR THE USE OF AN ARABINASE ENZYME PREPARATION FROM *ASPERGILLUS TUBINGENSIS* GPA41 AS A PROCESSING AID IN FOOD PRODUCTION

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Shin Nihon Chemical Co., Ltd.
19-10 Showa-cho, Anjyo
Aichi 446-0063
Japan

DATE:
19 June 2020

GRAS Notice for the Use of an Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 as a Processing Aid in Food Production

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GRAS Notice for the Use of an Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 as a Processing Aid in Food Production

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Shin Nihon Chemical Co., Ltd. (Shin Nihon) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that an arabinase enzyme preparation derived from a non-genetically modified production strain of *Aspergillus tubingensis* (*A. tubingensis* GPA41), also referred to as Sumizyme AG, which is intended for use as a processing aid in the processing of fruits and vegetables and as a filtration aid during down-stream wine and fruit juice production based on the enzyme's ability to aid with the degradation of plant cell walls, is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Shin Nihon's view that these notified uses are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Shin Nihon, the undersigned hereby certifies that all data and information presented in this Notice represents a complete and balanced submission that is representative of the generally available literature. Shin Nihon considered all unfavorable, as well as favorable, information that is publicly available and/or known to Shin Nihon and that is pertinent to the evaluation of the safety and GRAS status of arabinase enzyme preparation as described herein.

Signed,

June 19. 2020

Nobuo Okado
Director, Quality Assurance Department
Shin Nihon Chemical Co., Ltd.
nokado@snc-enzymes.co.jp

Date

1.1 Name and Address of Notifier

Shin Nihon Chemical Co., Ltd.
19-10 Showa-cho, Anjo
Aichi 446-0063
Japan

1.2 Common Name of Notified Substance

The subject of this Notice is an arabinase (EC 3.2.1.99) enzyme preparation from non-genetically modified *A. tubingensis* GPA41.

1.3 Conditions of Use

Shin Nihon intends to market an arabinase enzyme preparation intended for use during the processing of fruits and vegetables; it may also subsequently be used in various finished food and beverage (non-alcoholic and alcoholic) applications such as fruit fillings, vegetable purees, and fruit- or vegetable-based beverages, including wines. In addition to the intended use in the processing of fruits and vegetables, the arabinase enzyme preparation is also intended for addition to fruit juices and wines during end-stage production for the purpose of aiding with filtration of the final beverage product. As described in detail in Sections 2.1.1.3 and 2.5.1, the intended uses of the arabinase enzyme preparation are based on the ability of arabinase to hydrolyze α-(1,5)-arabinofuranosidic linkages found in the plant cell wall constituent arabinan, a α-(1,5)-linked polysaccharide of arabinose.

For the processing of fruits and vegetables, the enzyme preparation containing arabinase is proposed for use at levels of up to 500 ppm or approximately 24 mg total organic solids (TOS)/kg food substrate [based on an average TOS content of 4.7% as per analysis of 3 independent batches of the glycerol-formulated arabinase preparation from *Aspergillus tubingensis* GPA41 (Table 2.1.3.1-2)]. The processed fruits and vegetables may in turn be added to a range of foods and beverages, resulting in final inclusion levels of 9.6 to 24 mg TOS/kg food, based on inclusion levels of 40 to 100% of the enzyme-treated fruit or vegetable ingredient in the final food product.

When the arabinase enzyme preparation is added to fruit juices and wine as a filtration aid during end-stage production , the maximum recommended level is 500 ppm, equivalent to approximately 24 mg TOS/kg food.

As presented in Table 1.3-1, considering therefore use of the arabinase enzyme preparation for fruit and vegetable processing and during fruit juice and wine production, the maximum level of the enzyme preparation that could potentially be present in foods containing ingredients prepared with the enzyme and/or foods prepared with the use of the enzyme is 48 mg TOS/kg food or beverage.

Table 1.3-1 Maximum Levels of Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 Potentially Present in Foods Upon the Use of Ingredients Processed with the Enzyme Preparation or Use of the Enzyme Preparation

Food Use[a]	Use Level for Application of Arabinase Enzyme Preparation in Fruit/Vegetable Processing (ppm [as mg TOS/kg food substrate])	Use Level of Arabinase-Processed Fruit/Vegetable Ingredient Added to Food (%)[b]	Fruit and Vegetable Processing	Wine/Juice Production	Cumulative
			A Maximum Level of Arabinase Enzyme Preparation Present in Foods, as Consumed, that Contain Arabinase-Processed Fruit and Vegetable Ingredients (mg TOS/kg food)[c]	B Maximum Level of Arabinase Enzyme Preparation Added as a Filtration Aid During Wine/Juice Production (mg TOS/kg food)[c]	A + B Maximum Amount of Arabinase Enzyme Preparation Potentially Present in Foods, as Consumed[c]
Solid Foods					24 mg TOS/kg food
Fruit-Based Desserts	500 (24[d])	60	14.4	-	
Fruit Fillings for Pastries	500 (24[d])	40	9.6	-	
Fruit purees and pastes	500 (24[d])	100	24	-	
Vegetable purees and pastes	500 (24[d])	70	16.8	-	
Non-Milk Beverages					48 mg TOS/kg beverage[e]
Fruit Drinks and Ades	500 (24[d])	40	9.6	-	
Fruit Juices and Smoothies	500 (24[d])	100	24	24	
Fruit-Based Nectars	500 (24[d])	40	9.6	-	
Vegetable Juices	500 (24[d])	100	24	-	
Wine and wine beverages	500 (24[d])	100	24	24	

ppm = parts per million; TOS = total organic solids.

[a] The food uses and use levels for processed fruits and vegetables described in this table are some of the representative uses that have been reported by the manufacturers of these ingredients. This list is not comprehensive but indicative of the recommended applications of the arabinase-treated fruits and vegetables.

[b] Amount of enzyme-processed fruit/vegetable ingredient used in the final food product application; based on standard recipes obtained from the United States Food and Nutrient Database for Dietary Studies (FNDDS) 2015-2016 (USDA ARS, 2019).

[c] Considering both arabinase-processed fruits and vegetables added to final foods and arabinase added as a filtration aid during the production of fruit juice and wine. For fruits and vegetables processed with arabinase, it is assumed that 100% of the arabinase from *A. tubingensis* GPA41 used in the processing (24 mg TOS/kg food substrate) is carried over into the food products to which these ingredients are added. In reality, the processing conditions involved in the manufacture of the arabinase-treated fruits and vegetables involve, in some cases, treatment at high temperatures, which will inactivate the enzyme. Although in applications where the enzyme preparation is used in the filtration of fruit juices and wine products the enzyme would be added after possible treatment at high temperatures, the filtration membranes [ultrafiltration in the case of juice production and microfiltration (following suspension and/or precipitation of the enzyme by binding with tannins and polyphenols) in the case of wine production] may further reduce the transfer of arabinase into the final food. In the case of wine, racking or soutirage, a traditional wine production method whereby wine is moved from one barrel to another using gravity, also may further reduce enzyme residues in the final wine product.

[d] Based on average TOS content of the glycerol-formulated arabinase enzyme preparation from *A. tubingensis* GPA41 of 4.7% (Table 2.1.3.1-2).

[e] Fruit juice and wine products may be treated twice with arabinase: once during processing of the fruit (24 mg TOS/kg processed fruit and vegetables) and once during production of juice or wine products (24 mg TOS/kg fruit juice or wine). Therefore, the final cumulative use level is 48 mg TOS/kg fruit juice or wine product, the maximum amount of the enzyme preparation potentially present in non-milk beverages.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2019a), Shin Nihon has concluded that the intended uses of arabinase preparation from *A. tubingensis* GPA41 as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Shin Nihon Chemical Co., Ltd.
19-10 Showa-cho, Anjyo
Aichi 446-0063
Japan

Should the FDA have any questions or additional information requests regarding this Notification, Shin Nihon will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is Shin Nihon's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the Freedom of Information Act, 5 U.S.C. 552.

Part 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2.1.1 Identity of the Enzyme

2.1.1.1 Names and Systematic Numbers

Arabinase is identified by the following names and systematic numbers:

Accepted Name:	Arabinase
Systematic Name:	5-α-L-Arabinan 5-α-L-arabinanohydrolase
Synonyms:	Arabinan endo-1,5-α-L-arabinanase; endo-1,5-α-L-arabinanase; endo-α-1,5-arabanase; endo-arabanase; 1,5-α-L-arabinan 1,5-α-L-arabinanohydrolase; arabinan endo-1,5-α-L-arabinosidase (misleading); arabanase

International Union of Biochemistry and Molecular Biology (IUBMB) Number: [Enzyme Commission (EC) Number]	EC 3.2.1.99
Chemical Abstracts Service (CAS) Number:	75432-96-1

2.1.1.2 Amino Acid Sequence and Molecular Mass

The full length amino acid (aa) sequence of the arabinase enzyme from *A. tubingensis* GPA41 is as follows:

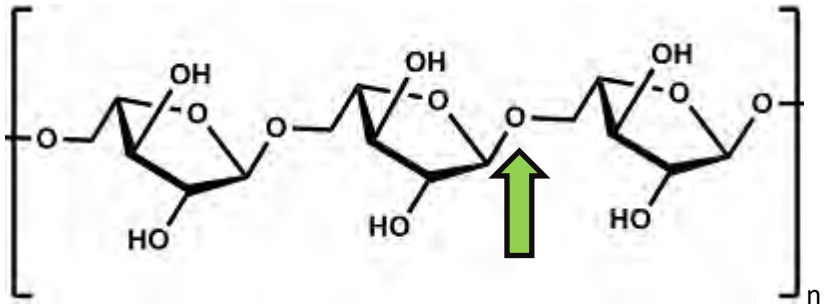
MYQLLSVASV PLLASLVHGY ADPGACSGVC TTHDPGLIRR ESDGTYFLFS TGNKISYVSA SSIEGPWTSV GSMLPDGSSI DLDGNDDLWA PDVSYVDGLY YVYYAVSTFG SQDSAIGLAT SETMEYGSWT DHGSTGIASS SAKIYNAIDP NLIYADGTYY INFGSFWDDI YQVPMKSTPT AAASSSYNLA YDPSGTHAEE GSYMFQYGDY YYLFYSAGIC CGYDTSMPAS GEEYHIKVCR STSPTGDFVD SDGTACTDGG GTMVLESHGE VYGPGGQGVY DDPNLGPVLY YHYMNTTIGY ADSDAQFGWN TIDFSSGWPV V

Arabinase derived from *A. tubingensis* GPA41 is not modified by a post-translational process or by technological procedures, and it is not protein engineered. Based on the amino acid sequence, the calculated molecular mass of arabinase is 34.5 kDa.

2.1.1.3 Enzyme Properties

(endo)-Arabinase[1] is characterized by its ability to hydrolyze (1→5)-α-arabinofuranosidic linkages (BRENDA Professional, 2020) (see Figure 2.1.1.3-1). The enzyme is therefore able to catalyze the endohydrolysis of α-(1,5)-arabinofuranosidic linkages in α-(1,5)-linked polysaccharides of arabinose, also known as arabinans, which are integral plant cell wall constituents, to produce oligomers of shorter length. Arabinase is most active on linear α-1,5-L-arabinan and acts more slowly on the α-(1,5)-linked arabinan backbone in branched arabinans. It belongs to the broader group of hemicellulases, which are enzymes capable of degrading the hemicellulose fraction of plant cell walls (BIOTOL, 1991).

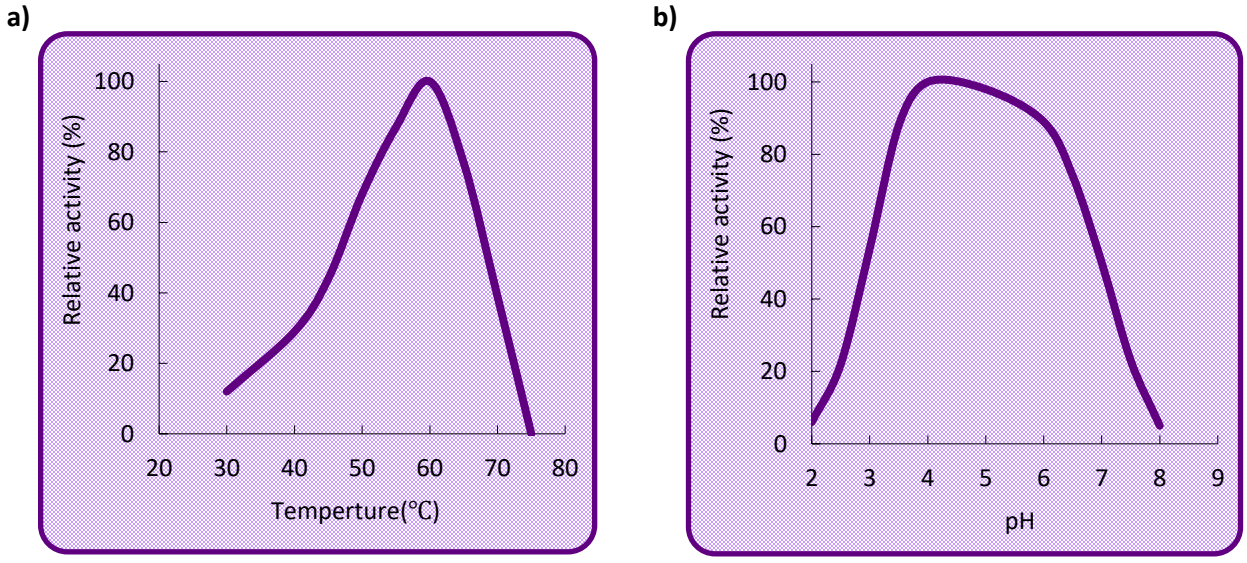
Figure 2.1.1.3-1 Activity of Arabinase on Linear α-(1,5)-L-Arabinan



[1] *Versus* the exo-splitting enzyme, arabinosidase (EC 3.2.1.55), which degrades branched arabinan to a linear chain by splitting off terminal α-(1,3)-linked L-arabinose side chains (BIOTOL, 1991).

The optimum reaction temperature and pH conditions for the activity of arabinase enzyme derived from *A. tubingensis* GPA41 were determined experimentally. As shown in Figure 2.1.1.3-2(a), the arabinase enzyme was determined to have maximum activity at a temperature of 60°C. Enzyme activity showed a progressive decline at temperatures below and above 60°C, with minimal activity observed at 30°C and no enzyme activity observed at 75°C. As shown in Figure 2.1.1.3-2(b), arabinase was determined to have maximum activity at pH in the range of 4 to 5. Enzyme activity showed a progressive decline at pH below 4 and above 5, with little activity observed at pH 2 and 8. Optimal functionality of arabinase from *A. tubingensis* GPA41 was therefore shown to occur at a pH of 3 to 4.5 and at a temperature of 50 to 60°C. The effective temperature and pH ranges for arabinase as intended for use in food and beverage processing are 30 to 60°C and 3 to 6.5, respectively.

Figure 2.1.1.3-2 Activity of Arabinase from *Aspergillus tubingensis* GPA41 as a Function of a) Temperature; and b) pH



2.1.2 Characterization of the Enzyme Source

The arabinase enzyme is derived from non-genetically modified strain of *A. tubingensis* (*A. tubingensis* GPA41), a filamentous fungi species, belonging to *Aspergillus* section *Nigri* (the black aspergilli) (Samson *et al*., 2004, 2007) (see Table 2.1.2-1 for taxonomic classification of *A. tubingensis* GPA41). Other common aspergilli species belonging to the *Aspergillus* section *Nigri* include *A. niger*, *A. carbonarius*, and *A. aculeatus* (Samson *et al*., 2004; Fog Nielsen *et al*., 2009; de Vries *et al*., 2017). Morphologically, *A. tubingensis* is indistinguishable from *A. niger*, which is one of the most common microorganisms used for the production of food enzymes (see Section 6.2.1). Distinction between *A. tubingensis* and *A. niger* can only be achieved by advanced molecular methods (Susca *et al.*, 2007). Since until recently, the species were practically indistinguishable, it may be expected that in some cases a production organism that was previously classified as *A. niger* could in fact have been *A. tubingensis* (EFSA, 2009). Shin Nihon has indicated this specific production strain to have been isolated from a food source (*i.e.*, fruit). *A. tubingensis* GPA41 was selected as the production strain based on its capacity to produce high levels of arabinase activity, its viability, and its suitability for industrial food production, including the lack of mycotoxin production (see Section 2.1.3.3).

The strain was taxonomically identified as belonging to the species *A. tubingensis* by the Centraalbureau Voor Schimmelcultures-Institute of the Royal Netherlands Academy of Arts and Sciences (CBS-KNAW) (renamed in 2017 to 'Westerdijk') Fungal Biodiversity Centre. Identification was made based on molecular analysis. Genomic DNA from the production strain as provided by Shin Nihon was extracted and a part of the calmodulin gene and the β-tubulin gene were amplified. The obtained amplicons were sequenced, analyzed, and compared with sequences deposited in the National Center of Biotechnology Information (NCBI) nucleotide database (GenBank) and local databases of the CBS-KNAW Fungal Biodiversity Centre.

A. tubingensis GPA41 has been deposited at the National Institute of Technology and Evaluation (NITE)'s Biological Resource Center (NBRC) under No. NITE SD 00284. The production strain is also stored and maintained at Shin Nihon's microbial collection (see Section 2.2.3).

Table 2.1.2-1 Taxonomic Classification of *Aspergillus tubingensis* GPA41

Class	Scientific Classification
Kingdom	Fungi
Division	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Section	*Aspergillus* sec. *Nigri*
Species	*Aspergillus tubingensis*
Strain	*Aspergillus tubingensis* GPA41

2.1.3 Composition of the Enzyme Preparation

2.1.3.1 Composition

The ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 is mainly composed of water (approximately 85%), with the protein and ash content of approximately 12% and 0.5%, respectively. No diluents, stabilizers, or preservatives are added to the ultra-filtered concentrate. Consistent with the specifications presented in Table 2.3.1-1, the TOS content of the ultra-filtered concentrate is in the range of 5 to 20%. Compositional analysis for 4 non-consecutive batches of the ultra-filtered concentrate, including the batch used for the toxicological studies (Lot No. 120127T), is presented in Table 2.1.3.1-1.

Table 2.1.3.1-1 Compositional Analyses for the Ultra-Filtered Concentrate of Arabinase from *Aspergillus tubingensis* GPA41

Parameter	Batch Number			
	130128T[a]	140902T[a]	180306T[a]	120127T[a,b]
Water (%)[c]	86.3	88.0	82.0	84.7
Protein (%)[d]	11.4	9.8	15.0	10.6
Ash (%)[e]	0.4	0.5	0.6	0.5
Total Organic Solids (TOS) (%)[f]	13.3	11.5	17.4	14.8
Enzyme Activity (U/g)[g]	680	550	887	667
Enzyme Activity (U/mg TOS)	5.1	4.8	5.1	4.5

[a] Production dates – Lot No. 130128T: 28 January 2013; Lot No. 140902T: 02 September 2014; Lot No. 180306T: 06 March 2018; Lot No. 120127T: 27 January 2012.
[b] This batch was used as the test article for the product-specific toxicological studies conducted (see Section 6.3.2).
[c] Loss on drying (JECFA, 2006b).
[d] Nitrogen determination (Kjeldahl Method).
[e] Determined as total ash (JECFA, 2006b).
[f] Total organic solids = 100% - (A+W+D), where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients.
[g] Internal method used to assess arabinase activity (endo-arabinase assay) developed by Shin Nihon (see Section 2.3.1 and Table 2.3.1-1).

For the preparation of the commercially available enzyme preparation, the ultra-filtered arabinase concentrate may be formulated with glycerol. The content of glycerol in the commercial enzyme preparation is approximately 50%. Glycerol (glycerin) is affirmed as GRAS for use in foods, with no limitation other than Good Manufacturing Practice (GMP) in the U.S. (21 CFR §182.1320 – U.S. FDA, 2019a). The specific activity (and accordingly the TOS content) of the formulated enzyme preparation may be adjusted based on end-product needs and uses. Typically, the formulated enzyme preparation will be standardized to have an activity in the range of approximately 210 to 250 U/g, and not less than 200 U/g. No other stabilizers, preservatives, or diluents are added to formulate the final commercial enzyme preparation.

Compositional analysis of representative glycerol-formulated products is presented in Table 2.1.3.1-2.

Table 2.1.3.1-2 Compositional Analyses for Formulated Arabinase from *Aspergillus tubingensis* GPA41 Product*

Parameter	Batch Number		
	130128-01[a]	140902-12[a]	180306-03[a]
Glycerol (%)	46	47	49
Water (%)[b]	49.1	47.9	46.4
Protein (%)[c]	4.1	4.2	3.7
Ash (%)[d]	0.1	0.2	0.3
Total Organic Solids (TOS) (%)[e]	4.8	4.9	4.3

Table 2.1.3.1-2 Compositional Analyses for Formulated Arabinase from *Aspergillus tubingensis* GPA41 Product*

Parameter	Batch Number		
	130128-01[a]	**140902-12[a]**	**180306-03[a]**
Enzyme Activity (U/g)[f]	244	235	217
Enzyme Activity (U/mg TOS)	5.1	4.8	5.1

* The formulated products (Lot Nos. 130128-01, 140902-12, and 180306-03) were prepared from the ultra-filtered arabinase concentrate batches for which compositional analyses are presented above in Table 2.1.3.1-1 (Lot Nos. 130128T, 140902T, and 180306T, respectively).
[a] Production dates – Lot No. 130128-01: 28 January 2013; Lot No. 140902-12: 02 September 2014; Lot No. 180306-03: 06 March 2018.
[b] Loss on drying (JECFA, 2006b).
[c] Nitrogen determination (Kjeldahl Method).
[d] Determined as total ash (JECFA, 2006b).
[e] Total organic solids = 100% - (A+W+D), where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients.
[f] Internal validated method used to assess arabinase activity (endo-arabinase assay), which was developed by Shin Nihon (also see Table 2.3.1-1).

The use of the arabinase preparation from *A. tubingensis* GPA41, based on TOS levels in the final food, is not to exceed the maximum recommended use levels of 48 mg TOS/kg food (from up to 24 mg TOS/kg food substrate from use in the processing of fruits and vegetables and up to 24 mg TOS/kg food in fruit juice and wine production – see Section 2.5.2).

2.1.3.2 Secondary Enzyme Activity

Arabinase enzyme preparation from *A. tubingensis* GPA41 is characterized by the activity of arabinase which catalyzes the endohydrolysis of α-(1,5)-arabinofuranosidic linkages. However, in addition to arabinase activity, the arabinase preparation also possesses endo-β-galactanase activity. (endo)-β-Galactanase is recognized for its ability to act on galactooligosaccharides and galactan but not arabinan. The arabinase enzyme preparation from *A. tubingensis* GPA41 is intended to be used only for its arabinase activity and specifically to hydrolyze arabinan (see Section 2.1.1.3).

2.1.3.3 Residual Metabolites from the Production Microorganism

Certain filamentous fungi are known as potential producers of toxic secondary metabolites such as mycotoxins. Some strains of *A. tubingensis* have been shown to produce ochratoxin A (Medina *et al.*, 2005; Chiotta *et al*., 2013; Logrieco *et al*., 2014; Lahour *et al*., 2017). However, absence of ochratoxin A production was confirmed for other *A. tubingensis* strains (Samson *et al*., 2004; Fog Nielsen *et al*., 2009; Storari *et al*., 2012; Palumbo and O'Keeffe, 2013; Lamboni *et al*., 2016) and in fact, some 'non-toxigenic' isolates of *A. tubingensis* have been shown to inhibit the production of mycotoxins by other toxigenic fungi, including certain *A. niger* strains (Aukkasarakul *et al*., 2014). Furthermore, no producers of fumonisin B_2 or B_4 were identified among several tested strains of *A. tubingensis* (Logrieco *et al*., 2014; Lamboni *et al*., 2016).

Testing for the presence of mycotoxins (aflatoxins B_1, B_2, G_1, G_2; sterigmatocystin; zearalenone; ochratoxin A; and T-2 toxin) was conducted in the glycerol-formulated final product (Lot Nos. 130128-01, 140902-12, and 180306-03[2]). Lot No. 180306-03 also was analyzed for fumonisins B_1 and B_2 levels. As summarized in Table 2.1.3.3-1, results of the analysis for the finished enzyme product showed no detectable levels of mycotoxins, at the respective limits of quantitation.

Table 2.1.3.3-1 Summary of Mycotoxin Analysis for Glycerol-Formulated Arabinase from *Aspergillus tubingensis* GPA41 Enzyme Preparation*

Parameter	LOD/LOQ	Batch Number		
		130128-01[a]	140902-12[b]	180306-03[c]
Aflatoxin (B_1, B_2, G_1, G_2)	LOQ: 0.5 ppb	ND	ND	ND
Sterigmatocystin	LOQ: 100 ppb	ND	ND	ND
Zearalenone	LOQ: 100 ppb	ND	ND	ND
Ochratoxin A	LOD: 0.5 ppb	ND	ND	ND
T-2 toxin	LOQ: 0.1 ppm	ND	ND	ND
Fumonisin (B_1 and B_2)	LOQ: 0.5 ppm	NA	NA	ND

LOD = limit of detection; LOQ = limit of quantification; NA = not available; ND = not detected; ppb = parts per billion.
Production dates – [a] 28 January 2013; [b] 02 September 2014; [c] 06 March 2018.

The production strain, *A. tubingensis* GPA41, was also subject to extrolite analysis. Results of the analysis confirmed *A. tubingensis* GPA41 not to be a producer of ochratoxin A; aflatoxins B1, B2, G1, or G2; deoxynivalenol; zearalenone; sterigmatocystin; T-2 toxin; or fumonisin B_1 or B_2, as well as several other mycotoxins and secondary metabolites[3]. Additionally, as discussed in Section 6.2.2.1, certain species of *Aspergilli* have been identified as producers of secondary metabolites with poorly described biological activity (EFSA, 2007, 2009). Other secondary metabolites that have been associated with *A. tubingensis* include pyranonigrin, naphtho-γ-pyrones, asperazine, nigragillin, and a number of malformins (Samson *et al*., 2004, 2007; EFSA, 2007, 2009; Fog Nielson *et al.,* 2009; Lamboni *et al*., 2016). Of these less common secondary metabolites of *A. tubingensis*, nigragillin, naphtho-γ-pyrones, and malformins (particularly malformin C) were classified as potentially toxic (EFSA, 2017a; Vadlapudi *et al*., 2017). Therefore, *A. tubingensis* GPA41 as used in the manufacture of the arabinase enzyme preparation was additionally tested for the production of nigragillin, malformins, and naptho-γ-pyrones. Results of the analytical testing confirmed absence of production of these secondary metabolites by the production organism.

[2] Formulated lots which correspond to Lot Nos. 130128T, 140902T, and 180306T of the ultra-filtered concentrate of arabinase, respectively (see Table 2.3.2-1 for batch analysis results).
[3] Alternariol methyl ether; alternariol; 3-Ac-DON; diacetoxyscirpenol; HT-2 toxin; α- and β-zearalenol; cyclopiazonic acid; roquefortine C; citrinin; mycophenolic acid, penitrem A, penicillic acid.

2.2 Manufacturing

2.2.1 Raw Materials and Processing Aids

The materials used in the manufacture of the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 are listed in Table 2.2.1-1. All raw materials and processing aids are food-grade and comply with purity criteria and limits established in the Food Chemicals Codex (FCC) or its equivalent, wherever applicable. All filtration aids are those commonly used by the food industry in the purification of food ingredients and are permitted for use in the U.S. for such purposes. For commercial applications, the arabinase may be formulated with suitable food-grade excipients and/or carriers, including glycerol. Glycerol (glycerin; CAS No. 56-81-5) is GRAS for use as a food substance in accordance with GMP (21 CFR §182.1320 – U.S. FDA, 2019a).

Table 2.2.1-1 Raw Materials and Processing Aids Used in the Manufacture of Ultra-Filtered Concentrate of Arabinase from *Aspergillus tubingensis* GPA41

Material	Function	Regulatory Status
Culture (Fermentation) Medium		
Wheat Bran	Nutrient (carbon, nitrogen, and vitamin source) in culture medium (seed and main fermentation medium)	Food ingredient
Yeast Extract	Nutrient (carbon, nitrogen, and vitamin source) in culture medium (for seed culture only)	Food ingredient
Silicone polymer	Anti-foaming agent (for seed culture only)[a]	Permitted as a defoaming agent (21 CFR §173.340 – U.S. FDA, 2019a)
Processing Aid		
Hydrochloric Acid	pH adjustment	GRAS as a buffer/neutralizing agent when used in foods in accordance with GMP (21 CFR §182.1057 – U.S. FDA, 2019a)
Sodium Hydroxide	pH adjustment	GRAS as a pH control agent when used in foods in accordance with GMP (21 CFR §184.1763 – U.S. FDA, 2019a)
Phosphoric Acid	pH adjustment	GRAS when used in foods in accordance with GMP (21 CFR §182.1073 – U.S. FDA, 2019a)
Filtration/Purification Aids		
Polyvinylidene difluoride (PV) membrane	Microfiltration filter	Permitted for use as an indirect food additive (as a resin may be safely used as articles or components of articles intended for repeated use in contact with food) (21 CFR §177.2510 – U.S. FDA, 2019a)
Polyethersulfone membrane	Ultrafiltration (UF) filter	Permitted for use as an indirect food additive (as a resin may be safely used as articles or components of articles intended for repeated use in contact with food) (21 CFR §177.2440 – U.S. FDA, 2019a)

Table 2.2.1-1 Raw Materials and Processing Aids Used in the Manufacture of Ultra-Filtered Concentrate of Arabinase from *Aspergillus tubingensis* GPA41

Material	Function	Regulatory Status
Diatomaceous earth	Ceramic filtration filter	Used in food processing as a filtration aid; as a component of composite filtration media is the subject of GRAS Notice 87, to which FDA had no questions; GRAS as a filter aid in food processing (SCOGS Report No. 61 [FASEB, 1979])

CFR = Code of Federal Regulations; GMP = Good Manufacturing Practices; GRAS = Generally Recognized as Safe.
[a] The main fermentation step during which the arabinase is secreted by the production strain (*A. tubingensis* GPA41) takes place during solid-state fermentation (see Section 2.6.2), and thus anti-foaming agents are not required at this stage.

2.2.2 Manufacturing Process

The arabinase preparation is manufactured in compliance with current GMP and the principles of Hazard Analysis and Critical Control Points (HACCP). Details of the implemented HACCP system are presented in Section 2.2.3.

A schematic overview of the manufacturing process for the ultra-filtered arabinase concentrate from *A. tubingensis* GPA41 is provided in Figure 2.2.2-1. The enzyme concentrate is produced from food-grade materials using quality-controlled fermentation and purification/recovery processes.

The production organism, *A. tubingensis* GPA41, is well controlled and monitored. Storage and maintenance of the production strain is based on a well-defined 2-tiered cell banking system of pure-culture ampoules, consisting of a master cell bank (MCB) and a working cell bank (WCB). A WCB is only accepted for production runs if it meets established enzyme productivity and the production strain quality control criteria.

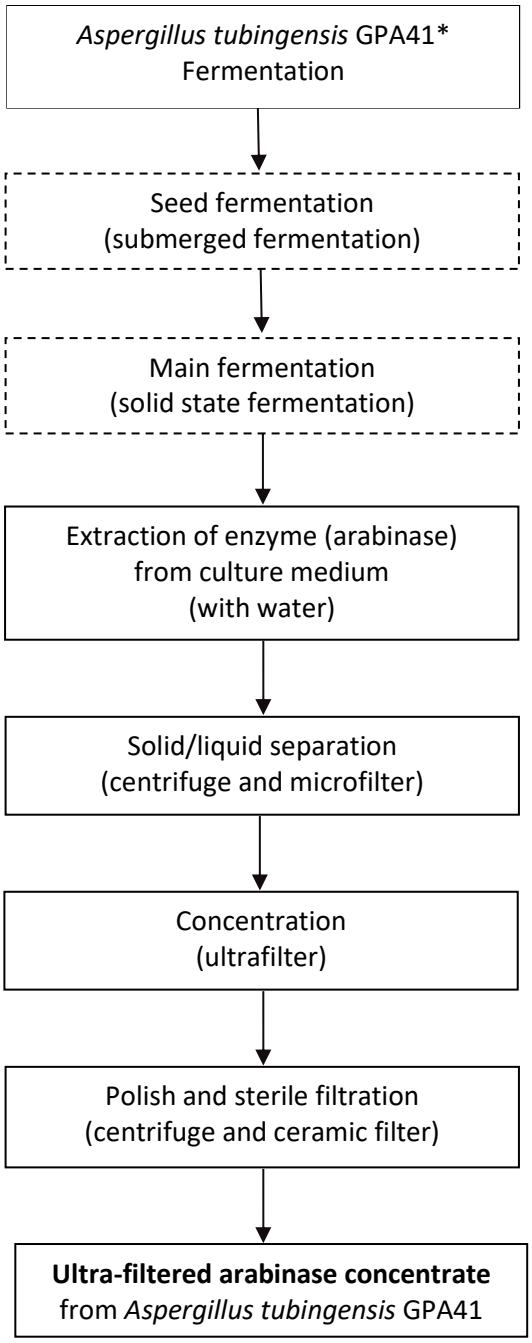
The seed inoculum is grown in a wheat bran- and yeast extract-based medium under submerged fermentation. Subsequently, the main fermentation medium is inoculated with the *A. tubingensis* GPA41 seed inoculum and the main culture is cultivated in a wheat bran-only medium under solid-state. Prior to the main fermentation, the seed culture is assessed again for the production strain and for microbial contamination. Prior to use, the culture media are sterilized under appropriate temperatures for pre-determined amounts of time.

During fermentation, arabinase is secreted from *A. tubingensis* GPA41 (the production strain) into the culture medium. At the completion of the main fermentation, the enzymatic activity is not less than 50 U/g (see Table 2.2.3-1).

The fermentation stage is followed by a series of recovery and purification steps, including extraction, solid/liquid separation, concentration, and polish and sterile filtration. The enzyme is extracted from the fermentation medium with water once fermentation is complete. This is followed by solid/liquid separation, which involves centrifugation and microfiltration with a polyvinylidene difluoride membrane (pore size of 0.45 µm). The filtrate containing the enzyme is then concentrated by ultrafiltration (UF) using a polyethersulfone membrane. The molecular weight cut-off for the UF filter is 5,000 Da; thus, content of substances of molecular weight below the cut-off is reduced in the retentate solution, which contains the enzyme. Finally, the concentrate undergoes germ and sterile filtration to remove insoluble materials and any potential contaminating microorganisms and residual amounts of the production strain. This step involves the addition of a filtration aid (diatomaceous earth) to the concentrated filtrate, centrifugation, and passage through a ceramic filter (pore size 0.2 µm). The product obtained at this stage is an ultra-filtered

concentrate of the arabinase enzyme. The ultra-filtered concentrate is tested to ensure compliance with the established specifications (see Section 2.3.1), including a limit of not less than 450 U/g for enzyme activity, prior to its release. The ultra-filtered concentrate is formulated with suitable carriers such as glycerol to provide a product with an arabinase activity in the range of approximately 210 to 250 U/g and not less than 200 U/g (see Table 2.1.3.1-2). The final glycerol-formulated enzyme preparation is a brown colored liquid with a slight characteristic odor.

Figure 2.2.2-1 Schematic Overview of the Manufacturing Process of Ultra-Filtered Arabinase Concentrate from *Aspergillus tubingensis* GPA41



* Ampule from working cell bank.

2.2.3 Quality Control

A HACCP plan is implemented during the manufacture of the arabinase preparation. Critical control points have been identified and measures are in place for the prevention of the identified hazards. Quality control steps during both the fermentation and purification processes ensure production of a high quality and consistent product.

Items that are monitored and controlled at each stage of the production process and the corresponding acceptance criterion are listed in Table 2.2.3-1. Measures are in place to ensure absence of microbial contamination and to ensure that residual amounts of the production strain (*A. tubingensis* GPA41) are not transferred to the final product. Additionally, the fermentation conditions are strictly maintained to ensure they are optimal for mycelium growth and enzyme production. The seed culture is also assessed for the production strain and microbial contamination. During the main (solid-state) fermentation, the temperature is strictly controlled and enzyme activity is assessed. Absence of microbial contamination, as well as absence of the production strain is verified during the final stages of production (*i.e.*, mold: not detected).

Ultimately, each manufactured batch of the ultra-filtered enzyme concentrate is analyzed for conformity with the specifications as set out in Table 2.3.1-1.

Table 2.2.3-1 Quality Control Steps in the Production of Ultra-Filtered Arabinase Concentrate from *Aspergillus tubingensis* GPA41

Quality Control Step	Items Measured or Controlled[a]	Criterion
Production Strain	N/A	Stored at -80°C in production facility (microbial collection at Shin Nihon) and at NBRC
Seed Fermentation	Production strain (mold: *A. tubingensis* GPA41)	NLT 10^3 CFU/g
	Microbial contamination (total aerobic plate count)	Not detected
Main Fermentation	Temperature (continuously monitored and controlled)	29 to 31°C
	Enzyme activity (endo arabinase assay)	NLT 50 U/g or U/mL (±3 SD)
Concentration with Ultrafiltration Membrane[b]	Enzyme activity (endo arabinase assay)	NLT 450 U/g
Filtration with Ceramic Filter	Production strain (mold: *A. tubingensis* GPA41)	Not detected
	Microbial contamination (total aerobic plate count)	NMT 50,000 CFU/g
	Enzyme activity (endo arabinase assay)	NLT 450 U/g

CFU = colony-forming units; N/A = not applicable; NBRC = Biological Resource Center, National Institute of Technology and Evaluation; NLT = not less than; NMT = not more than; SD = standard deviation.
[a] Methods of analysis are listed in Table 2.3.1-1.
[b] While this step is not a critical control point, arabinase activity is monitored at this stage.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Food-grade specifications have been established for the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 and are presented in Table 2.3.1-1. The specifications for arabinase concentrate from *A. tubingensis* GPA41 comply with the current purity limits for food enzyme preparations as established in the latest edition of the FCC (2019a,b) and by the Joint FAO/WHO Committee on Food Additives (JECFA, 2006a). All methods of analysis are nationally or internationally recognized or have been internally validated by Shin Nihon.

Enzymatic activity of the concentrate (*i.e.*, arabinase activity) is measured using an internal method (*i.e.*, endo-arabinase assay) developed by Shin Nihon whereby 1 unit of activity (U) is defined as the amount of enzyme required to release 1 µmole arabinose reducing-sugar equivalents from linear arabinan per minute under the conditions of the assay.

Table 2.3.1-1 Specifications for Ultra-Filtered Concentrate of Arabinase from *Aspergillus tubingensis* GPA41

Parameter	Specification Limit	Method of Analysis
Compositional Parameters		
Enzyme Activity (U/g)	≥450[a]	Endo-arabinase assay (internal)[b]
Total Organic Solids (TOS) (%)	5 to 20	Calculation[c]
Heavy Metals		
Arsenic (mg/kg)	NMT 3	Japan's Specifications and Standards for Food Additives (9th Edition), B General Tests, Arsenic Limit Test (MHLW, 2018)
Lead (mg/kg)	NMT 5	Japan's Specifications and Standards for Food Additives (9th Edition), B General Tests, Lead Limit Test (AAS) (MHLW, 2018)
Microbiological Criteria		
Total Aerobic Plate Count (CFU/g)	NMT 50,000	BAM – Chapter 3: Conventional plate count method (U.S. FDA, 2001)
Coliforms (CFU/g)	NMT 30	BAM – Chapter 4: Conventional method for coliforms (U.S. FDA, 2002)
Escherichia coli (in 25 g)	Negative	BAM – Chapter 4: Conventional method for *E. coli* (U.S. FDA, 2002)
Salmonella species (in 25 g)	Negative	AOAC Method 989.13 (AOAC, 2000)
Others		
Antibiotic Activity	Negative	Antibiotic activity (JECFA, 2006c)[d]

AAS = atomic absorption spectrophotometry; AOAC = Association of Analytical Communities; BAM = Bacteriological Analytical Manual; CFU = colony-forming units; NMT = not more than.

[a] The specific activity (and accordingly the TOS content) of the formulated enzyme preparation may vary depending on the needs of Shin Nihon's customers. Typically, the formulated enzyme preparation will be standardized to have a minimum guaranteed activity of 200 U/g. Although the levels of enzyme activity (and hence TOS values) is variable, the use of the enzyme preparations containing arabinase from A. *tubingensis* GPA41 will not exceed the maximum recommended use levels of 48 mg TOS/kg food (from up to 24 mg TOS/kg food substrate from use in processing of fruits and vegetables and up to 24 mg TOS/kg food in fruit juice and wine production).

[b] The enzyme activity is defined as the amount of arabinase which liberates 1 mg of substrate residue (arabinose reducing-sugar equivalents) from linear arabinan per minute (expressed as U).

[c] Total organic solids = 100% - (A+W+D), where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients.

[d] Tested in formulated product.

2.3.2 Batch Analysis

Three non-consecutive batches of the ultra-filtered concentrate of arabinase derived from *A. tubingensis* GPA41 (Lot Nos. 130128T, 140902T, and 180306T) were analyzed to verify that the manufacturing process produces a consistent product that meets the specifications defined in Section 2.3.1 (Table 2.3.1-1). Results of the batch analysis are summarized in Table 2.3.2-1 and confirm production of a product consistent with the established specifications.

Table 2.3.2-1 Batch Analyses for the Ultra-Filtered Concentrate of Arabinase from *Aspergillus tubingensis* GPA41

Parameter	Specification	Manufacturing Batch Number		
		130128T[a]	140902T[b]	180306T[c]
Compositional Parameters				
Assay (Activity) (U/g)	NLT 450	680	550	887
Total Organic Solids (%)	5 to 20	13.3	11.5	17.4
Heavy Metals				
Arsenic (mg/kg)	NMT 3	≤3	≤3	≤3
Lead (mg/kg)	NMT 5	≤5	≤5	≤5
Microbiological Parameters				
Total Viable Plate Count (CFU/g)	NMT 50,000	≤10	≤10	≤10
Coliforms (CFU/g)	NMT 30	≤30	≤30	≤30
Escherichia coli	Negative (in 25 g)	Negative	Negative	Negative
Salmonella species	Negative (in 25 g)	Negative	Negative	Negative
Others				
Antibiotic Activity	Negative	Negative[d]	Negative[d]	Negative[d]

CFU = colony-forming units; NLT = not less than; NMT = not more than.
Production dates – [a] 28 January 2013; [b] 02 September 2014; [c] 06 March 2018.
[d] Tested in the glycerol-formulate product (Lot Nos. 130128-01, 140902-12, and 180306-06 corresponding to ultra-filtered concentrate Lot Nos. 130128T, 140902T, and 180306T, respectively).

2.4 Stability

2.4.1 Bulk Stability

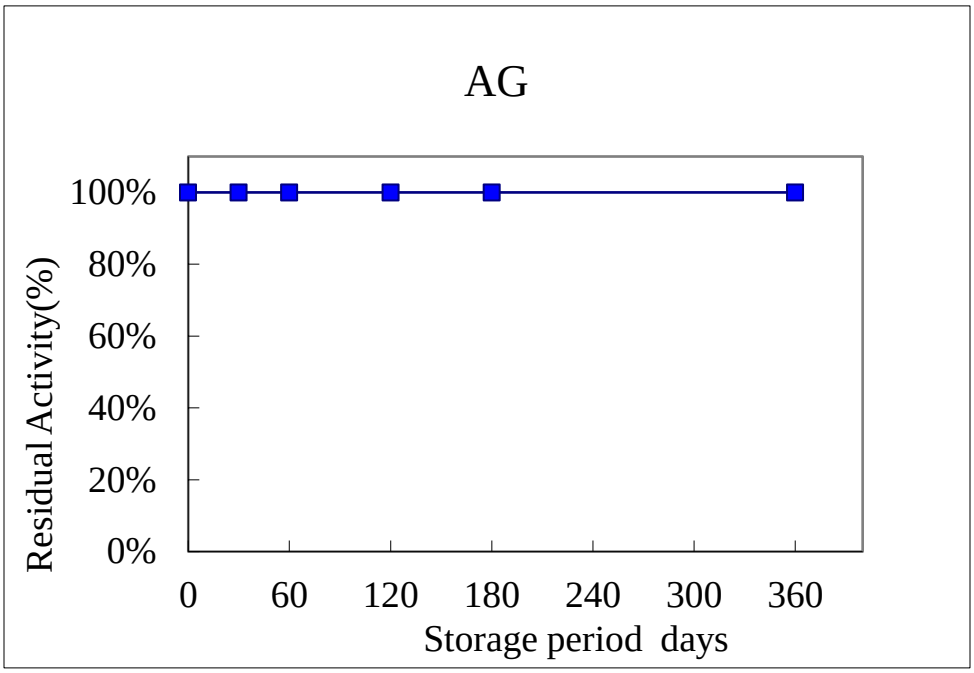
It is recommended that that the enzyme preparation be stored under cool temperatures (refrigeration to room temperatures) and dark conditions. A minimum shelf-life of 12 months has been established for the glycerol-formulated enzyme preparation when it is stored under ambient temperatures (20 to 25°C) in appropriate packaging (airtight containers). The stability data supporting the 12-month shelf-life of the enzyme preparation under the indicated storage conditions are provided in Table 2.4.1-1 and Figure 2.4.1-1. The stability study was conducted with a representative liquid arabinase preparation formulated with glycerol (AG-L200 G ARB; Lot No. 120209-05) and characterized by an arabinase activity of approximately 200 U/g. The enzyme preparation was stored at a temperature of 20 to 25°C for up to 360 days (approximately 12 months). No loss of enzyme activity was observed during storage of the enzyme preparation at any time point, for up to 12 months.

Table 2.4.1-1 Stability of a Representative Sample of Arabinase Preparation Formulated with Glycerol During Bulk Storage

Parameter Evaluated	Time Point (Day)					
	0 (Baseline)	30	60	120	180	360
Residual Activity[a]	100	100	100	100	100	100

[a] % of initial activity.

Figure 2.4.1-1 Stability of a Representative Sample of Arabinase Preparation Formulated with Glycerol (AG-L200 G ARB – Lot No. 120209-05) During Bulk Storage



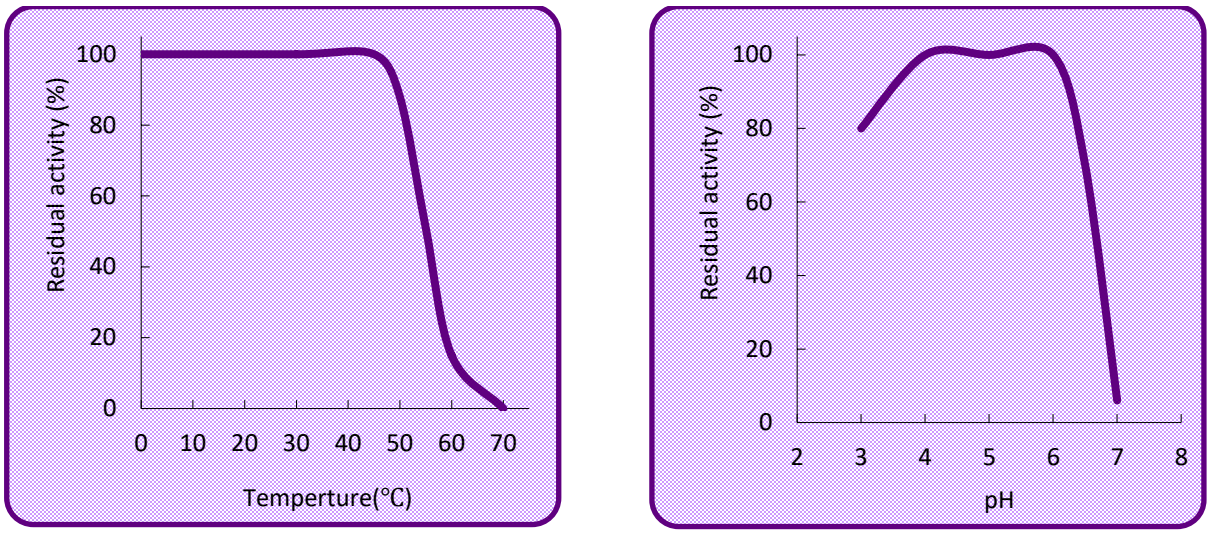
2.4.2 Thermo- and pH-Stability

Stability studies were conducted to evaluate the thermo- and pH stability of arabinase from *A. tubingensis* GPA41 under a range of temperatures and pH conditions.

To assess thermostability, residual arabinase activity of enzyme test solutions (2 U arabinase/mL; pH 4) pre-incubated at temperatures ranging from 0 to 70°C was measured using the endo-arabinase assay (see Table 2.3.1-1). As shown in Figure 2.4.2-1(a), arabinase activity was relatively stable at temperatures of 0 to 50°C. As temperature increased, enzyme stability decreased rapidly, with no activity observed at a temperature of 70°C. Similarly, the pH stability of arabinase was investigated by measuring the residual arabinase activity of enzyme test solutions (2 U arabinase/mL) pre-incubated for 1 hour at 40°C, with the pH adjusted to 3, 4, 5, 6, 6.5, or 7. As shown in Figure 2.4.2-1(b), arabinase activity was relatively stable at pH values ranging from 3 to 6.5, with a rapid decline in enzymatic activity observed at pH 7.

Figure 2.4.2-1 Stability of Arabinase from *Aspergillus tubingensis* GPA41 Following Incubation Under Varying a) Temperature; and b) pH Conditions

a) **b)**



2.5 Intended Technical Effects

2.5.1 Technological Function

Traditionally, one of the main applications of arabinase in food and beverage processing is for the reduction of haze in fruit juices (Sharma *et al*., 2017). Shin Nihon intends to use the enzyme preparation for the processing of fruits and vegetables intended for use in various applications such as fruit fillings, vegetable purees, and fruit- or vegetable-based beverages. The enzyme preparation also may be added for processing of grapes intended for wine production. Additionally, the enzyme preparation may be further used during downstream fruit juice and wine production to aid with end-stage filtration. The proposed uses of the enzyme preparation are consistent with the ability of arabinase to degrade arabinose polysaccharides (arabinans) which are structural components of fruit and vegetable cell walls. As described in Section 2.1.1.3, arabinase is a plant cell-wall-degrading enzyme, which breaks down the cell wall polysaccharide, arabinan, *via* endohydrolysis. The cell wall-degrading ability of arabinase can aid with the 'softening' of fruit or vegetable pieces during the preparation of pastes or purée or with the release (extraction) of juice from fruits and vegetables (*e.g*., during juice squeezing, membrane filtration). As such, arabinase may be added at various stages of fruit or vegetable processing and juice or wine making. The processed fruit or vegetable may be the final food product as consumed (*e.g*., ready-to-eat applesauce) or used as an ingredient of other final food products.

The enzyme preparation is not intended to perform any technological function in the final food product. In many cases, any residual arabinase that remains following addition of the enzyme preparation during the processing of fruits and vegetables will be heat-denatured. As described in Section 2.4.2, the enzyme is inactivated at temperatures ≥70°C. The final processing steps for some of the finished food ingredients and/or food products will involve high temperatures, such as those which occur during pasteurization or sterilization. For example, fruit and vegetable juices and other fruit and vegetable preparations such as pastes and purée are typically thermally treated (pasteurized) to minimize pathogenic microbial contamination. In such cases, arabinase will be heat-denatured as a result of the final food processing, and

the enzyme will not have any technological effect on the final foods as consumed. In cases where the enzyme preparation will be used during down-stream production of juices or wines, some removal of the enzyme from the final food product is anticipated to occur as a result of the final filtration steps. Specifically, the enzyme preparation is primarily added to aid with end stage filtration of the juice or wine (*i.e*., ultrafiltration and microfiltration, respectively), minimizing accumulation of various substances on the surface of the filtration membranes (*i.e*., membrane fouling), which blocks the filtration membranes and disturbs filtration efficacy.

2.5.2 Food Applications and Use Levels

The arabinase enzyme preparation from *A. tubingensis* GPA41 (Sumizyme AG) is intended for use in the processing of fruits and vegetables, which are subsequently added to finished food and beverage products, as well as during downstream production of juices and wines. For the purposes of the dietary exposure assessment, it is assumed that the amount of enzyme added on a mg TOS basis per kg of substrate is equivalent to the amount of enzyme present per kg of processed fruit or vegetable used in final foods.

The maximum recommended level of the enzyme preparation from *A. tubingensis* GPA41 that will be added during the processing of fruits and vegetables is 500 ppm or approximately 24 mg TOS/kg food substrate. The processed fruits and vegetables are in turn added to a range of foods and beverages. The representative final food uses to which the enzyme-processed fruit and vegetable ingredients may be added and the use levels of the processed fruits and vegetables in final foodstuffs are summarized in Table 2.5.2-1. Addition of fruits and vegetables processed with arabinase to final foods as consumed at maximum use levels ranging from 40 to 100% would result in levels of the arabinase enzyme preparation in the final foodstuff ranging from approximately 9.6 to 24 mg TOS as arabinase/kg food.

Table 2.5.2-1 Summary of Food Uses and Use Levels of Fruits and Vegetables Processed with the Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 in Conventional Food and Beverage Products

Food Use[a]	Use Level for Application of Arabinase Enzyme Preparation in Fruit/Vegetable Processing (ppm [as mg TOS/kg food substrate])	Use Level of Arabinase-Processed Fruit/Vegetable Ingredient Added to Food (%)[b]	Amount of Arabinase Enzyme Preparation Potentially Present in Foods, as Consumed, that Contain Arabinase-Processed Ingredients (TOS/kg food)[c]
Solid Foods			
Fruit-Based Desserts	500 (24[d])	60	14.4
Fruit Fillings for Pastries	500 (24[d])	40	9.6
Fruit Purees and Pastes	500 (24[d])	100	24
Vegetable Purees and Pastes	500 (24[d])	70	16.8
Non-Milk Beverages			
Fruit Drinks and Ades	500 (24[d])	40	9.6
Fruit Juices and Smoothies	500 (24[d])	100	24
Fruit-Based Nectars	500 (24[d])	40	9.6
Vegetable Juices	500 (24[d])	100	24
Wine and Wine Beverages	500 (24[d])	100	24

Table 2.5.2-1 Summary of Food Uses and Use Levels of Fruits and Vegetables Processed with the Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 in Conventional Food and Beverage Products

Food Use[a]	Use Level for Application of Arabinase Enzyme Preparation in Fruit/Vegetable Processing (ppm [as mg TOS/kg food substrate])	Use Level of Arabinase-Processed Fruit/Vegetable Ingredient Added to Food (%)[b]	Amount of Arabinase Enzyme Preparation Potentially Present in Foods, as Consumed, that Contain Arabinase-Processed Ingredients (TOS/kg food)[c]

[a] The food uses and use levels for processed fruits and vegetables described in this table are some of the representative uses that have been reported by the manufacturers of these ingredients. This list is not comprehensive but indicative of the recommended applications of the arabinase-treated fruits and vegetables.
[b] Amount of enzyme-processed fruit/vegetable ingredient used in the final food product application. Based on standard recipes obtained from the U.S. Food and Nutrient Database for Dietary Studies (FNDDS) 2015-2016 (USDA ARS, 2019).
[c] Assumes 100% of the arabinase from *A. tubingensis* strain GPA41 used in the processing of fruits and vegetables (500 ppm or 24 mg TOS/kg food substrate) is carried over into the food products in which these ingredients will be added. In reality, the processing conditions involved in the manufacture of the arabinase-treated ingredients may involve treatment at high temperatures which will inactivate the enzyme, thereby ensuring that the enzyme will not have any technological effect in the foods containing the ingredients that are consumed.
[d] Based on average TOS content of the glycerol-formulated arabinase enzyme preparation from *A. tubingensis* GPA41 of 4.7% (see Table 2.1.3.1-2).

For the additional use of the arabinase enzyme preparation during juice or wine production as a filtration aid, the maximum recommended level of the enzyme preparation that will be added is 500 ppm, equivalent to approximately 24 mg TOS/kg fruit juice or wine. In juices and wines, therefore, the cumulative maximum recommended use level of the enzyme preparation (from initial use in fruit processing at 500 ppm, as per Table 2.5.2-1), followed by subsequent use of the enzyme preparation during production of the fruit juice or wine (500 ppm) is 1,000 ppm, equivalent to approximately 48 mg TOS/kg of fruit juice or wine.

Cumulative maximum levels of the arabinase enzyme preparation from *A. tubingensis* GPA41 that could potentially be present in the consumed product from use of the enzyme preparation in both fruit and vegetable processing and juice and wine production are summarized in Table 2.5.2-2.

Table 2.5.2-2 Maximum Levels of Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 Potentially Present in Foods Processed with the Enzyme Preparation

Food Use	A Maximum Level of Arabinase Enzyme Preparation from Processed Fruit and Vegetables Added to Foods (mg TOS/kg food)	B Maximum Level of Arabinase Enzyme Preparation Added as a Filtration Aid During Wine/Juice Production (mg TOS/kg food)	A + B Maximum Amount of Arabinase Enzyme Preparation Potentially Present in Foods, as Consumed[a]
Solid Foods			24 mg TOS/kg food
Fruit-Based Desserts	14.4	-	
Fruit Fillings for Pastries	9.6	-	
Fruit purees and pastes	24	-	
Vegetable purees and pastes	16.8	-	
Non-Milk Beverages			48 mg TOS/kg beverage[b]
Fruit Drinks and Ades	9.6	-	
Fruit Juices and Smoothies	24	24	
Fruit-Based Nectars	9.6	-	
Vegetable Juices	24	-	
Wine and wine beverages	24	24	

TOS = total organic solids.

[a] Considering both arabinase-processed fruits and vegetables added to final foods and arabinase added as a filtration aid during the production of fruit juice and wine. For fruits and vegetables processed with arabinase, it is assumed that 100% of the arabinase from *A. tubingensis* GPA41 used in the processing (24 mg TOS/kg food substrate) is carried over into the food products to which these ingredients are added. In reality, the processing conditions involved in the manufacture of the arabinase-treated fruits and vegetables involve, in some cases, treatment at high temperatures, which will inactivate the enzyme. Although in applications where the enzyme preparation is used in the filtration of fruit juices and wine products, the enzyme would be added after possible treatment at high temperatures, the filtration membranes [ultrafiltration in the case of juice production and microfiltration (following suspension and/or precipitation of the enzyme by binding with tannins and polyphenols) in the case of wine production] may further reduce the transfer of arabinase into the final food. In the case of wine, racking or soutirage, a traditional wine production method whereby wine is moved from one barrel to another using gravity, also may further reduce enzyme residues in the final wine product.

[b] Fruit juice and wine products may be treated twice with arabinase, first during processing of the fruit (24 mg TOS/kg processed fruit and vegetables) and second during production of juice or wine products (24 mg TOS/kg fruit juice or wine). Therefore, the final cumulative use level is 48 mg TOS/kg fruit juice or wine product, the maximum amount of the enzyme preparation potentially present in non-milk beverages.

For the purposes of the dietary exposure assessment described in Part 3 below, it is assumed that the ingredients (*i.e.*, fruits and vegetables) treated with the enzyme preparation (Sumizyme AG) are added to foods intended for the general population at an inclusion rate of 100%. Similarly, it is assumed that fruit juice and wine treated with the enzyme preparation to aid with end-stage filtration during production will have 100% market penetration at the maximum specified level of use. This is a conservative estimate to account for any variations that may occur from the maximum use levels that are typically reported for the arabinase-treated ingredients and beverage products. Assuming further that 100% of the enzyme preparation (Sumizyme AG) used in the manufacture of the processed ingredients is carried over into the final foods (*i.e.*, there is no removal and/or inactivation), and considering the additional application of the enzyme preparation (Sumizyme AG) during production of juices and wines, the maximum level of the enzyme preparation that could potentially be present in foods containing ingredients prepared with the enzyme and/or foods prepared with the use of the enzyme is 48 mg TOS/kg food or beverage (see Table 2.5.2-2).

Part 3. §170.235 Dietary Exposure

Potential human exposure to the arabinase enzyme preparation under the proposed conditions of use was estimated. The assessment was performed using the 'Budget' method.

3.1 Overview of the Budget Method

The potential human exposure to the arabinase enzyme preparation as derived from *A. tubingensis* GPA41 has been estimated using the Budget Method, which is a widely-accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2009). The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods and of non-milk beverages; (ii) the level of presence of the substance in solid foods and in non-milk beverages; and (iii) the proportion of solid foods and of non-milk beverages that may contain the substance (FAO/WHO, 2009). The levels of anticipated exposure to food enzymes that are derived using the Budget Method are thus considered to be conservative estimates (EFSA, 2009, 2014; FAO/WHO, 2009).

The results of this assessment are described in Section 3.2 below.

3.1.1 Assumptions of the Budget Method

Level of Consumption of Solid Foods and Non-Milk Beverages

The Food and Agriculture Organization of the United Nation/World Health Organization (FAO/WHO) report on the *Principles and Methods for the Risk Assessment of Chemicals in Food* (FAO/WHO, 2009) specifies the standard values for food intakes to be 0.05 and 0.1 kg/kg body weight/day for solid foods and non-milk beverages, respectively.

Level of Presence of Food Enzyme in Solid Foods and Non-Milk Beverages

To estimate the exposure to the enzyme preparation from its intended uses in foods containing ingredients made with the enzyme (fruits and vegetables), it is assumed that the entire enzyme preparation added during processing will be present in the final foods as consumed (*i.e.*, assuming no removal and/or inactivation). Thus, the amount of enzyme assumed to be present in solid foods and non-milk beverages is based on the maximum level of the enzyme used in the production of ingredients. It is anticipated that all of the enzyme preparation added (*i.e.*, 24 mg TOS/kg) remains in the ingredients, which are conservatively assumed to be added to final foods at levels of up to 100%, as described above in Section 2.5.2. However, in the case of fruit juice and wine products, the enzyme preparation is also intended to be used during down-stream production to aid with filtration, resulting in the amount of arabinase added to fruit juice and wine products being doubled. Therefore, the maximum amount of the arabinase enzyme preparation that could potentially be present in non-milk beverages containing ingredients made with the enzyme preparation and produced with the aid of the enzyme preparation (as a filtration aid) is 48 mg TOS/kg food.

Proportion of Solid Foods and Non-Milk Beverages that May Contain the Food Enzyme

According to the FAO/WHO report, the default proportions that are typically assumed are that 12.5% of all solid foods and 25% of all non-milk beverages consumed will contain the food enzyme (FAO/WHO, 2009). However, the proportion of solid foods containing the food enzyme may be increased to 25% in cases where the substance (or in this case, the ingredients made with the enzyme) is used in a wide range of food categories (FAO/WHO, 2009). As mentioned, the foods listed in Table 2.5.2-1 are just some of the representative uses in which the ingredients made with the enzyme preparation could potentially be added. However, since the substance is not used in a wide range of food categories, the proportion of solid foods that are assumed to contain the enzyme was not increased to 25% for the TMDI assessment.

3.2 Theoretical Maximum Daily Intake of Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41

Based on conservative assumptions for the Budget Method described in Section 3.1.1 above, the TMDI of the arabinase enzyme preparation (Sumizyme AG), from its intended use in the processing of fruits and vegetables, and in the clarification of fruit juice and wine during production, was calculated to be 1.35 mg TOS/kg body weight/day, as shown in Table 3.2-1.

Table 3.2-1 Estimated TMDI of Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 from Use in the Production of Foods Intended for the General Population, Including Foods that Contain Ingredients Made with the Enzyme Preparation

Products	A Level of Consumption of Foods (kg/kg bw/day)	B Proportion of Foods Containing Arabinase Enzyme Preparation (%)	C Level of Consumption of Foods Containing Arabinase Enzyme Preparation (kg/kg bw/day)[a]	D Maximum Level of Arabinase Enzyme Preparation in Foods (mg TOS/kg)[b]	Exposure to Arabinase Enzyme Preparation (mg TOS/kg bw/day)[c]
Solid Foods	0.05	12.5	0.00625	24	0.15
Non-Milk Beverages	0.1	25	0.025	48	1.2

bw = body weight; TMDI = theoretical maximum daily intake; TOS = total organic solids.
[a] Calculation: (A)*(B/100).
[b] Maximum amount of arabinase enzyme preparation potentially present in foods, as consumed (Table 2.5.2-2).
[c] Calculation: (C)*(D).

3.3 Summary and Conclusions

The potential human exposure to the arabinase enzyme preparation (Sumizyme AG) was calculated assuming that all of the enzyme used in the processing of fruits and vegetables remains in the final foods containing these ingredients. However, in some cases, the arabinase will be inactivated during the final stages of processing of the food ingredient prepared with the enzyme preparation and/or of the final food product containing the enzyme-treated ingredient. For example, treatment of the ingredient or final food product at high temperatures, such as during pasteurization or sterilization of juices, would denature and inactivate the enzyme. Furthermore, although the additional use of the arabinase enzyme preparation at filtration in the case of fruit juice would occur after possible treatment at high temperatures, the ultrafiltration membranes also are anticipated to reduce the transfer of arabinase into the final food. In the case of wine production, microfiltration of the wine also may aid in the reduction of the transfer of residual enzyme into the final food as the enzyme may be suspended and/or precipitated by binding with tannins

and polyphenols. Enzyme residues in the final wine product may be further reduced by racking or soutirage, a traditional wine production method whereby wine is moved from one barrel to another using gravity.

A number of other conservative assumptions are also made during the exposure assessment to ensure there is no underestimation of the potential exposure to the enzyme preparation (Sumizyme AG), including:

- It was assumed that maximum use levels of the enzyme preparation (Sumizyme AG) are used in the processing of fruits and vegetables (500 ppm) and during juice and wine production (500 ppm), whereas in reality actual use levels are lower;

- It was assumed that the arabinase-processed ingredients (fruits and vegetables) are added to foods and beverages intended for the general population at levels of up to 100%, which is unlikely to be the case (for example, fruit smoothies may contain ingredients other than arabinase-processed fruits); and

- It was assumed that none of the enzyme is denatured or removed.

Using the budget method, the TMDI of the arabinase enzyme preparation (Sumizyme AG) from the consumption of foods containing ingredients made with the enzyme preparation (arabinase-treated fruits and vegetables), while also taking into consideration additional use of the enzyme preparation during fruit juice and wine production, was estimated at 1.35 mg TOS/kg body weight/day in the general population.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the arabinase enzyme preparation from *A. tubingensis* GPA41 (Sumizyme AG).

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

The safety of arabinase enzyme preparation from *A. tubingensis* GPA41 was assessed according to the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC) (IFBC, 1990), which are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods. The determination of safety of the enzyme preparation for use in food followed the decision tree developed by Pariza and Johnson (2001) for evaluating the safety of microbially derived food enzymes (refer to Attachment A of Appendix A).

The primary consideration in evaluating enzyme safety is the safety of the production strain. As such, the potential toxigenicity and pathogenicity, as well as ability to produce antibiotics of the production strain, *A. tubingensis* GPA41, was examined as part of the evaluation of the safety of the arabinase enzyme preparation. A discussion of the safety of *A. tubingensis* GPA41 is provided below in Section 6.2, which included consideration of the history of use of the production organism, potential for secondary metabolite production (mycotoxins and antibiotic activity) by the organism, and its pathogenicity. In addition to the consideration of the safety of the production organism, the safety of the enzyme preparation itself also was assessed. This included examination of the history of use of arabinase in food processing as described in Section 6.3.1. Furthermore, a series of Good Laboratory Practice (GLP)-compliant toxicological studies were conducted with the ultra-filtered concentrate of arabinase of *A. tubingensis* GPA41, including a 90-day oral rat toxicity study and a battery of *in vitro* and *in vivo* genotoxicity assays, that also were considered in support of the safety of the enzyme preparation. Results of the available studies are presented in Section 6.3.2. A NOAEL of 6,900 U/kg body weight/day, equivalent to 1,530 mg TOS/kg body weight/day, was determined for the arabinase concentrate as derived from *A. tubingensis* GPA41 under the conditions of the subchronic toxicity study, which was the highest dose tested in the subchronic toxicity study. Collective evaluation of the results of the available short-term genotoxicity assays confirmed that the arabinase concentrate derived from *A. tubingensis* GPA41 does not possess genotoxic potential. The results of the toxicological studies conducted with the ultra-filtered arabinase concentrate from *A. tubingensis* GPA41 were published in a peer-reviewed journal (Okado *et al*., 2020). Additional considerations related to the safety of arabinase as derived from *A. tubingensis* GPA41 (*i.e.*, potential allergenicity and toxicogenicity of the enzyme) also were addressed and are presented in Section 6.3.3.

The available data supporting the safety of arabinase enzyme preparation from *A. tubingensis* GPA41 are summarized below.

6.2 Safety of the Production Strain

6.2.1 History of Safe Use (*Aspergillus tubingensis*)

Aspergilli species belonging to the *Aspergillus* section *Nigri*, including *A. tubingensis* and other related species such as *A. niger* and *A. aculeatus,* are recognized as a production organism for food enzymes (Pariza and Johnson, 2001; EFSA, 2007, 2009). Within the section *Nigri*, *A. tubingensis* (alongside sister species *A. luchuensis*) was found to be related to *A. niger* (Samson *et al*., 2004, 2007; de Vries *et al*., 2017). Taxonomic classification of the *Aspergillus* section *Nigri* has been a matter of contention and formerly, *A. tubingensis* and *A. niger* were indistinguishable (EFSA, 2009). However, since the development of more advanced molecular methods, the '*A. niger* complex' has been divided into 2 separate species: *A. niger* and *A. tubingensis*. Therefore, while there is limited information available related to the use of *A. tubingensis* as a source organism for food enzymes specifically, it may be reasonably expected that in some cases where a microorganism was previously classified as *A. niger* the organism could in fact have be *A. tubingensis*, and thus it is likely that at least some enzymes used in food processing thought to be produced by *A. niger* strains, are in fact products of *A. tubingensis.*

The U.S. FDA has not objected to the use of *A. tubingensis* strains (Mosseray RH3544 and DuPont IB strain 1M341) as the gene-donor organism in the production of transgenic pectin esterase, polygalactouronase, and lipase (expressed in *Trichoderma reesei*), which were determined as GRAS for use in various food applications in the U.S. [GRAS Notice (GRN) Nos. 000557, 000558, and 000808 respectively] (U.S. FDA, 2015a,b, 2019b).

Although as a fungal strain capable of producing secondary metabolites of potential toxicological concern, *A. tubingensis* does not qualify for the Qualified Presumption of Safety (QPS)[1] status as granted to eligible microorganisms by the European Food Safety Authority EFSA (see Section 6.2.2.1 below), *A. tubingensis* is recognized as a source organism in the production of enzymes in the EU (see Sections 2.1.3.3 and 6.2.2.1). Specifically, EFSA considered the safety of *A. tubingensis* strains (MUCL 39199 and ATCC SD6740) in the production of endo-1,3(4)-β-glucanase and α-galactosidase for use in animal feed (EFSA 2018, 2020).

6.2.2 Secondary Metabolites

6.2.2.1 Toxicogenicity

As discussed in Section 2.1.3.3, some species of filamentous fungi, including *Aspergilli* species, are recognized producers of major food mycotoxins such as ochratoxin A. Accordingly, Shin Nihon provided analytical data demonstrating that the production strain used in the manufacture of the arabinase preparation (*A. tubingensis* GPA41) does not produce major mycotoxins such as aflatoxins, sterigmatocystin, zearalenone, ochratoxin A, T-2 toxin, and fumonisins B_1 and B_2. Similarly, absence of these mycotoxins also was confirmed in the glycerol-formulated arabinase enzyme preparation.

In addition to the potential production of major mycotoxins, *Aspergilli* including *A. tubingensis* also have been identified as producers of secondary metabolites with poorly described biological activity, such as pyranonigrin, nigragillin, naphtho-γ-pyrones, and asperazine (Samson *et al*., 2004, 2007; EFSA, 2007, 2009). Of these metabolites with less well described toxicity profiles, production of nigragillin, naphtho-γ-pyrones, and malformins could present a potential safety concern (Samson *et al*., 2004, 2007; Fog Nielson *et al.*, 2009; Lamboni *et al*., 2016; EFSA, 2017a; Vadlapudi *et al*., 2017). Based on the potential for production of secondary metabolites with unknown toxicity profiles, EFSA considers *A. tubingensis* along with other species of the *Aspergillus* section *Nigri* (*A. niger, A. foetidus, A. tubingensis, A. aculeatus*) as not eligible for QPS status (EFSA, 2007, 2009, 2010, 2011, 2012, 2013). It is recognized however that many of the *Aspergilli* have long histories of apparent safe use for food applications. Recently, enzyme preparations produced using *A. tubingensis* strains were evaluated by EFSA (albeit for use in animal feeds) and considered as safe for their intended uses based on the demonstration of absence of production of secondary metabolites such as naphthopyrones, nigragillin, and pyranonigrin (as well as ochratoxin A) (EFSA, 2018, 2020). Similarly, analytical data confirming absence of malformin C, nigragillin, and aurasperone (a naphtho-γ-pyrone) were considered as part of the evaluation of safety by EFSA of enzyme preparations produced by related *Aspergilli* species, *A. niger* or *A. oryzae* (EFSA, 2015a,b, 2017b,c). Accordingly, as presented in Section 2.1.3.3, the source organism, *A. tubingensis* GPA41, also was tested for production of less ubiquitous secondary metabolites of potential concern, including nigragillin, malformins, naptho-γ-pyrones, which were not detected.

Overall, *A. tubingensis* GPA41 was confirmed not to produce major food-borne mycotoxins as well as other secondary metabolites with less well understood toxicity profiles. Results of mycotoxin testing conducted with the formulated enzyme preparation also were negative, confirming absence of mycotoxins in the final enzyme product. Available analytical results therefore confirm the arabinase concentrate as derived from *A. tubingensis* GPA41 to be free of potential toxic secondary metabolites. This conclusion is also supported by the results of the subchronic toxicity study summarized in Section 6.3.2, which demonstrate that oral exposure to the ultra-filtered enzyme concentrate at doses far in excess of the daily intakes estimated under the proposed conditions of use does not result in systemic toxicity in rats.

6.2.2.2 Antibiotic Production

The arabinase enzyme preparation does not exhibit any antibiotic activity. To ensure that the enzyme product is free of any antibiotic activity, every batch of arabinase enzyme preparation is tested for antibiotic activity as per the specifications (see Section 2.3.1). Results of the confirmatory batch analysis (refer to Table 2.3.2-1) show compliance with the specifications for absence of antibiotic activity as presented in Section 2.3.1.

6.2.3 Pathogenicity

As discussed by Pariza and Johnson (2001), "*Food enzyme preparations rarely contain viable production organisms. Hence the issue of pathogenicity is largely moot as regards food enzyme production strains*". Nevertheless, it is common practice to characterize the pathogenic potential of the production strain. Reports of *A. tubingensis* isolates obtained from humans are generally not common in the literature, although this may be attributable in part to the difficulties associated with differentiating among the different species within the black *Aspergillus* group based on morphological features alone (Gautier *et al*., 2016). However, a few recent studies were identified in the literature in which subjects with suspected or confirmed non-invasive and invasive aspergillosis were recruited in order to assess among others efficacy of antifungal agents or diagnostic methods in which molecular identification techniques were used to differentiate among the *Aspergillus* species. Study participants consisted largely of immune compromised subjects or individuals with underlying health conditions (*e.g*., hematological disorders, chronic obstructive pulmonary disease, sarcoidosis, transplant patients, autoimmune diseases, malignancies) and other predisposing factors for infections (*e.g*., prolonged corticosteroid use). *A. tubingensis* was recovered from patient samples (*e.g*., respiratory tract, skin, or blood samples) in a number of instances (Balajee *et al*., 2009; Arabatzis *et al*., 2011; Pagiotti *et al*., 2011; Colozza *et al.*, 2012; Hsiue *et al.*, 2012; Szigeti *et al*., 2012a,b; Alastruey-Izquierdo *et al*., 2013; Gheith *et al.,* 2014; Shahi *et al*., 2015; Gautier *et al*., 2016; Aller-García *et al*., 2017; Brun *et al*., 2017). Generally, the frequency with which *A. tubingensis* was identified in the human samples appeared comparable or lower to that for *A. niger* (Balajee *et al*., 2009; Arabatzis *et al.,* 2011; Colozza *et al*., 2012; Hsiue *et al.*, 2012; Szigeti *et al*., 2012b; Alastruey-Izquierdo *et al*., 2013; Gheith *et al*., 2014; Shahi *et al*., 2015). A case of invasive *A. tubingensis* infection, successfully treated with antimicrobial agents, was reported in an immuno-compromised male following tooth extraction (Bathoorn *et al*., 2013). Two further case reports of treatable keratitis caused by *A. tubingensis* also were identified in seemingly otherwise healthy non-immunocompromised males (Kredics *et al*., 2009).

Overall, cases of human *A. tubingensis* infections are rare and not usually acquired under normal conditions. Although *A. tubingensis* has the ability to infect humans, it is generally considered an opportunistic pathogen predominantly only affecting individuals with other underlying health conditions. The use of *A. tubingensis* as the source organism in the production of arabinase is therefore not anticipated to pose an increased risk of pathogenicity for the general population (consistent with the proposed use of the enzyme for fruit and vegetable processing or during wine or juice production). It is important to note that the manufacturing process of the ultra-filtered arabinase concentrate involves a number of purification/ filtration steps and quality controls to ensure that the production organism (*A. tubingensis* GPA41) is not transferred to the enzyme preparation.

6.2.4 Toxicological Studies with another Enzyme Preparation Derived from a Related Strain of *Aspergillus tubingensis* (CTM 507)

A 4-week oral toxicity study was identified in which the potential toxicity of a glucose oxidase preparation from another strain of *A. tubingensis* (*A. tubingensis* CTM 507) was assessed (Kriaa *et al*., 2015). Groups of male Wistar rats provided a heat-inactivated glucose oxidase preparation (enzyme activity not reported) derived from *A. tubingensis* CTM 507 or commercially available glucose oxidase *via* gavage at 0.4 mg/kg body weight/day. A further group received only the standard diet (control). No adverse effects were observed in rats following oral administration of the *A. tubingensis*-derived enzyme preparation, thus providing further support for the safety of the *A. tubingensis* species for use in the production of enzymes intended for human consumption.

6.3 Safety of the Enzyme Preparation

6.3.1 Existing Authorizations for Arabinase

Although there are no existing authorizations for the use of arabinase in food in the U.S., use of arabinase as a clarifying agent in juice processing, attributable to its arabinan-degrading ability, appears to be widely recognized (Aehle, 2007; Duvetter *et al*., 2009; Ceci and Lozano, 2010; BMG, 2012; Sharma *et al*., 2017).

The Food Standards Australia New Zealand (FSANZ) lists 'endo-arabinase' (EC 3.2.1.99) from *A. niger* as a processing aid (Schedule 18) and specifically as a permitted enzyme of microbial origin (FSANZ, 2020). In the European Union (EU), 2 applications for the authorization of the food enzyme arabinase from *A. niger* have been submitted to the European Commission [by Advanced Enzyme Technologies Ltd. and by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP)] (EFSA, 2016). The review of the applications by EFSA is in progress.

In Japan, the use of arabinase is approved under the name of hemicellulase; a monograph for arabinase is available in the 9th Edition of the Japan's Specifications and Standards for Food Additives. While presently *A. niger* is listed as a source organism for the enzyme, addition of *A. tubingensis* is expected in an amendment to the 9th Edition or in the next edition.

Additionally, 'arabinanase' from *A. niger* also is included in the Codex Inventory of Substances Used as Processing Aids (IPA) (CCFA, 2018), and appeared on the AMFEP list of commercially used enzymes (AMFEP, 2009). The enzyme also is listed for use in wine production by the International Organisation of Vine and Wine (OIV, 2012, 2017).

6.3.2 Toxicological Studies

The potential toxicity of arabinase from *A. tubingensis* GPA41 was assessed in a battery of standard toxicological tests, consisting of a repeated-dose 90-day oral toxicity study conducted in rats and a series of mutagenicity/genotoxicity assays, including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, an *in vivo* mammalian erythrocyte micronucleus test, and an *in vivo* comet assay. All tests were performed in compliance with the Organisation of Economic Co-operation and Development (OECD) Principles of GLP (OECD, 1998a) and in accordance with the OECD Guidelines for the Testing of Chemicals. Toxicology tests were performed with a single representative batch of ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 (Lot No. 120127T – also see Table 2.1.3.1-1), before the addition of other components used to prepare the formulated enzyme preparation (*e.g.*, glycerol). The test

material lot was characterized by an arabinase activity of 690 U/mL (667 U/g)[4] [consistent with the specification limit of ≥450 U/g for enzyme (arabinase) activity as per Table 2.3.1-1] and a TOS content of 15.3% (w/v) [14.8% (w/w)[5]], which corresponded to 4.5 U/mg TOS.

6.3.2.1 Subchronic Toxicity

The ultra-filtered concentrate of arabinase was administered to male and female Crl:CD (SD) [SPF] rats (10/sex/group) by gavage at dose levels of 0 (distilled water), 69, 690, or 6,900 U/kg body weight/day (corresponding to approximately 0, 15, 153, and 1,530 mg TOS/kg body weight/day, respectively, based on 4.5 U/mg TOS) for 90 or 91 days (Okado *et al*., 2020). The doses were selected based on the results of a previous 2-week dose-range finding study in which no adverse effects were reported following administration of the test material at doses of up to 6,900 U/mL. The subchronic toxicity study was conducted according to GLP and consistent with OECD Guideline No. 408 (OECD, 1998b). Animals were 5 weeks old at study initiation, with body weights ranging from 151 to 176 g for males and 122 to 147 g for females. General condition of the animals was monitored on a daily basis. Body weight and food consumption were measured weekly. Urinalysis, ophthalmological examination, hematology, clinical chemistry, organ weight measurements, macroscopic examination, and microscopic examination were conducted at end of treatment. Animals scheduled for necropsy were euthanized by exsanguination. Histopathological examination was conducted on tissue samples from control and high-dose group animals.

No early deaths were observed throughout the study period. One low-dose male exhibited visible back trauma on Days 30 to 37, with the injury resolving without any lasting effects. Body weights, body weight gain, and food consumption of test animals were comparable to controls throughout the study period. Clinical chemistry analysis revealed significantly higher potassium levels in high-dose (1,530 mg TOS/kg body weight/day) males (4.59±0.11 mmol/L *versus* 4.35±0.18 mmol/L). Since the variability in plasma potassium levels was not accompanied by any changes in urine potassium, or variations in other electrolytes and individual animal values were all noted to be within the laboratory's historical data, the difference was considered as not related to the administration of the enzyme preparation. All other statistically significant differences between test and control values in hematological and clinical chemistry parameters showed no dose-response and none were considered to be related to administration of the enzyme preparation. Urinalysis and ophthalmological examinations were unremarkable.

In high-dose males, relative (to body weight) weights of the spleen (0.145±0.010% *versus* 0.168±0.020%; 14%) and thymus (0.055±0.15% *versus* 0.073±0.015%; 25%) were significantly lower compared to controls. In females, absolute kidney weights were higher in the high-dose group (1.92±0.23 g *versus* 1.69±0.15 g); a clear dose-response was not apparent. The organ weight variations may have been the result of slightly greater (albeit not statistically significant) body weights in males and females of the high-dose group.

Macroscopic variations in high-dose males consisted of single incidences of a cyst in the spleen, a brown patch in the lungs, a brown and a red patch in the liver, and a dilated renal pelvis. In high-dose females, a nodule in the stomach, a diverticulum in the ileum, and a nodule on the Zymbal's gland (auditory sebaceous gland) were observed. Histopathological observations that occurred only in the high-dose group with no correlates in the control group included edema in the glandular stomach in 2 males and 2 females of the high-dose group, as well as regeneration of acinar cells in the pancreas in 2 males and 1 female. The nodule identified on the Zymbal's gland of the high-dose female was histologically confirmed to be an adenoma. The gross abnormalities observed in the spleen, lungs, liver, and kidneys (males) and stomach and ileum (females) were isolated findings. The histological variabilities of the glandular stomach and pancreas in the high-dose animals were slight focal changes, with the severity and morphological characteristics consistent

[4] Note that values presented in Table 2.1.3.1-1 were expressed as % (w/w) rather than % (w/v).

with those typically seen for this strain and age of rats and were therefore determined to be spontaneous findings. Zymbal's gland adenoma had been previously identified as an isolated finding in other repeat-dose oral rat studies conducted at the laboratory. Although Zymbal's gland tumors are generally not considered common spontaneous lesions in rats, incidences in control animals of toxicological studies are documented (Dinse *et al*., 2010; Rudmann *et al*., 2012; Weber, 2017). Therefore, the single occurrence of adenoma of the Zymbal's gland in a female rat in this study also was considered a spontaneous finding. Overall, it was concluded that no adverse effects related to the oral administration of the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 were observed in the study at doses up to 1,530 mg TOS/kg body weight/day (or 6,900 U/kg body weight/day), the highest dose tested.

Based on the result of this study, the highest dose tested of 1,530 mg TOS/kg body weight/day was determined as the NOAEL, which is several-fold greater than the theoretical maximum daily intake of 1.35 mg TOS/kg body weight/day (see Table 3.2-1) resulting from the proposed uses of the arabinase enzyme preparation from *A. tubingensis* GPA41 and thus supports the safety of the arabinase enzyme preparation under the intended conditions of use as described herein.

6.3.2.2 Mutagenicity/Genotoxicity

A battery of short-term *in vitro* and *in vivo* assays was conducted with the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 to assess the potential genotoxicity of the enzyme preparation. The studies are summarized in Table 6.3.2.2-1 with the results discussed below.

Table 6.3.2.2-1 Summary of Results of *In vitro* and *In vivo* Genotoxicity Studies Conducted with Arabinase from *Aspergillus tubingensis* GPA41[a] (Okado *et al*., 2020)

Test System	Test Object	Concentration/Dose	Result
In vitro			
Bacterial Reverse Mutation Test	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537; *E. coli* WP2uvrA	Pre-Incubation (Main): WP2uvrA: 0, 4.31, 8.63, 17.3, 34.5, or 69.0 U/plate (0, 0.96, 1.9, 3.8, 7.7, and 15.3 mg TOS/plate, respectively)[b] (±S9) TA98: 0, 2.16, 4.31, 8.63, 17.3, 34.5, or 69.0 U/plate (0, 0.48, 0.96, 1.9, 3.8, 7.7, and 15.3 mg TOS/plate, respectively)[b] (+S9) TA1537: 0, 4.31, 8.63, 17.3, 34.5, or 69.0 U/plate (0, 0.96, 1.9, 3.8, 7.7, and 15.3 mg TOS/plate, respectively)[b] (+S9)	Positive (TA98; 34.5 and 69.0 U/plate) Negative (all others)
		Modified Method (Main and Confirmatory)[c]: TA98, TA100, TA1535: 0, 2.16, 4.31, 8.63, 17.3, 34.5, or 69.0 U/plate (0, 0.96, 1.9, 3.8, 7.7, and 15.3 mg TOS/plate, respectively)[b] (±S9) TA1537: 0, 2.16, 4.31, 8.63, 17.3, 34.5, or 69.0 U/plate (0, 0.96, 1.9, 3.8, 7.7, and 15.3 mg TOS/plate, respectively)[b] (-S9)	Negative
Chromosome Aberration Test	CHL fibroblasts	Short-Term[d]: 0, 28.3, 35.3*, 44.2*, 55.2*, or 69.0 U/mL (0, 6.3, 7.8, 9.8, 12.3, and 15.3 mg TOS/mL, respectively)[b] (-S9)	Positive
		0, 22.6, 28.3, 35.3, 44.2*, 55.2*, or 69.0* U/mL (0, 5.0, 6.3, 7.8, 9.8, 12.3, and 15.3 mg TOS/mL, respectively)[b] (+S9)	Positive (55.2 and 69.0 U/mL)
		Continuous[e]: 0, 1.07, 1.79, 2.98, 4.97, 8.28, 13.8, or 23.0 U/mL (0, 0.24, 0.40, 0.66, 1.1, 1.8, 3.1, and 5.1 mg TOS/mL, respectively)[b] (-S9)	Negative[f]

Table 6.3.2.2-1 Summary of Results of *In vitro* and *In vivo* Genotoxicity Studies Conducted with Arabinase from *Aspergillus tubingensis* GPA41[a] (Okado *et al*., 2020)

Test System	Test Object	Concentration/Dose	Result
In vivo			
Micronucleus Test	Rat; Crl:CD(SD) [SPF] 6M/group	Oral (gavage); 0, 1,730, 3,450, or 6,900 U/kg bw/day (383, 767, and 1,530 mg TOS/kg bw/day, respectively)[b]; twice (with a 24-hour interval)	Negative
Comet Assay	Rats; Crl:CD(SD) (glandular stomach and duodenal cells) 6M/group	Oral (gavage); 0, 383, 765, or 1,530 mg TOS/kg bw/day; twice (with a 21-hour interval)	Negative

+S9 = with metabolic activation; - S9 = without metabolic activation; bw = body weight; CHL = Chinese hamster lung; h = hours; M = male animals; TOS = total organic solids.
* Concentrations selected for assessment of chromosomal aberrations.
[a] Ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41; Lot number: 120127T (arabinase activity of 690 U/mL and TOS content of 15.3%).
[b] Based on 4.5 U/mg TOS.
[c] 'Treat-an-wash' method applied in case of positive results, which were deemed to be a result of co-presence of free amino acids in the culture medium in the preliminary and/or concentration-finding assays [also in the main study with TA98 (+S9)].
[d] 6-hour treatment, followed by 18-hour expression period.
[e] 24-hour incubation period.
[f] At concentrations of 13.8 or 23.0 U/mL (3.1 and 5.1 mg TOS/mL) significant incidence of c-mitosis impeded analysis.

Bacterial reverse mutation test

The potential mutagenicity of ultra-filtered concentrate of arabinase was evaluated in a bacterial reverse mutation assay using *Salmonella typhimurium* strains TA100, TA98, TA1535, and TA1537, as well as *Escherichia coli* strain WP2*uvrA* in the presence and absence of metabolic activation (Okado *et al*., 2020). The study was conducted in accordance with OECD Guideline No. 471 (OECD, 1997) and the Principles of GLP (OECD, 1998a). Distilled water served as a negative control for all strains. One of the following compounds was employed as a positive control for assays conducted in the absence of metabolic activation: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), sodium azide (NaN3), or 9-aminoacridine hydrochloride (9-AA). For assays conducted in the presence of metabolic activation, 2-aminoanthracene (2-AA) was used as the positive control.

In the preliminary assay and concentration-finding assay, which were conducted using the pre-incubation method, an increase in revertant colonies of twice or more compared to the negative control values was observed in *S. typhimurium* strains TA100 and TA1535 in the presence and absence of metabolic activation. In addition, a similar increase in revertant colonies was observed in *S. typhimurium* strain TA1537 in both the preliminary and concentration-finding assays and in *S. typhimurium* strain TA98 in the concentration-finding assay, in the absence of metabolic activation. These findings were determined to be due to the co-presence of free amino acids and therefore for conditions testing positive in the screening assays (TA100 and TA1535 with and without metabolic activation, and TA1537 and TA98 without metabolic activation), the main study was conducted using a modified pre-incubation method involving a washing step to remove free amino acids released into the culture medium (*i.e*., the 'treat-and-wash' method). The treat-and-wash method is considered to be a valid modified method and is recommended in cases where free amino acids may be present in the test substance, and may be responsible for the overall increase in the bacterial growth and thus greater potential for the occurrence of spontaneous mutations (Thompson *et al*., 2005; EFSA, 2014). This was followed by a confirmatory assay conducted under the same conditions. For strains and/or conditions testing negative in the screening assays (*E. coli* WP2*uvrA* with and without metabolic activation and TA98 and TA1537 with metabolic activation), the main study also followed the pre-incubation method.

In the main study following the standard (pre-incubation) method, growth inhibition was observed at concentrations of ≥17.3 U/plate (≥3.8 mg TOS/plate). An increase in revertant colonies twice or more compared to negative controls was observed only in *S. typhimurium* TA98 at concentrations of 34.5 and 69.0 U/plate (7.7 and 15.3 mg TOS/plate, respectively). Therefore, in the case of *S. typhimurium* TA98, the modified 'treat-and-wash' method also was used to assess the potential mutagenicity of the ultra-filtered concentrate of arabinase in the presence of metabolic activation.

Testing of *S. typhimurium* TA100, TA1535, and TA98 in the presence and absence of metabolic activation and *S. typhimurium* TA1537 in the absence of metabolic activation was carried out using the modified 'treat-and-wash' method. Under the modified method, no growth inhibition was observed and the number of revertant colonies was less than 2 times that of the negative controls. To determine reproducibility of the modified method, a further confirmatory study was conducted. Similar results were observed in the confirmatory study. Based on the results of this study, it was concluded that the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 did not cause gene mutation and that the increases in the number of revertant colonies observed in the assay using the standard method were due to the presences of free amino acids.

<u>*In Vitro* Chromosomal Aberration Test</u>

The clastogenic potential of ultra-filtered concentrate of arabinase was investigated in an *in vitro* chromosomal aberration test conducted in cultured Chinese hamster lung (CHL) fibroblasts (Okado *et al*., 2020). The study was conducted in accordance with OECD Guideline No. 473 (OECD, 2014a), and the Principles of GLP (OECD, 1998a). Following preliminary testing to determine the mitotic index, as well as an appropriate concentration range, an assay was conducted wherein cells were incubated with the test article for 6 hours in the absence or presence of metabolic activation, followed by an 18-hour expression period ('short-term treatment'). A further assay also was conducted wherein cells were incubated with the test article for 24 hours in the absence of metabolic activation ('continuous treatment'). Physiological saline served as the negative control while mitomycin C (MMC) and cyclophosphamide (CP) were used as the positive controls. One hundred metaphase cells per plate were examined microscopically for chromosomal or chromatid-type aberrations. The number of polyploid cells (38 chromosomes or more) also was counted in 200 metaphases for each concentration.

Considering growth rate reductions of less than 50%[5], the concentrations that were selected for assessment of chromosomal aberrations in the short-term assay were 44.2, 55.2, and 69.0 U/mL and 35.3, 44.2, and 55.2 U/mL, with and without metabolic activation, respectively. Compared to controls, statistically significant and concentration-dependent increases in the incidence of chromosome aberrations were observed under the conditions of the short-term assay; however, the increases in the number of cells with aberrations were notably greater in the absence of metabolic activation.

In the assay involving continuous treatment, analysis of cells was considerably impeded by the presence of c-mitosis (mitosis with disturbed spindle function) at all concentrations. Testing under the conditions of the continuous treatment assay was therefore repeated at lower concentrations. At concentrations of up to 8.28 U/mL (1.8 mg TOS/mL), no significant effects on the incidence of chromosome aberrations, incidence of polyploid cells, or cell growth rates were observed. However, at the 2 highest concentrations (13.8 and 23.0 U/mL or 3.1 and 5.1 mg TOS/mL), analysis was again prevented by significant incidences of c-mitosis.

Based on the results of the study, it was concluded that ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 induces structural chromosome aberrations in CHL cell lines as observed under the conditions of the short-term treatment assay; it was noted however that in comparison to known clastogens (including the positive controls used in the study, MMC and CP), the clastogenic activity of the arabinase concentrate was considered to be low. Thus, *in vivo* testing was performed in order to confirm that the enzyme preparation is non-clastogenic.

In Vivo Mammalian Erythrocyte Micronucleus Test

The genotoxic potential of ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 was further investigated in an *in vivo* micronucleus assay conducted in Crl:CD (SD) [SPF] male rats (Okado *et al*., 2020). This study was conducted in accordance with OECD Guideline No. 474 (OECD, 2014b), as well as the principles of GLP. Rats (6/group) were administered the ultra-filtered arabinase concentrate by gavage at dose levels of 0 (control), 1,730, 3,450, and 6,900 U/kg body weight/day (383, 767, and 1,530 mg TOS/kg body weight/day, respectively) for 2 consecutive days. Distilled water served as the negative control while cyclophosphamide was used as a positive control. The general condition of test and negative control animals were observed at 1, 24, 25, and 48 hours after initial dosing. Twenty-four hours after the final administration, animals were euthanized (*via* CO_2 inhalation) and bone marrow samples were prepared. Additionally, animal body weights also were measured immediately before animals were euthanized. No clinical signs of toxicity or adverse effects on body weight gain were observed in the food enzyme groups. There were no significant differences in the frequency of micronucleated cells in the animals receiving the ultra-filtered arabinase concentrate when compared to the negative control group. In addition, no significant differences in the ratio of immature erythrocytes to the total number of analyzed erythrocytes were observed in the test groups compared to the negative control group. In contrast, marked increases in the incidence of micronucleated cells and a decrease in the ratio of immature erythrocytes to the total number of analyzed erythrocytes was observed in the positive control group compared to the negative control group. Based on the results of this study, it was concluded that ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 did not induce micronucleated erythrocytes in rat bone marrow cells and was therefore considered to be non-clastogenic *in vivo*.

[5] The relative cell growth rates were 50% or less at concentrations of ≥55.2 U/mL in the absence of metabolic activation and at concentrations of ≥69.0 U/mL in the presence of metabolic activation

In Vivo Comet Assay

In order to confirm the negative results obtained in the *in vivo* micronucleus assay, and also to consider the potential site-of-contact genotoxicity concerns of the intact enzyme on rapidly dividing cells in the mucosa of the stomach and upper small intestine, particularly since no evidence of toxicity to the target organ (bone marrow cells) was observed in the *in vivo* micronucleus assay, a further *in vivo* study was undertaken in which stomach and duodenum cells were assessed for possible DNA damage. It is generally recognized that site-of-contact tissues may be more appropriate for evaluation of a possible genotoxic effect for highly reactive substances which are not systemically available and where no kinetic evidence of systemic exposure is obtained (EFSA, 2011; OECD, 2016).

As such, the genotoxic potential of ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 was further investigated in an *in vivo* mammalian alkaline comet assay in Crl:CD (SD) [SPF] male rats (Okado *et al*., 2020). Pre-absorptive cells of the stomach and duodenum, which would be expected to come into direct contact with the test article immediately following ingestion, were examined in the assay. The study was conducted in accordance with OECD Guideline No. 489 (OECD, 2016), and the Principles of GLP (OECD, 1998a). Groups of 6 rats were administered ultra-filtered arabinase concentrate by gavage at doses of 383, 765, and 1,530 mg TOS/kg body weight/day for 2 consecutive days at 21-hour intervals. Distilled water served as the test article solvent and negative control, while ethyl methanesulfonate (EMS) served as the positive control. Clinical observations were performed at 1, 21 (prior to the second administration), 22, and 24 (just prior to necropsy) hours following the first administration. Body weight measurements were obtained before necropsy. Three hours after the second administration, animals were euthanized by CO_2 inhalation and were subjected to resection of the glandular stomach and duodenum. Macroscopic examinations of the glandular stomach and duodenum were unremarkable in all treated animals. No adverse effects were observed, and body weight gain was normal among the animals. No statistically significant increases in the % tail DNA or hedgehog frequency in stomach or duodenum cells were observed in animals gavaged with the ultra-filtered arabinase concentrate, compared to the negative control group. Positive controls showed increases in the % tail DNA of stomach and duodenum cells. Based on the results of this study, it was concluded that ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 lacks genotoxic potential.

6.3.3 Additional Safety Considerations

6.3.3.1 Allergenicity of Arabinase Protein from A. tubingensis *GPA41*

As discussed by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances".* To confirm that the arabinase as derived from *A. tubingensis* GPA41 does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted using the AllergenOnline database Version 18B (available at http://www.allergenonline.org; updated 23 March 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2018). The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. A full-length alignment search of AllergenOnline was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). No matches were identified from searching with the full amino acid sequence of arabinase.

A second homology search was conducted according to the approach outlined by the FAO/WHO (2001) and the Codex Alimentarius Commission (Codex Alimentarius, 2009). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length arabinase amino acid sequence from *A. tubingensis* GPA41. The 80-amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility (FAO/WHO, 2001). Using this search strategy, no matches with greater than 35% identity in the sliding window of 80 amino acids were identified. A third homology search conducted using the exact 8-mer approach also did not produce any matches.

Based on the results of the sequence homology searches, arabinase from *A. tubingensis* GPA41 is not expected to produce an allergenic response following consumption of foods with potential enzyme residues from the use of the enzyme preparation. Additionally, there is no evidence from the available scientific literature indicating allergenicity to arabinase in consumers of foods to which the enzyme has been added. Furthermore, the enzyme would be inactivated and denatured under some of the proposed conditions of food processing. Therefore, the use of the arabinase enzyme preparation is not anticipated to pose any allergenicity concerns in consumers.

6.3.3.2 Toxigenic Potential of Arabinase

To confirm that the arabinase enzyme protein does not harbor any toxic potential, the Basic Local Alignment Search Tool (BLAST) program maintained by the NCBI was used to conduct a sequence alignment query of the arabinase amino acid sequence against downloaded protein sequences obtained from a curated database of venom proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot[6]) using FASTA, a sequence alignment tool. BLAST searches also were conducted against a UniProt-maintained curated database of virulence proteins and toxins (UniProtKB/Swiss-Prot/TrEMBL[7]). A sequence alignment of ≥35% identity was used as a threshold for identification as a positive alignment (Codex Alimentarius, 2003; Goodman *et al.,* 2008; Goodman and Tetteh, 2011). All sequence matches with known toxins of ≥35% had low query coverages (3 to 5%) paired with high E-values (4.0 to 9.8). No sequence matches with ≥35% identity to known virulence factors were identified. Therefore, the arabinase enzyme protein was not considered to share homology or structural similarity with any known animal venom proteins and toxins or virulence factors (Pearson, 2000; Bushey *et al.,* 2014).

6.4 GRAS Panel Evaluation

Shin Nihon has concluded that arabinase enzyme preparation derived from a non-genetically modified production strain of *Aspergillus tubingensis* (designated as strain GPA41), as described herein, is GRAS for use as a processing aid in the processing of fruits and vegetables and in the production of fruit juice and wine, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of the production strain (*A. tubingensis* GPA41), the arabinase enzyme preparation, and the enzyme arabinase and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Dr. David Brusick, Ph.D., A.T.S.

[6] The UniProtKB/Swiss-Prot Tox-Prot database is available at: http://www.uniprot.org/uniprot/?query=taxonomy%3A%22Metazoa+[33208]%22+AND+%28keyword%3Atoxin++OR+annotation%3A%28type%3A%22tissue+specificity%22+AND+venom%29%29+AND+reviewed%3Ayes&sort=score.

[7] The UniProtKB/Swiss-Prot/TrEMBL database is available at: http://www.uniprot.org/uniprot/?query=keyword:KW-0843.

(Toxicology Consultant), and Dr. Michael W. Pariza, Ph.D. (University of Wisconsin-Madison). For the purposes of the GRAS Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined under 21 CFR § 170.3(i) (U.S. FDA, 2019a).

The GRAS Panel, convened by Shin Nihon, independently and critically evaluated all data and information presented herein, and also concluded arabinase enzyme preparation derived *A. tubingensis* GPA41 is GRAS for use as a processing aid in the processing of fruits and vegetables and in the production of fruit juices and wines, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses is presented in Appendix A.

6.5 Conclusion

Based on the above data and information presented herein, Shin Nihon has concluded that the intended food uses of arabinase enzyme preparation derived from a non-genetically modified production strain of *Aspergillus tubingensis* (designated as strain GPA41), as described in Section 1.3, is GRAS based on scientific procedures. General recognition of Shin Nihon's GRAS conclusion is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of the arabinase preparation from *A. tubingensis* GPA41 in food, who similarly concluded that the proposed uses of the enzyme preparation as a processing aid as described herein are GRAS.

The arabinase enzyme preparation from *A. tubingensis* GPA41 therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

Part 7. §170.255 List of Supporting Data and Information

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	§	Section Title
170 Secondary direct food additives permitted in food for — human consumption	170.3	Definitions
	170.30	Secondary direct food additives permitted in food for human consumption. Section §170.30—Eligibility for classification as generally recognized as safe (GRAS)
173—Secondary direct food additives permitted in food for human consumption	173.340	Defoaming agents
177—Indirect food additives: polymers	177.2440	Polyethersulfone resins
	177.2510	Polyvinylidene fluoride resins
182—Substances generally recognized as safe	182.1057	Hydrochloric acid
	182.1073	Phosphoric acid
	182.1320	Glycerin
184—Direct food substances affirmed as generally recognized as safe	184.1763	Sodium hydroxide

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APPENDIX A
Consensus Statement

GRAS Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of an Arabinase Enzyme Preparation from *Aspergillus tubingensis* GPA41 for Use as a Processing Aid in Food Production

22 January 2020

INTRODUCTION

Shin Nihon Chemical Co., Ltd. (Shin Nihon) intends to market an arabinase enzyme preparation derived from non-genetically modified *Aspergillus tubingensis* GPA41 (hereinafter referred to Sumizyme AG) for use as a processing aid in fruit and vegetable applications in the United States (U.S.). The enzyme preparation also is intended for use during juice and wine production. An Expert Panel of independent scientists (the GRAS Panel), qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened by Shin Nihon to conduct a critical and comprehensive evaluation of the available pertinent data and information concerning the arabinase enzyme preparation (Sumizyme AG). The GRAS Panel was asked to determine whether the intended uses of the enzyme preparation (Sumizyme AG) would be Generally Recognized as Safe (GRAS), based on scientific procedures. For purposes of the GRAS Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2018).

The GRAS Panel consisted of the below-signed qualified scientific experts: Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine); David Brusick, Ph.D., A.T.S. (Toxicology Consultant); and Michael W. Pariza, Ph.D. (University of Wisconsin-Madison). The GRAS Panel was selected and convened in accordance with the Food and Drug Administration (FDA)'s guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S., FDA 2017). Shin Nihon ensured that all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in food safety, toxicology, and microbiology. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or "appearance issues" were identified. The GRAS Panel received an honorarium as compensation for their time; the honorarium provided to the GRAS Panel was not contingent upon the outcome of their deliberations. The GRAS Panel, independently and collectively, critically evaluated a supporting dossier submitted by Shin Nihon ["*Documentation to Support the Use of an Arabinase Enzyme Preparation from Aspergillus tubingensis GPA41 as a Generally Recognized as Safe (GRAS) Processing Aid in Food Production*", dated 06 October 2019]. This dossier contains a comprehensive package of data and information, including the method of manufacture, product specifications and analytical data, stability, intended conditions of use, estimated intake of the arabinase enzyme preparation (Sumizyme AG) based on all intended uses, and a summary of the available scientific information and data pertinent to the safety of the arabinase enzyme preparation, including safety of the production organism. Information was provided by Shin Nihon. Also, a search of the published literature through October of 2017 was conducted to identify any additional information relevant to the safety of the arabinase enzyme preparation. Following their independent and collaborative critical evaluation of the data and information, the GRAS Panel convened *via* teleconference on 17 October 2019. The GRAS Panel reviewed their findings and following discussion

unanimously concluded that the intended uses described herein of the arabinase enzyme preparation (Sumizyme AG), meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the basis for the GRAS Panel's conclusion is provided in the following section.

SUMMARY AND BASIS FOR GRAS

The enzyme preparation is primarily characterized by the activity of the enzyme arabinase (EC 3.2.1.99) but also possesses endo-β-galactanase activity. Arabinase catalyzes the endohydrolysis of (1→5)-α-arabinofuranosidic linkages in polysaccharides of arabinose. Arabinose polysaccharides, commonly referred to as arabinans, are structural constituents of plant cell walls. Arabinase functions as a plant cell wall-degrading enzyme and can aid in the 'softening' of fruit or vegetable pieces for use in other finished food applications such as fruit fillings or vegetable purees. Arabinase also is recognized for use in the clarification of juices.

Arabinase is secreted by *A. tubingensis* GPA41 during fermentation. The production strain, *A. tubingensis* GPA41, is non-genetically modified and was isolated from fruit. The production strain was taxonomically identified as belonging to the filamentous fungi species *A. tubingensis*, by Westerdijk Fungal Biodiversity Institute[1] at the Royal Netherlands Academy of Arts and Sciences and has been deposited at the National Institute of Technology and Evaluation's Biological Resource Center. Morphologically, *A. tubingensis* is indistinguishable from *Aspergillus niger* and distinction can only be achieved by advanced molecular methods. *A. tubingensis* GPA41 was selected as the production organism based on its capacity to produce high levels of arabinase.

Shin Nihon's arabinase enzyme preparation is manufactured in compliance with cGMP and a Hazard Analysis and Critical Control Points system is implemented. All raw materials and processing aids are food-grade and are permitted for use in the U.S. for such purposes. The production strain is stored and maintained by Shin Nihon using a well-defined two-tiered cell banking system which consists of a master cell bank and a working cell bank. The manufacture of the enzyme preparation begins with cultivation of a seed culture, which is then used to inoculate a main culture that is carried out under solid-state fermentation. During fermentation, arabinase is secreted from *A. tubingensis* GPA41 into the culture medium and a series of filtration and purification steps are subsequently applied to obtain an ultra-filtered concentrate of arabinase. The ultra-filtered concentrate is not directly sold to manufacturers, but depending on the needs of Shin Nihon's customers, is formulated with a suitable carrier (*e.g.,* standardized with glycerol) to a final arabinase activity in the range of approximately 210 to 250 U/g, and not less than 200 U/g. A number of quality control steps are implemented during the manufacture of the enzyme preparation to ensure a high quality and consistent product. The fermentation conditions are strictly maintained to ensure they are optimal for mycelium growth and enzyme production. Additionally, analyses are conducted to ensure that residual amounts of the *A. tubingensis* production strain are not transferred to the enzyme preparation, as indicated by the absence of mold.

[1] Previously known as the Centraalbureau Voor Schimmelcultures Fungal Biodiversity Centre (CBS).

Shin Nihon has demonstrated that the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 is composed mainly of water (approximately 85%), with the protein and ash content reported at approximately 12 and 0.5%, respectively. No diluents, stabilizers, or preservatives are added to the ultra-filtered concentrate. The specifications for the ultra-filtered concentrate comply with the food-grade specifications for food enzyme preparations established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and those stated in the Food Chemicals Codex. The ultra-filtered concentrate is characterized by a total organic solids (TOS) content in the range of 5 to 20% and is specified to contain an arabinase activity of not less than 450 U/g. The GRAS Panel reviewed analytical data of 3 non-consecutive production batches of the ultra-filtered arabinase concentrate (Lot Nos. 130128T, 140902T, and 180306T), all of which demonstrated that the manufacturing process consistently results in a product that complies with the established specifications. In addition to TOS content and arabinase activity, which ranged from 11.5 to 17.4% and 550 to 887 U/g, respectively, analysis for heavy metals, microbiological contaminants, and antibiotic activity also showed compliance with final product specifications.

The stability of the formulated enzyme preparation (*i.e.,* standardized with glycerol) was examined following storage of samples under ambient temperatures (20 to 25°C) in air-tight containers for up to 360 days. No loss of enzyme activity was reported, and Shin Nihon established a minimum shelf-life of 12 months for the arabinase enzyme preparation. Shin Nihon also conducted additional studies to assess the pH- and thermo-stability of arabinase. Arabinase activity was shown to be relatively stable at temperatures of up to 50°C and pH values ranging from 3 to 6.5. Rapid declines in arabinase activity were reported at pH 7 and as temperatures increased above 50°C, with no activity detected following incubation at a temperature of 70°C. Temperatures of 50 to 60°C and pH from 3 to 4.5 were determined as optimal activity conditions. The arabinase enzyme preparation may be used to aid in the 'softening' of fruit or vegetable pieces or with the release (extraction) of juice from fruits and vegetables. Specifically, the enzyme preparation is intended for use in the processing of fruits and vegetables, for subsequent use in applications such as fruit fillings or vegetable purees and pastes, or during fruit or vegetable juice squeezing. The enzyme preparation may also be used during end-stage juice or wine production to help with filtration. The arabinase enzyme preparation may be added at various stages of fruit or vegetable processing and juice or wine making (*e.g.,* during juice squeezing, membrane filtration). Technological effects of the enzyme on the final foods are limited by denaturation of the enzyme during the final processing steps that may involve high temperatures (*e.g.,* pasteurization or sterilization) as applied to the finished food ingredients and/or food products. Enzyme residues also may be reduced in the final beverage products as a result of end-stage filtration[2]. The maximum level at which the enzyme preparation is proposed for use for fruit and vegetable processing is 500 ppm or 24 mg TOS/kg food substrate (based on an average TOS content of 4.7%). These processed fruits and vegetables are in turn used in a wide range of foods including (but not limited to) fruit-based desserts, fruit fillings for pastries, fruit and vegetable purees and pastes, fruit and vegetable juices, fruit drinks and ades, fruit smoothies and fruit-based nectars, and wines and wine beverages. Based on the representative use levels for processed fruits and vegetables, the ingredients are added to final foods as consumed at maximum use levels up to 100%, equivalent to up to approximately 24 mg TOS/kg food. For use during production of fruit juices or wines as a filtration aid, the enzyme preparation also is proposed for addition at a level of 500 ppm or 24 mg TOS/kg food. Therefore, the cumulative maximum level of the enzyme preparation that could potentially be present in the consumed product is 48 mg TOS/kg food.

[2] In the case of juice production, while the arabinase enzyme preparation is added to aid with filtration (to break down buildup formed on the ultra-filtration membrane), the membrane also prevents the enzyme from filtering into the final juice product. In the case of wine production, transfer of the enzyme may be reduced by suspension and/or precipitation of the enzyme by binding with wine tannins and polyphenols and/or gravity filtration.

The potential human exposure to the enzyme preparation was estimated using the Budget Method, which is a widely-accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2009). For the purposes of the exposure assessment, it was assumed that 100% of the enzyme added during processing will remain in the final foods as consumed (*i.e.*, assuming no removal and/or inactivation during processing). The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, instead of using food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods (*i.e.*, 0.05 kg/kg body weight/day) and non-milk beverages (*i.e.,* 0.1 kg/kg body weight/day); (ii) the level of presence of the substance in solid foods (*i.e*., 24 mg TOS/kg food) and non-milk beverages (*i.e.*, 48 mg TOS/kg food); and (iii) the proportion of solid foods (*i.e.*, 12.5%) and non-milk beverages (*i.e.,* 25%) that may contain the substance (FAO/WHO, 2009). Based on these assumptions, the TMDI for the arabinase enzyme preparation, based on its uses in the processing of fruits and vegetables that are subsequently added to foods for the general population, and in the production of fruit juices and wines as a filtration aid, was calculated to be 1.35 mg TOS/kg body weight/day (0.15 and 1.2 mg TOS/kg body weight/day from consumption of solid foods and non-milk beverages, respectively).

The GRAS Panel recognized that the estimated intake values obtained are gross overestimations of the exposure to the enzyme preparation based upon the use of the conservative Budget Method (EFSA, 2009, 2014; FAO/WHO, 2009). In addition, it was assumed that the enzyme preparation will be used at the maximum use levels, the arabinase-processed ingredients (fruits and vegetables) are added to the consumed food or beverage products at levels of up to 100%, and all of the enzyme used during food processing will be present in the final foods as consumed (*i.e.*, no removal and/or inactivation). As mentioned, in reality the enzyme will be inactivated and/or denatured by high temperatures applied during the final processing steps of the arabinase-processed ingredients (fruits and vegetables) and/or the foods containing the enzyme-processed food ingredient. For example, fruit and vegetable juices and other fruit and vegetable preparations such as pastes, and purée are typically thermally treated (pasteurized) to minimize pathogenic microbial contamination. Furthermore, in the case of juices and wines, levels of enzyme residues will be reduced as a result of end-production filtration steps.

The GRAS Panel critically evaluated the data and information characterizing the safety of the enzyme preparation (Sumizyme AG). The safety of the enzyme preparation for use in foods was assessed according to the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC, 1990) which are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods. The decision tree developed by Pariza and Johnson (2001) for evaluating the safety of microbially derived food enzymes was applied to arabinase from *A. tubingensis* GPA41 to determine its acceptability for use in food under the conditions of use proposed herein (refer to Attachment A).

With respect to evaluating enzyme safety, the primary focus is the safety of the production microorganism. *A. tubingensis* is recognized as a production organism for food and feed enzymes (U.S. FDA, 2015a,b; EFSA, 2018). Furthermore, given the difficulties in differentiating between *A. niger* and *A. tubingensis*, it may also be anticipated that some enzymes previously considered to be products of *A. niger*, may in fact have been products of *A. tubingensis*. Despite the common use of aspergilli in the food industry, it has been noted that certain filamentous fungi have the capacity to produce secondary metabolites, including well-established mycotoxins and other less known metabolites of poorly described biological activity and possible safety concern (*e.g*., nigragillin, naphtho-γ-pyrones, and malformins) (Pariza and Johnson, 2001; Samson *et al*., 2004, 2007; Fog Nielson, 2009; Lamboni *et al*., 2016; EFSA, 2017). However, analysis demonstrated that the production organism, *A. tubingensis* GPA41, does not produce any such mycotoxins,

including ochratoxin A, which has been shown to be produced by some *A. tubingensis* strains. The source organism also was tested for production of nigragillin, naphtho-γ-pyrones, and malformins and found to be non-detectable. In addition, representative batches of the formulated enzyme preparation (Sumizyme AG) have been analyzed for mycotoxins, including aflatoxins B_1, B_2, G_1, G_2, sterigmatocystin, zearalenone, ochratoxin A, T-2 toxin, and fumonisins B_1 and B_2, which were confirmed to be absent. Absence of antibiotic activity also was confirmed in the glycerol-formulated enzyme preparation. Several cases of *A. tubingensis* infections in humans have been reported; however, *A. tubingensis* is generally considered an opportunistic agent which affects individuals with other underlying health conditions and use of *A. tubingensis* GPA41 as the source organism in the production of arabinase is not anticipated to pose an increased risk of pathogenicity for the general population. *A. tubingensis* GPA41, the production strain used in the manufacture of the arabinase enzyme preparation (Sumizyme AG), is considered a safe source organism. The production strain was concluded to be derived from an organism that has an established history of safe use in the production of enzymes intended for food use, can be regarded as non-pathogenic to the general population, and is not a producer of mycotoxins or antibiotic substances. The GRAS Panel also recognized that a series of purification steps are employed during the manufacture of the enzyme preparation to ensure that the production strain is not transferred into the final enzyme preparation product.

Additional support for the safety of the enzyme preparation (Sumizyme AG) was derived from the results of a battery of toxicological studies, which consisted of a repeated-dose 90-day oral toxicity study conducted in rats and a series of mutagenicity/genotoxicity assays, including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, an *in vivo* mammalian erythrocyte micronucleus test, and an *in vivo* comet assay. Testing was conducted with a single representative batch of ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 (Lot No. 120127T), meeting the product specifications and characterized by an activity of 690 U/mL and a TOS content of 15.3% (corresponding to 4.5 U/mg TOS). The sub-chronic oral toxicity study in rats was conducted in accordance with Good Laboratory Practice (GLP) and Organisation for Economic Co-operation and Development (OECD) Guideline No. 408 (OECD, 1998a,b). Male and female Crl:CD (SD) [SPF] rats (10/sex/group) were orally administered the enzyme preparation (Sumizyme AG) by gavage at doses of 0 (distilled water), 69, 690, or 6,900 U/kg body weight/day (corresponding to approximately 0, 15, 153, and 1,530 mg TOS/kg body weight/day, respectively, based on 4.5 U/mg TOS) for 90 or 91 days (Sugi, 2014 [unpublished]; Okado *et al*., 2019). The doses were selected based on the results of a preliminary 2-week dose-range finding study, in which no adverse effects were reported following the administration of the test material at doses of up to 6,900 U/mL. General condition of the animals was monitored on a daily basis. Body weight and food consumption were measured weekly. Urinalysis, ophthalmological examination, hematology, clinical chemistry, organ weight measurements, macroscopic examination, and microscopic examination were conducted at the end of treatment.

No deaths were reported throughout the study period. Body weights, body weight gain, and food consumption of test animals were comparable to controls throughout the study period. Although some statistically significant hematological variations were reported, none were dose-dependent. In high-dose males, potassium levels were significantly increased compared to controls. The difference was considered not related to the administration of the enzyme concentrate given absence of any accompanying changes in urinary potassium or other electrolytes. Individual animal values were within the laboratory's historical control range. All other statistically significant clinical chemistry variations also occurred in the absence of a dose-response. Urinalysis and ophthalmological examinations were unremarkable. A significant decrease in relative thymus and spleen weights of high-dose males and increase in absolute kidney weights in high-dose females was reported; however, this may have been the result of slightly greater (albeit not statistically significant) body weights in males and females of the high-dose group. Gross abnormalities were reported in the spleen (cyst), lungs (brown patch), liver (a brown and red patch), and renal pelvis (dilation) of high-dose males and in the stomach (nodule), ileum (diverticulum) of high-dose females), but were deemed to be

isolated findings. Histological variabilities of the glandular stomach (edema) and pancreas (regeneration of acinar cells) in the high-dose animals consisted of slight focal changes, with the severity and morphological characteristics consistent with those typically seen for this strain and age of rats and were therefore determined to be spontaneous findings. Macroscopic examination revealed a nodule in the Zymbal's gland (auditory sebaceous gland) of a high-dose female, which was histopathologically confirmed as an adenoma. Although Zymbal's gland tumors are generally not considered common spontaneous lesions in rats, incidences in control animals of toxicological studies are documented (Dinse *et al*., 2010; Rudmann *et al*., 2012; Weber, 2017). Zymbal's gland adenoma also had been previously identified as an isolated finding in other repeat-dose oral rat studies conducted at this laboratory. Therefore, the single occurrence of adenoma of the Zymbal's gland in a female rat in this study was considered a spontaneous finding. It was concluded that no adverse effects related to the oral administration of the ultra-filtered concentrate of arabinase from *A. tubingensis* GPA41 were reported in the study at doses up to 1,530 mg TOS/kg body weight/day (or 6,900 U/kg body weight/day), the highest dose tested. The no-observed-adverse-effect level (NOAEL) was 1,503 mg/kg body weight/day.

A bacterial reverse mutation assay was conducted in accordance with OECD Guideline No. 471 (OECD, 1997) and the Principles of GLP (OECD, 1998a) to assess the potential mutagenicity of the enzyme preparation using *Salmonella typhimurium* strains TA100, TA98, TA1535, and TA1537, as well as *Escherichia coli* strain WP2*uvrA* in the presence and absence of metabolic activation (Kasamoto, 2014a [unpublished]; Okado *et al*., 2019). Increases in revertant colonies of twice or more compared to the negative control were reported in several tester strains during the preliminary study, concentration-finding study, and the main test which were performed using the pre-incubation method. In the main test, an increase in growth inhibition was reported. These findings were determined to be the result of the co-presence of free amino acids released into the culture medium (higher presence of free amino acids may be responsible for the overall increase in the bacterial growth and, thus greater potential for spontaneous mutations to occur). Therefore, an additional main study and confirmatory study were conducted using the 'treat-and-wash' method, which is a recognized and acceptable modification of the pre-incubation method for testing of proteinous substances involving a washing step to remove free amino acids released into the culture medium (Thompson *et al*., 2005; EFSA, 2014). Using the treat-and-wash method, no positive responses were reported in either the main or confirmatory treat-and-wash studies with the arabinase concentrate. Similar results, which were also attributed to the co-presence of free amino acids, were reported when the Ames assay was performed with a different enzyme preparation (nuclease P1 from *Penicillium citrium*, also a Shin Nihon product) (Okado *et al*., 2016). As such, the ultra-filtered arabinase concentrate was concluded to be not mutagenic and the increase in the number of revertant colonies under conditions of the standard method was deemed a result of free amino acids in the culture medium.

The clastogenic potential of the enzyme preparation (Sumizyme AG) was investigated in an *in vitro* chromosomal aberration test conducted in cultured Chinese hamster lung (CHL) fibroblasts (Kasamoto, 2014b [unpublished]; Okado *et al*., 2019) in accordance with OECD Guideline No. 473 (OECD, 2014a) and the Principles of GLP (OECD, 1998a). The CHL fibroblasts were incubated with the ultra-filtered arabinase concentrate for either 6 hours (short-term treatment, followed by an 18-hour expression period) in the absence and presence of metabolic activation or for 24 hours (continuous treatment) in the absence of metabolic activation at concentrations of up to 15.3 mg TOS/mL. Under the conditions of the short-term assay, the arabinase concentrate induced structural chromosome aberrations in CHL cells; testing with S9 was noted to somewhat attenuate the positive response. Although testing under continuous treatment conditions produced negative results, cells with analyzable mitosis were only available at concentrations of up to 5.1 mg TOS/mL; at higher concentrations, cells displayed c-mitosis (disturbance of spindle function) and thus could not be analyzed. Based on the results of the *in vitro* study, potential clastogenicity could not be excluded and additional *in vivo* testing was conducted. The ultra-

filtered arabinase concentrate was tested *in vivo* in the mammalian erythrocyte micronucleus test in male Crl:CD (SD) [SPF] rats in accordance with OECD Guideline No. 474 (OECD, 2014b) as well as the principles of GLP (Kasamoto, 2014c [unpublished]; Okado *et al*., 2019). Rats (6/group) were administered the concentrate by gavage at doses of 0, 384, 767, or 1,530 mg TOS/kg body weight/day for 2 consecutive days. Cyclophosphamide served as a positive control. The concentrate did not induce micronucleated erythrocytes in rat bone marrow cells and was therefore considered to be non-clastogenic *in vivo*. In order to address potential site-of-contact genotoxicity, a further *in vivo* comet assay was conducted using stomach and duodenum cells. Groups of 6 male Crl:CD (SD) [SPF] rats were administered the arabinase concentrate by gavage at doses of 0, 383, 765, or 1,530 mg TOS/kg body weight/day for 2 consecutive days at 21-hour intervals (Tanaka, 2017 [unpublished]; Okado *et al*., 2019 [study conducted in accordance with OECD Guideline for the Testing of Chemicals No. 489 (OECD, 2016) and the Principles of GLP (OECD, 1998a)]). Distilled water served as the test article solvent and negative control, while ethyl methanesulfonate served as the positive control. No adverse effects were reported, and body weight gain was normal among the animals. No statistically significant increases in the % tail DNA or hedgehog frequency in stomach or duodenum cells were reported in the treated animals compared to the negative control group. Results obtained in the comet assay further confirmed absence of any genotoxic potential of the concentrate in pre-absorptive cells which would be expected to come into direct contact with the test article immediately following ingestion. Based on the results of the *in vivo* micronucleus and comet assays, arabinase concentrate derived from *A. tubingensis* GPA41 was concluded as lacking genotoxic potential.

As reported by Pariza and Foster (1983): "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances*". To confirm that arabinase as derived from *A. tubingensis* GPA41 does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted using the AllergenOnline database (Version 18B – available at http://www.allergenonline.org; updated 23 March 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2018). No matches were identified from searching with the full amino acid sequence of arabinase. Additionally, no matches were identified when the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length arabinase amino acid sequence from *A. tubingensis* GPA41, as per the approach outlined by the FAO/WHO (2001) and the Codex Alimentarius (2009). The 80-amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20), and significant homology is defined as an identity match of greater than 35%. No matches were identified using this search strategy. Another search using the exact 8-mer approach, also did not produce any matches. Therefore, arabinase from *A. tubingensis* GPA41 is not expected to pose any allergenicity concerns. This is further supported by the fact that no evidence of allergenicity to arabinase was identified in the literature. The amino acid sequence of arabinase from *A. tubingensis* GPA41 also was screened against known toxins in an electronic databases maintained by UniProt (using the Basic Local Alignment Search Tool [BLAST] program). No sequence matches with ≥35% identity to known virulence factors were identified. The absence of toxigenic potential of *A. tubingensis* GPA41-derived arabinase was further confirmed based on the results of the sequence homology searches.

Based on the weight of evidence, including history of safe use of *A. tubingensis* as a source organism for enzyme production, demonstrated lack of pathogenic and toxicogenic potential for the production strain, and results of a series of toxicological studies conducted with the ultra-filtered enzyme concentrate, including a 90-day study in which absence of any adverse effects at the highest dose tested was confirmed, Shin Nihon's arabinase from *A. tubingensis* GPA41 can be concluded to pose no safety concerns under the intended conditions of use.

The totality of scientific evidence reviewed demonstrates that the intended uses of Shin Nihon's arabinase from *A. tubingensis* GPA41 (Sumizyme AG), manufactured consistent with cGMP and meeting appropriate food-grade specifications, are safe and suitable. The data and information summarized in this report demonstrate that the intended uses of the enzyme preparation (Sumizyme AG) as a processing aid in food production are GRAS, based on scientific procedures.

CONCLUSION

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to the safety of the intended conditions of use for Shin Nihon's arabinase enzyme preparation produced by fermentation from *Aspergillus tubingensis* GPA41 (Sumizyme AG) and meeting appropriate established specifications, and concluded that it is safe and suitable for consumption at the maximum levels specified for use in select foods.

We further unanimously conclude that the proposed uses of Sumizyme AG, meeting appropriate food-grade specifications presented in the supporting dossier and produced consistent with current Good Manufacturing Practice (cGMP), are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

[redacted]

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ATTACHMENT A
Determining the Safety of Shin Nihon's Arabinase from *Aspergillus tubingensis* GPA41 for Use in Food Based on the Decision Tree Developed by Pariza and Johnson (2001)

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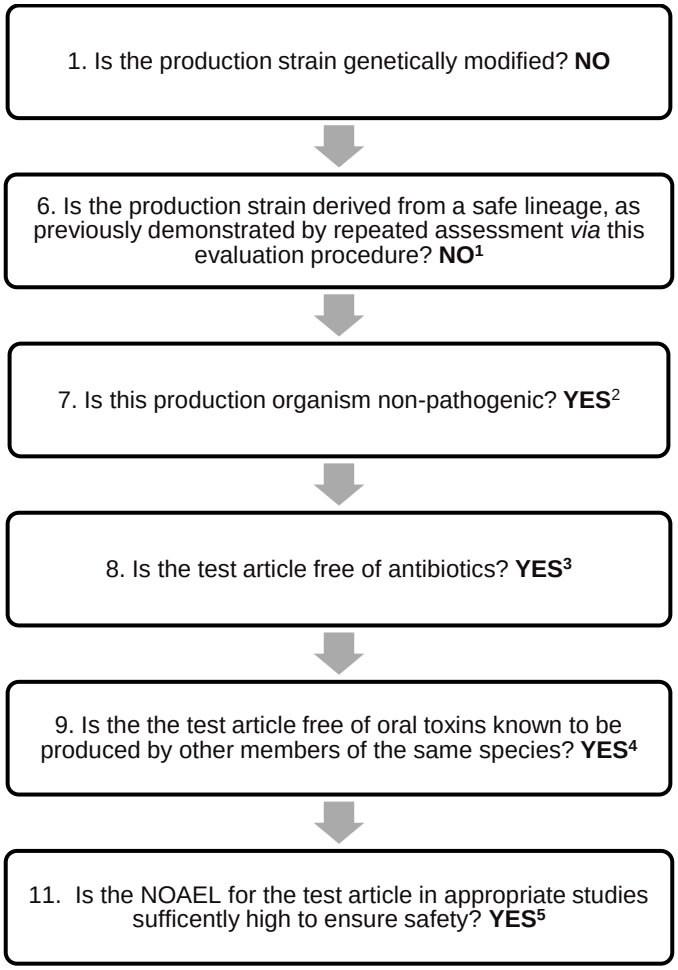
Figure A-1 Determining the Safety of Shin Nihon's Arabinase from *Aspergillus tubingensis* GPA41 for Use in Food



NOAEL = no-observed-adverse-effect level.
Modified from Pariza and Johnson (2001).

[1] The production strain for the arabinase enzyme preparation is *A. tubingensis* GPA41. *A. tubingensis* is a filamentous fungus belonging to *Aspergillus* section *Nigri* (the black aspergilli). Although *A. tubingensis* is recognized as an existing source organism for the production of enzymes and it may be expected that some enzymes previously considered to be produced by *A. niger* are possibly products of *A. tubingensis*, *Aspergillus* species have not been granted Qualified Presumption of Safety (QPS) status (by the European Food Safety Authority) in consideration of the production of secondary metabolites with unknown toxicities/biological profiles.

[2] Yes: Regarded as an opportunistic pathogen; pathogenic potential limited to individuals with underlying health conditions.

[3] Yes: Based on the absence of antibiotic activity in the arabinase enzyme preparation as produced from *A. tubingensis* GPA41.

[4] Yes: Based on extrolite analysis provided for *Aspergillus niger* GPA41; analysis indicates levels below 'minimum detection limit', including for aflatoxins, ochratoxin A, fumonisin B1 and B2, and malformins.

[5] Yes: A NOAEL of 1,530 mg TOS/kg body weight/day was determined for the enzyme on the basis of a 90-day study in rats (Sugi, 2014a [unpublished] – Experiment No. F076 [365-188]; Okado *et al.*, 2019 [unpublished manuscript]). A large margin of safety exists between the NOAEL and the estimated level of exposure to Sumizyme AG from foods (1.35 mg TOS/kg body weight/day).