RECEIVED
MAY 15 2023
OFFICE OF FOOD ADDITIVE SAFETY

11 May 2023

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 Notice for *beta*-Lactoglobulin Produced From *Aspergillus oryzae*

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Imagindairy Ltd. [Nahum Het St 7, Tirat Carmel 3508504, Israel], as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that *beta*-lactoglobulin produced from *Aspergillus oryzae*, is GRAS on the basis of scientific procedures, for use in various conventional food and beverage products across multiple categories; these food uses of *beta*-lactoglobulin are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Imagindairy's GRAS conclusion is enclosed for review by the agency.

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,

Tomer Gold
VP Research & Development
Imagindairy Ltd.
Email: RA@imagindairy.com
Cell: +972-54-3314033

GRAS NOTICE FOR *BETA*-LACTOGLOBULIN PRODUCED FROM *ASPERGILLUS ORYZAE*

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:
Imagindairy Ltd.
Nahum Het St 7, Tirat Carmel
3508504, Israel

DATE:
11 May 2023

GRAS Notice for *beta*-Lactoglobulin Produced from *Aspergillus oryzae*

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

GRAS Notice for *beta*-Lactoglobulin Produced from *Aspergillus oryzae*

PART 1 §170.225 SIGNED STATEMENTS AND CERTIFICATION

In accordance with Title 21 of the *Code of Federal Regulations* (CFR) §170 Subpart E consisting of §170.203 through 170.285, Imagindairy Ltd. ("Imagindairy") hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of *beta*-lactoglobulin, as manufactured by Imagindairy, in various conventional food and beverage products as described in Section 1.3 below, are not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Imagindairy's view that these notified uses of β-lactoglobulin are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Imagindairy, the undersigned hereby certifies that all data and information presented in this GRAS Notice represent a complete and balanced submission that is representative of the generally available literature. Imagindairy considered all unfavorable as well as favorable information that is publicly available and/or known to Imagindairy and that is pertinent to the evaluation of the safety and GRAS status of β-lactoglobulin as a food ingredient for addition to various conventional food and beverage products, as described herein.

Signed,

May 11, 2023

Tomer Gold
VP Research & Development
Imagindairy Ltd.
Email: RA@imagindairy.com
Cell: +972-54-3314033

Date

1.1 Name and Address of Notifier

Imagindairy Ltd.
Nahum Het St 7, Tirat Carmel
3508504, Israel

1.2 Common Name of Notified Substance

β-Lactoglobulin, BLG

Non-Animal Whey Protein

1.3 Conditions of Use

Imagindairy's β-lactoglobulin produced *via* fermentation of *A. oryzae* is intended to be used as a non-animal protein source in foods and beverages that currently use protein from milk or plants. Food categories and use levels for which Imagindairy's β-lactoglobulin is intended for use are provided in Table 1.3-1 below.

The ingredient is not intended for use in infant formula, and the proposed food categories do not include food uses that are subject to the oversight by the U.S. Department of Agriculture (USDA) and its Food Safety Inspection Service (*i.e.*, meat and poultry or meat and poultry containing foods).

Table 1.3-1 Food Uses and Use Levels of β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Food Category	Food Use	Maximum β-Lactoglobulin Use Levels
Nutritional Products	Meal replacements and supplements	15%
	Powdered nutritional beverages	25%
	Nutritional bars	35%
	Electrolyte-type sports drinks	6%
	Performance nutritional beverages, high protein	25%
Dairy and Dairy-based Products	Fluid milk, powdered milk, flavored milk, milk-based drinks and drink mixes (*e.g.*, dairy smoothies, hot chocolate from mix), milk substitutes	6%
	Cream, half & half, cream cheese, cheese spread, whipped cream	15%
	Yogurt and fermented milk products	8%
	Spreads, dips	10%
	Cream substitutes	15%
	Frozen dairy desserts and mixes	10%
	Cheese used primarily as ingredients (*e.g.* ricotta cheese)	15%
	Semi-hard cheese (*e.g.*, feta, Camembert, brie)	25%
Sugar-based Products	Desserts and Mousses	5%
	Confections (including chocolate confections)	10%
	Coatings and fillings	10%
	Cookies and brownies, crackers, popcorn, potato chips, tortilla chips, hard pretzels/snack mix	5%
	Doughnuts, toaster pastries, muffins	10%
Dressings	Salad dressings	5%
	Minor main entrée sauces (*e.g.*, Alfredo sauce, white sauce, cheese sauce)	6%
Baked Goods	French toast, crepes, pancakes, bagels, scones, biscuits, croissants	10%
	Breads & rolls, English muffins, pizza crust	10%
Egg Products	Egg substitutes	10%

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) (U.S. FDA, 2022a), Imagindairy has concluded that the intended uses of β-Lactoglobulin from *A. oryzae* 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 Notice 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:

Imagindairy Ltd.
Nahum Het St 7, Tirat Carmel
3508504, Israel

Should the U.S. FDA have any questions or additional information requests regarding this GRAS Notice, Imagindairy will supply these data and information upon request.

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

It is Imagindairy's view that all data and information presented in Parts 2 through 7 of this GRAS Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential; therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, Title 5 of the *United States Code* 552 (5 U.S.C. 552).

PART 2 §170.230 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

β-Lactoglobulin is the major whey protein component in bovine milk and is a common ingredient in many foods (Barbiroli *et al.*, 2022). It is usually present in milk at concentrations of 2 to 3 g/L, making up roughly 9% of the total protein content of bovine milk (Kontopidis *et al.*, 2004). Imagindairy's β-lactoglobulin is a highly purified protein (≥85% of total protein) produced by fermentation of the fungal strain *Aspergillus oryzae*, a common production organism for the industrial production of food enzymes. The final product is a white to off-white to yellowish powder.

Table 2.1-1 Chemical Identity of β-Lactoglobulin

Common Name	β-lactoglobulin
Common Abbreviation	BLG, β-Lg, b-Lg, B-LG
Trade Name	N/A
Molecular Formula	N/A
Molecular Weight	36.6 kDa (dimer); 18.3 kDa (monomer)
CAS Registry Number	9045-23-2
CAS Name	β-Lactoglobulin
IUPAC Name	N/A
IUPAC Abbreviations	N/A
Other Abbreviations	N/A

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry; N/A = not applicable.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

2.2.1.1 Host Strain

A. oryzae strain RIB40, a filamentous fungus, is used as a base strain in the construction of the production strain of β-lactoglobulin (strain Ao_st0002). *A. oryzae* is a Biosafety Level 1 organism, as classified by the American Type Culture Collection, and has a long-history of safe use in the production of food enzymes (Barbesgaard *et al*., 1992). The production strain *A. oryzae* Ao_st0002 was constructed from the recipient host strain *A. oryzae* Ao_st0044. The recipient host strain *A. oryzae* Ao_st0044 was constructed from the base strain *A. oryzae* RIB40 by deletion of the endogenous orotidine-5'-monophosphate decarboxylase gene (*pyrG*) and LigD. Deletion of *pyrG* results in an uracil auxotrophic Strain Ao_st0044 as the gene *pyrG* is an established homolog to URA3 in *Saccharomyces cerevisiae*. Therefore, uracil is used as a selection marker for Strain Ao_st0044 . The *A*. *oryzae* strain RIB40 has been deposited in an international culture collection, World Data Centre for Microorganisms (WDCM), as WDCM 139 and was obtained from a previous collection of microorganisms in 2019. According to the record, the original isolation of the strain from horsebean (otherwise known as "fava bean"), collected in Kyoto, Japan, was reported in 1950 by Murakami *et al.* (1950) and deposited into the collection in 1973. Hence the strain was collected, isolated, and deposited prior to the Convention on Biological Diversity (CBD, 1993) and its Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (CBD, 2011) and is not under the regulations based on these international agreements.

2.2.1.2 Construction of the Production Strain

Imagindairy employs genetic modification practices that are commonly used and well defined in order to obtain both a pure product and optimize expression of β-lactoglobulin. *A. oryzae* is frequently used as a production organism because of its characterized secretion capacity, genetic tractability, and fast growth.

The Ao_st0002 production strain is constructed by transformation of 2 or more expression cassettes to the recipient strain Ao_st0044. The expression cassette consists of a nucleic acid sequence of the β-lactoglobulin gene from the domestic cow (*Bos taurus*) fused to a secretion signal flanked by a promoter and a terminator sequence. The fusion of the secretion signal was designed with the addition of a cleavage site, removing the secretion signal with no trace of the signal or the cleavage site. Thus, the resulting amino acid sequence is identical to the bovine β-lactoglobulin isoform B protein. The nucleic acid sequence of β-lactoglobulin was optimized to induce maximal levels of expression without changing the native amino acid sequence of the protein. The expression cassette also contains the coding sequence of the *Aspergillus niger* orotidine-5'-monophosphate decarboxylase gene (*pyrA*) homolog of the *A. oryzae pyrG* (GenBank: X96734.2). *pyrA* is utilized to regain the uracil production used as a selection marker from the *pyrG* deleted auxotrophic Ao_st0044 strain. In addition, the expression cassette contains a series of well characterized transcriptional elements such as promoters, terminators, and transcription factors to promote the expression of the β-lactoglobulin gene from endogenous elements of non-toxigenic relative species or synthetic sources.

No antibiotic selection markers or origin of replication sequences were used in the construction process of the production strain Ao_st0002 or the recipient host strain Ao_st0044.

2.2.1.3 Genomic Stability of the Production Strain

Imagindairy is currently conducting a multi-generational genomic stability study of its production strain. Genomic stability is being assessed by growing the fungi from spores of the production strain on solid media plates until the production of new spores. Spores of Ao_st0002 were collected (representing a new generation of the fungi) and placed on a fresh solid media plate to grow. Genomic DNA was isolated from the spores and copy number of the β-lactoglobulin gene was evaluated by quantitative polymerase chain reaction (qPCR). The copy-number of the β-lactoglobulin gene in the fifth generation was evaluated and compared to the copy-number of the mother strain (generation 0) by the qPCR delta-delta ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001). The results show a consistent copy-number of the β-lactoglobulin gene after 5 generations of the fungi with RQ = 1.109 ± 0.031 (normalized relative quantity), establishing a genetic stability of Ao_st0002 production strain for 5 generations.

2.2.2 Description of the Production Process

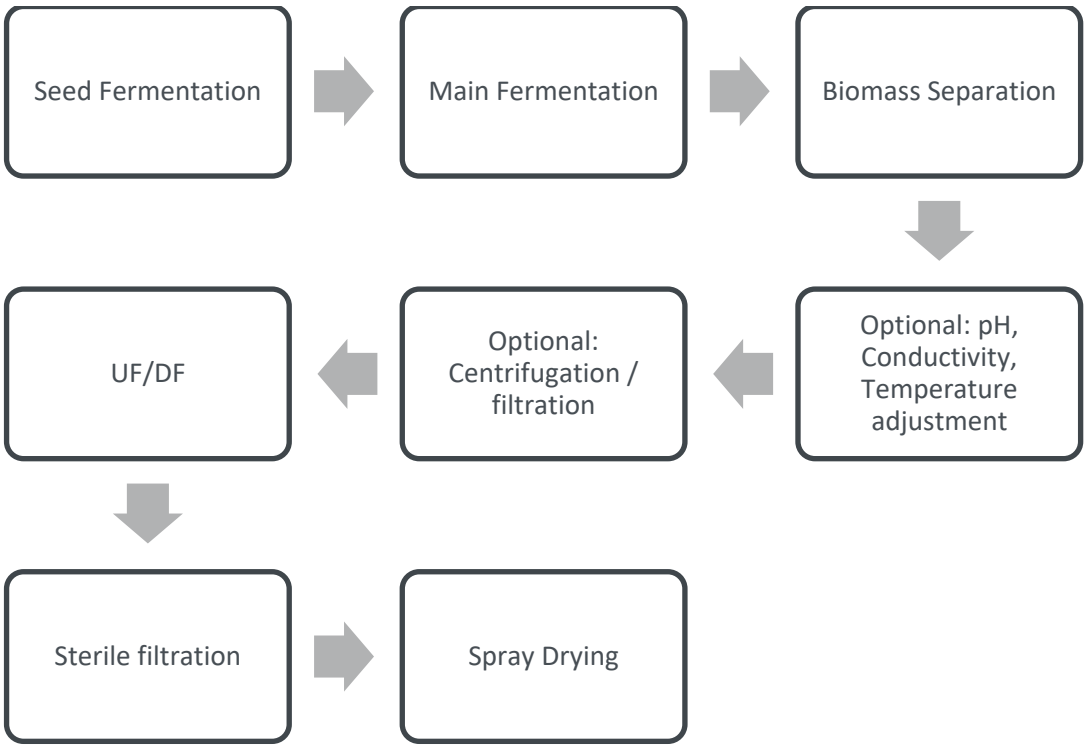
Imagindairy's commercial β-lactoglobulin is manufactured in compliance with current Good Manufacturing Practice (cGMP) and Hazard Analysis and Critical Control Points. All materials (raw materials, processing aids, filtration aids, and pH adjusters) are of a purity and quality suitable for their intended use; they are food grade and/or GRAS, or high-quality chemical or pharmaceutical grades (*e.g.* United States Pharmacopeia, National Formulary, or American Chemical Society grades) from approved suppliers.

Manufacturing Process

Imagindairy's β-lactoglobulin is manufactured by precision fermentation of genetically modified species of *A. oryzae* without expression induction by solvents such as methanol. A flow chart describing the process is

depicted in Figure 2.2.2-1 below. The fermentation process begins with the insertion of spore suspension stock vial seed into the seed fermentation stage to increase biomass amount. At the end of the seed fermentation stage, the culture is transferred to the main fermentation stage to produce β-lactoglobulin. Following the fermentation stage, the biomass is separated from the broth. The harvest then undergoes a series of purification steps: pH, conductivity, and temperature adjustment (optional); centrifugation and/or filtration to remove impurities; concentration and dialysis using ultrafiltration/diafiltration; sterile filtration; and spray drying. The final product is a white to off-white to yellowish powder consisting of ≥60% total protein, of which β-lactoglobulin is not less than 85%.

Figure 2.2.2-1 Flow Chart of the Manufacturing Process of β-Lactoglobulin *via* Fermentation of *Aspergillus oryzae*



DF = diafiltration; UF = ultrafiltration.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

The product specifications for Imagindairy's β-lactoglobulin produced *via* the fermentation of *A. oryzae*, including compositional, heavy metals, and microbial parameters, are presented in Table 2.3.1-1.

Table 2.3.1-1 Product Specifications for β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Parameter	Specification	Method
pH	6 to 8	AOAC 981.12
Composition		
Protein (Dumas/Kjeldahl) (%)	NLT 60	AOAC 986.06 (mod.), 992.15 (mod.), or 991.20

Table 2.3.1-1 Product Specifications for β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Parameter	Specification	Method
β-Lactoglobulin as % of protein (%)	NLT 85	SEC-HPLC/In-house method
Moisture (%)	NMT 10	AOAC 926.08, AOAC 927.05
Ash (%)	NMT 10	AOAC 945.46
Fat (%)	NMT 5	AOAC 989.05
Total carbohydrates (%)	NMT 30	21 CFR – Calculation
Heavy Metals		
Lead (ppm)	NMT 0.1	AOAC 2011.19, 993.14, and 2015.01 (mod.)
Arsenic (ppm)	NMT 0.1	AOAC 2011.19, 993.14, and 2015.01 (mod.)
Mercury (ppm)	NMT 0.1	AOAC 2011.19, 993.14, and 2015.01 (mod.)
Cadmium (ppm)	NMT 0.1	AOAC 2011.19, 993.14, and 2015.01 (mod.)
Microbiology		
Total plate count (CFU/g)	NMT 10,000	AOAC 966.23
Yeast (CFU/g)	NMT 100	FDA BAM Chapter 18 mod.
Mold (CFU/g)	NMT 100	FDA BAM Chapter 18 mod.
Coliforms (CFU/g)	NMT 10	FDA BAM Chapter 4/AOAC 991.14
Escherichia coli (CFU/g)	NMT 10	AOAC 991.14
Salmonella (CFU/g)	Absent in 25 g	AOAC RI-121501

AOAC = Association of Official Analytical Collaboration; BAM = *Bacteriological Analytical Manual*; CFR = *Code of Federal Regulations*; CFU = colony-forming units; FDA = Food and Drug Administration; mod. = modified; NLT = not less than; NMT = not more than; ppm = parts per million; SEC-HPLC = size exclusion high-performance liquid chromatography.

2.3.2 Batch Analysis

Data from the batch analysis of 3 non-consecutive lots of β-lactoglobulin produced *via* fermentation of *A. oryzae* have been provided in Tables 2.3.2-1 and 2.3.2-2 below and demonstrate that the manufacturing process described in Section 2.2 produces a consistent product that meets the established specifications.

Chemical Analysis

Table 2.3.2-1 Summary of the Chemical Product Analysis for 3 Lots of β-Lactoglobulin

Parameter	Specification	Manufacturing Lot ID51	Manufacturing Lot ID55	Manufacturing Lot AO035
pH	6 to 8	7.10	7.52	7.76
Composition				
Protein (%) (Dumas/Kjeldahl)	NLT 60	63.55	65.09	63.9
β-Lactoglobulin as % of protein (%)	NLT 85	95.9	96.5	98 .0
Moisture (%)	NMT 10	8.04	6.59	9.25
Ash (%)	NMT 10	4.88	3.15	3.62
Fat (%)	NMT 5	0.19	0.23	0.14
Total carbohydrates (%) (calculated)	NMT 30	23.34	24.94	23.09
Heavy Metals				
Lead (ppm)	NMT 0.1	0.022	0.014	0.10
Arsenic (ppm)	NMT 0.1	0.019	0.032	<0.01

Table 2.3.2-1 Summary of the Chemical Product Analysis for 3 Lots of β-Lactoglobulin

Parameter	Specification	Manufacturing Lot ID51	Manufacturing Lot ID55	Manufacturing Lot AO035
Mercury (ppm)	NMT 0.1	<0.005	<0.005	<0.005
Cadmium (ppm)	NMT 0.1	<0.005	<0.005	<0.005

NLT = not less than; NMT = not more than; ppm = parts per million.

Microbiological Analysis

Table 2.3.2-2 Summary of the Microbiological Analysis for 3 Lots of β-Lactoglobulin

Parameter	Specification	Manufacturing Lot ID51	Manufacturing Lot ID55	Manufacturing Lot AO035
Total plate count (CFU/g)	NMT 10,000	<10	10	<250 CFU/mL*
Yeast (CFU/g)	NMT 100	<10	<10	<10
Mold (CFU/g)	NMT 100	10	<10	<10
*Listeria monocytogenes***	Absent in 25 g	Not detected	Not detected	Not detected
Coliforms (CFU/g)	NMT 10	<10	<10	<10
Escherichia coli (CFU/g)	NMT 10	<10	<10	<10
Salmonella (CFU/g)	Absent in 25 g	Not detected	Not detected	Not detected

AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; NLT = not less than; NMT = not more than; SMEDP = Standard Methods for the Examination of Dairy Products.
* Tested using SMEDP 6.040 method.
** Test using AOAC-RI 080901.

2.3.2.1 Additional Analyses

Data from the batch analysis of 3 non-consecutive lots of Imagindairy's β-lactoglobulin produced *via* fermentation of *A. oryzae* demonstrate that various mycotoxins and aflatoxins are not produced during the manufacture of β-lactoglobulin (see Table 2.3.2.1-1).

Table 2.3.2.1-1 Summary of Aflatoxin Analyses for 3 Lots of β-Lactoglobulin

Parameter	Method	Manufacturing Lot ID51	Manufacturing Lot ID55	Manufacturing Lot AO35
Aflatoxins				
Aflatoxin HPLC (µg/kg)	HPLC [LOQ=1.00]	<1.00	<1.00	<1.00
Aflatoxin B1 (µg/kg)	HPLC [LOQ=0.25]	<0.25	<0.25	<0.25
Aflatoxin B2 (µg/kg)	HPLC [LOQ=0.25]	<0.25	<0.25	<0.25
Aflatoxin G1 (µg/kg)	HPLC [LOQ=0.25]	<0.25	<0.25	<0.25
Aflatoxin G2 (µg/kg)	HPLC [LOQ=0.25]	<0.25	<0.25	<0.25
Ochratoxin A (µg/kg)	HPLC [LOQ=1.00]	<1.00	<1.00	<1.00
Other Toxins				
Zearalenone (µg/kg)	MP 2415 rev 1 2021; [LOD = 10]	Not detected	Not detected	Not detected
Tricotecen (ug/kg)	MP 2415 rev 1 2021	Not detected	Not detected	Not detected
Deoxynivalenol (µg/kg)	MP 2415 rev 1 2021 [LOD = 20]	Not detected	Not detected	Not detected
Toxin HT2 (µg/kg)	MP 2415 rev 1 2021;	Not detected	Not detected	Not detected

Table 2.3.2.1-1 Summary of Aflatoxin Analyses for 3 Lots of β-Lactoglobulin

Parameter	Method	Manufacturing Lot ID51	Manufacturing Lot ID55	Manufacturing Lot AO35
	[LOD = 5]			
Toxin T2 (µg/kg)	MP 2415 rev 1 2021; [LOD = 2.5]	Not detected	Not detected	Not detected
Sum of toxin T2 and HT2 (µg/kg)		<10	<10	<10

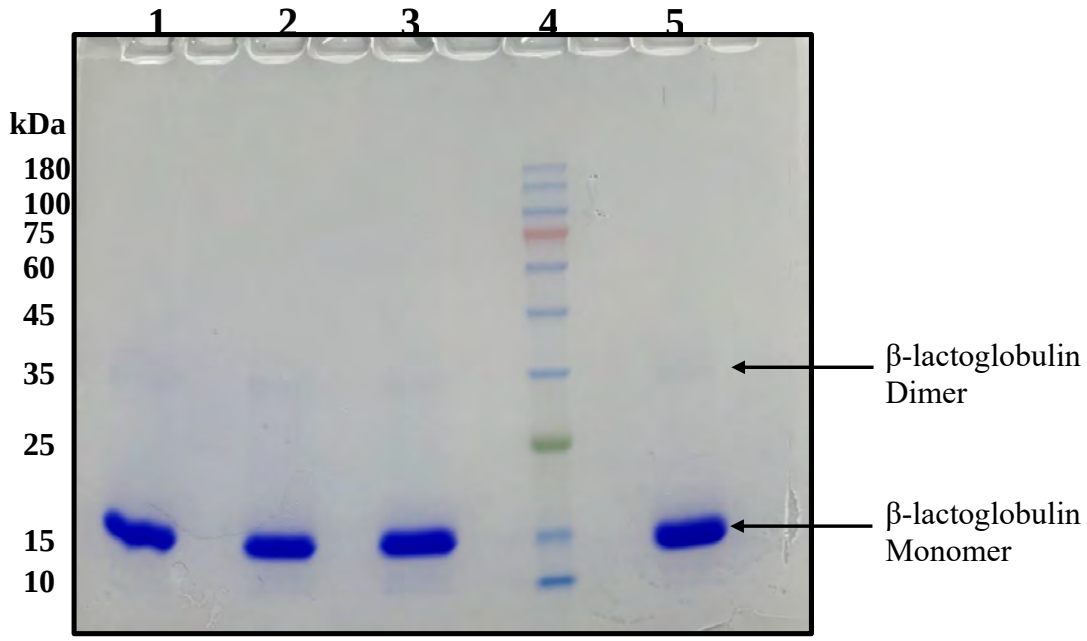
HPLC = high-performance liquid chromatography; LOD = limit of detection; NLT = not less than; NMT = not more than; LOQ = limit of quantitation.

2.3.3 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Analysis

As shown in Figure 2.3.3-1, Manufacturing Lots AO35, ID51, and ID55 of β-lactoglobulin powder originating from *A. oryzae* fermentation (Lanes 1 to 3, respectively) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and compared to a bovine β-lactoglobulin standard (Lane 5).

For the SDS-PAGE analysis, β-lactoglobulin powders were dissolved in 2 to 4 mg/mL of Milli-Q water. The samples were then diluted in loading dye (Sigma, Cat No. S3401) with addition of β-mercaptoethanol as a reducing agent (Sigma-Aldrich, M3148), and heated to 95˚C for 5 minutes. Next, 20 µg of AO35, ID51 and ID55 recombinant and bovine standard β-lactoglobulin (Sigma Aldrich) were loaded on Tris-glycine polyacrylamide gel. The run was then conducted at constant volts, stained with Coomassie for 30 minutes, and de-stained overnight. Results indicate that the produced β-lactoglobulin protein from *A. oryzae* is the same size as pure bovine β-lactoglobulin.

Figure 2.3.3-1 Coomassie Blue–Stained Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Analysis of the Recombinant β-Lactoglobulin



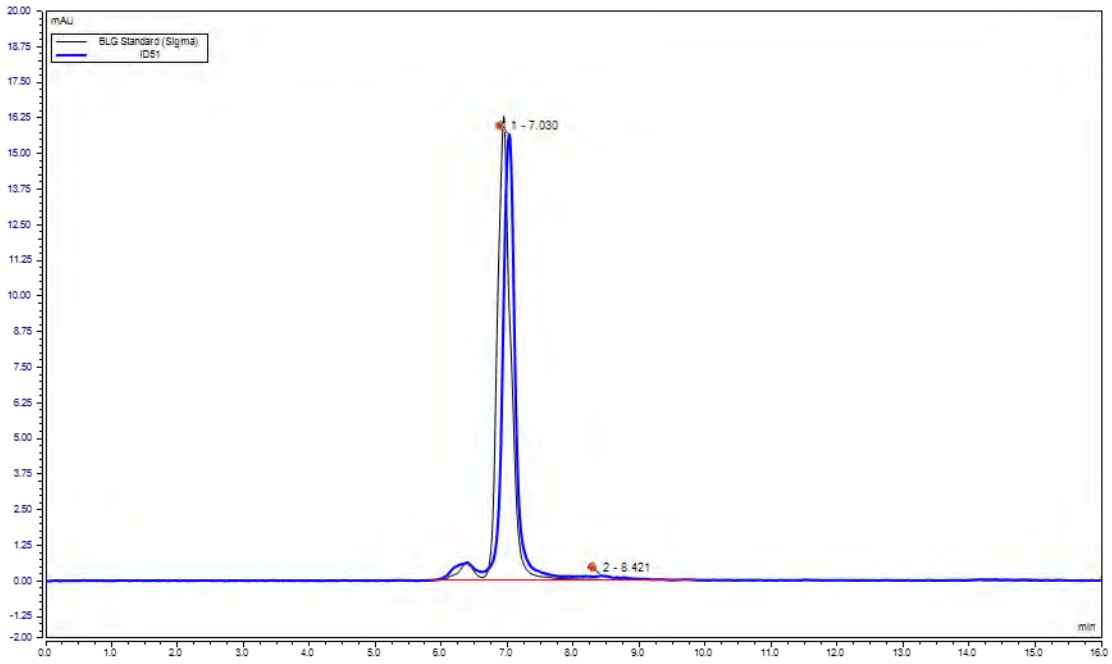
Note: Lot AO35 (Lane 1), Lot ID51 (Lane 2), Lot ID55 (Lane 3), Protein Marker PM2500 (Lane 4), bovine β-lactoglobulin standard (Sigma-Aldrich) (Lane 5). 20 µg of recombinant and standard β-lactoglobulin were loaded. Molecular weights are 18.3 and 36.6 kDa for β-lactoglobulin monomer and dimer, respectively.

2.3.4 Size Exclusion High-Performance Liquid Chromatography Analysis

Manufacturing Lots AO35, ID51, and ID55 of β-lactoglobulin powder produced by fermentation of *A. oryzae* Ao_st0002 were analyzed by size exclusion high-performance liquid chromatography (SEC-HPLC), which has been validated for its intended purpose, to assess its purity. The samples were analyzed by Waters BEH SEC column using a ThermoScientific HPLC device with an absorption detector. The results show 2.0, 4.1, and 3.5% impurities of total protein for Manufacturing Lots AO35, ID51, and ID55, respectively.

A representative chromatogram of Manufacturing Lot ID51 and bovine β-lactoglobulin standard (Sigma-Aldrich) is depicted in Figure 2.3.4-1 below.

Figure 2.3.4-1 **Representative Chromatogram of the Recombinant β-Lactoglobulin Lot ID51 (blue) and the Bovine β-Lactoglobulin Standard (Sigma-Aldrich) (black) Using Size Exclusion High-Performance Liquid Chromatography**



PART 3 §170.235 DIETARY EXPOSURE

3.1 History of Use in Food/Current Regulatory Status

β-Lactoglobulin was the subject of a favorable European Food Safety Authority (EFSA) safety review as a novel food pursuant to Regulation (EU) 2015/2283 (EFSA, 2022). The novel food (≥90% w/w dry matter protein), with β-lactoglobulin as the primary component (≥90% of total protein), was found to be safe at levels up to 25 g/100 g in isotonic and sports drinks, whey powder, probiotic milk-like drinks, and foods for special medical purposes (FSMP) (EFSA, 2022). In the U.S., β-lactoglobulin has been concluded to be GRAS at use levels up to 35% in conventional foods and has been the subject of 3 GRAS Notices (GRNs 863, 1005, and 1056 – U.S. FDA, 2020, 2022b,c). Of the 3, GRN 863 received a "no questions" letter from the U.S. FDA, while GRN 1005 withdrawn by the notifier, and GRN 1056 is pending FDA review.

3.2 Intended Use of β-Lactoglobulin and Levels of Use in Food and Beverages

Imagindairy's β-lactoglobulin produced *via* fermentation of *A. oryzae* is intended to be used as a non-animal protein source in foods that currently use protein from milk or plants. The intended use will be entirely substitutional with other β-lactoglobulin ingredients currently on the market, and its intended uses, use levels, and estimated cumulative daily intake will be incorporated by reference from GRN 1056, p. 15–21 (Remilk Ltd., 2022; U.S. FDA, 2022c). Examples of the typical food uses and use levels of Imagindairy's β-lactoglobulin, as well as the use levels from previous β-lactoglobulin GRAS Notices, are summarized in Table 3.2-1.

Table 3.2-1 Food Uses and Use Levels of β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Food Category	Food Use	Use Level Specified in GRN 863 (U.S. FDA, 2020)	Use Level Specified in GRN 1056 (U.S. FDA, 2022c)	Use Level Specified for Imagindairy's β-Lactoglobulin
Nutritional Products	Meal replacements and supplements	5 to 15%	15%	15%
	Powdered nutritional beverages	10 to 25%	25%	25%
	Nutritional bars	5 to 35%	35%	35%
	Sports beverages	5 to 20%	-	-
	Electrolyte-type sports drinks	-	6%	6%
	Performance nutritional beverages, high protein	-	25%	25%

Table 3.2-1 Food Uses and Use Levels of β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Food Category	Food Use	Use Level Specified in GRN 863 (U.S. FDA, 2020)	Use Level Specified in GRN 1056 (U.S. FDA, 2022c)	Use Level Specified for Imagindairy's β-Lactoglobulin
Dairy and Dairy-based Products	Milk products (including beverages, and coffee creamer)	1 to 15%	-	-
	Fluid milk, powdered milk, flavored milk, milk-based drinks and drink mixes (*e.g.*, dairy smoothies, hot chocolate from mix), milk substitutes	-	6%	6%
	Cream, half & half, cream cheese, cheese spread, whipped cream	-	15%	15%
	Yogurt and fermented milk products	1 to 5%	8%	8%
	Spreads, dips, and cream substitutes	1 to 5%	-	-
	Spreads, dips	-	10%	10%
	Cream substitutes	-	15%	15%
	Frozen dairy desserts and mixes	1 to 10%	Ice cream, frozen yogurt 8%	10%
	Cheese used primarily as ingredients (*e.g.* ricotta cheese)	-	15%	15%
	Semi-hard cheese (*e.g.*, feta, Camembert, brie)	-	25%	25%
Sugar-based Products	Desserts and Mousses	<5%	5%	5%
	Confections (including chocolate confections)	1 to 10%	10%	10%
	Coatings and Fillings	1 to 10%	10%	10%
	Snack Foods	1 to 10%		
	Cookies and brownies, crackers, popcorn, potato chips, tortilla chips, hard pretzels/snack mix	-	5%	5%
	Doughnuts, toaster pastries, muffins	-	10%	10%
Dressings	Salad Dressings	<5%	Creamy salad dressings 5%	5%
	Minor main entrée sauces (*e.g.*, Alfredo sauce, white sauce, cheese sauce)	-	6%	6%

Table 3.2-1 Food Uses and Use Levels of β-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Food Category	Food Use	Use Level Specified in GRN 863 (U.S. FDA, 2020)	Use Level Specified in GRN 1056 (U.S. FDA, 2022c)	Use Level Specified for Imagindairy's β-Lactoglobulin
Baked Goods	French toast, crepes, pancakes, bagels, scones, biscuits, croissants	-	10%	10%
	Breads & rolls, English muffins, pizza crust	-	10%	10%
Egg Products	Egg substitutes	-	10%	10%

GRN = GRAS Notice.

3.3 Estimated Intake of β-Lactoglobulin

3.3.1 Intake Estimates for β-Lactoglobulin

The intended use and use levels of Imagindairy's β-lactoglobulin will be entirely substitutional with other β-lactoglobulin preparations currently on the market; therefore, the cumulative estimated daily intake will be incorporated by reference from GRN 1056, p. 15–21 (Remilk Ltd., 2022; U.S. FDA, 2022c). Estimates of the intended intake of β-lactoglobulin were developed from food consumption records collected in the What We Eat in America component of the National Health and Nutrition Examination Surveys conducted in 2015–2016 and 2017–2018 (USDA, 2019, 2022; CDC, 2020a,b, 2022a,b). The maximum use level of β-lactoglobulin across applicable food categories was used to calculate the 2-day average intake estimates of β-lactoglobulin at the mean and 90th percentile of intake from proposed uses outlined in Table 3.2-1. In summary, both the *per capita* and per user cumulative mean intake of β-lactoglobulin in the population ages 2 years and older was 31.0 g/day, while the cumulative 90th percentile intake was 56.4 g/day. The estimated dietary intakes are highly conservative, as the maximum intended use level is assumed for all foods in each use category, and it is assumed that consumers are selecting products containing β-lactoglobulin at all consumption events.

β-lactoglobulin produced by Imagindairy is chemically identical to β-lactoglobulin present within traditional whey protein in terms of its nutrition and safety; thus, β-lactoglobulin products can be used in place of traditional whey protein or other protein products currently on the market. All uses of β-lactoglobulin will be entirely substitutional with other β-lactoglobulin-containing milk protein preparations (*e.g.*, whey protein isolates, concentrates) currently on the market; therefore, the intended uses of Imagindairy's β-lactoglobulin will not increase the overall intake of β-lactoglobulin or dietary protein.

PART 4 §170.240 SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with β-lactoglobulin.

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 evaluation of Imagindairy's β-lactoglobulin was conducted using scientific procedures and included hazard characterization of the production organism *A. oryzae*, and the β-lactoglobulin fermentation product. The safety of the production organism is reviewed in Sections 6.2 and 6.3, and data and information characterizing the safety of β-lactoglobulin are described in Section 6.4.

6.2 Safety of the Parental Organism

The safety and low risk associated with the commercial use of *A. oryzae* has long been well established (U.S. EPA, 1997), and *A. oryzae* has been consumed for centuries as part of fermented foods such as miso and sake (Allwood *et al.*, 2021). Globally, the production organism, *A. oryzae*, has a long history of safe use in food production. The food enzymes produced by *A. oryzae* include amylases, aminopeptidases, aspariginases, glucanases, glucose oxidases, laccases, lactases, lipases, phospholipase, pectinesterases, phytase, proteases, and xylanases (Amfep, 2015; U.S. FDA, 2018; FSANZ, 2022; Health Canada, 2023). In the U.S., *A. oryzae* has primarily been used as a production organism to derive many enzymes (GRNs 8, 10, 34, 43, 75, 90, 103, 106, 113, 122, 142, 201, 811, 979, and 982 – U.S. FDA, 2023). In France, *A. oryzae* is listed as an authorized source of various enzyme preparations (Article Annexe I C of *Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires* [JORF, 2006]). *A. oryzae* is included in the Danish *List of Microbial Cultures notified to the Danish Veterinary and Food Administration* (Danish Veterinary and Food Administration, 2016) as a notified microbial culture applied and is permitted for use in food. *A. oryzae* has also been notified to EFSA 51 times for its intentional direct use or its use as a source of food and feed additives, food enzymes, and plant protection products, and it is included in the Union list of novel foods (EU, 2017). Lastly, powdered *A. oryzae* grown with added minerals (calcium, iron, zinc, chromium, selenium, copper, magnesium, manganese, molybdenum, or combinations thereof) has been concluded GRAS and received a "no questions" letter from the U.S. FDA for use in conventional foods such as breakfast cereals, pastas, processed fruit and vegetable juices, soups, and nutritional drinks, at a level that provides 25% of the daily value for each mineral in the product, up to 250 mg powder per serving (GRN 829 – U.S. FDA, 2019).

A. oryzae is not considered to be pathogenic to humans and its long history of use indicates it is not a safety concern (Barbesgaard *et al.*, 1992; He *et al.*, 2019).

6.3 Safety of the Production Strain

Imagindairy employs genetic modification practices that are commonly used and well defined in order to obtain both a pure product and optimize expression of β-lactoglobulin. *A. oryzae* is frequently used as a production organisms because of its characterized secretion capacity, genetic tractability, and fast growth. No antibiotic selection markers or origin of replication sequences are used in the construction process of the production strain or the recipient host strain. Data from a multi-generational genomic stability study show a consistent copy-number of the β-lactoglobulin gene after 5 generations of the fungi establishing the genetic stability of the production strain. Additionally, Imagindairy provided data from the batch analysis of 3 non-consecutive lots of β-lactoglobulin demonstrating that various mycotoxins and aflatoxins produced by related Aspergillus spp. are not produced by the production organism. Taken together, it can be concluded that the production strain is safe for use in the production of Imagindairy's β-lactoglobulin.

6.4 Safety of β-Lactoglobulin

β-Lactoglobulin from bovine milk is a small protein with a molecular mass of approximately 18.3 kDa (Barbiroli *et al*., 2022). β-Lactoglobulin has been reported to be relatively pepsin-resistant in the stomach and has fast gastric emptying. Both *in vitro* and *in vivo* data have shown that β-lactoglobulin is rapidly digested in the intestine, which leads to the absorption of amino acids in the proximal intestine (Mahé *et al.*, 1995, 1996; Rieu *et al.*, 2007; Farnfield *et al.*, 2009; Sanchón *et al.*, 2018).

β-Lactoglobulin was the subject of a favorable EFSA safety review as a novel food pursuant to Regulation (EU) 2015/2283. The novel food (≥90% w/w dry matter protein), with β-lactoglobulin as the primary component (≥90% of total protein), was found to be safe at levels up to 25 g/100 g in isotonic and sports drinks, whey powder, probiotic milk-like drinks, and FSMP (EFSA, 2022). In the U.S., β-lactoglobulin has been concluded to be GRAS at use levels up to 35% in conventional foods and has been the subject of 3 GRAS Notices (GRNs 863, 1005, and 1056 – U.S. FDA, 2020, 2022b,c). Of the 3, GRN 863 received a "no questions" letter from the U.S. FDA, while GRN 1005 withdrawn by the notifier and GRN 1056 is pending FDA review. Lastly, a bovine β-lactoglobulin preparation (Arla Foods Inc., Lacprodan® BLG; total protein content ≥86%; β-lactoglobulin >90%) has been shown to be not genotoxic and showed no toxicity at up to doses of 1,000 mg/kg body weight/day, the highest dose tested, in a 90-day rodent subchronic toxicity study (Dybdahl *et al.*, 2021).

Traditional whey protein has a long history of safe use and its safety has been affirmed through many scientific reviews. The safety discussions and GRAS conclusions of whey protein are directly applicable to the GRAS conclusion of β-lactoglobulin since β-lactoglobulin is a component of whey protein4. Whey protein concentrate is GRAS affirmed in 21 CFR §184.1979(c) (U.S. FDA, 2022d). The regulation states that whey protein concentrate is the substance obtained by the removal of sufficient nonprotein constituents from whey so that the finished dry product contains not less than 25% total protein. Additionally, "whey protein" has been the subject of 2 GRAS Notices (GRNs 37 and 633) that received "no questions" letters from the U.S. FDA, while "concentrated milk proteins" has been the subject of GRN 504, which also received a "no questions" letter from the Agency (U.S. FDA, 2000, 2014, 2016). In general, these safety evaluations followed the same basic principle summarized in GRN 504:

> *Due to the long history of human consumption of milk, milk and milk proteins pose little toxicological concern to humans or animals. With the exception of certain sensitive populations (e.g., milk-allergic and lactose-intolerant individuals), we are not aware of adverse effects associated with consumption of concentrated milk proteins.*

As was the case in the previous GRAS Notices, Imagindairy's β-lactoglobulin is identical to the β-lactoglobulin found in cow's milk; therefore, the previous conclusions of the safety of β-lactoglobulin are of direct relevance to the safe use of Imagindairy's β-lactoglobulin as a food ingredient. Based on a literature search in April 2023, Imagindairy did not identify any new information to suggest that β-lactoglobulin is unsafe for use as a food ingredient. Imagindairy is aware that there exist sensitive consumers who are allergic to milk proteins such as β-lactoglobulin; however, consumption of milk-derived ingredients or β-lactoglobulin is not associated with any other adverse effects and does not pose any additional safety concerns besides its allergenic potential. Taken together, there is reasonable certainty that the use of Imagindairy's β-lactoglobulinis safe for consumption, and Imagindairy concludes its β-lactoglobulin is GRAS for its intended uses and use levels.

6.4.1 Toxicological Studies of Other β-Lactoglobulins

Toxicological studies on other preparations of β-lactoglobulin can be used as corroborative evidence supporting the safety of Imagindairy's β-lactoglobulin. The safety of a bovine β-lactoglobulin preparation by Arla Foods (Lacprodan® BLG; total protein content ≥86%; β-lactoglobulin >90%) was evaluated by a bacterial reverse mutation assay, an *in vitro* micronucleus assay, a 14-day repeated dose rodent toxicity study, and a 90-day repeated dose rodent subchronic toxicity study (Dybdahl *et al.*, 2021).

In an Organisation for Economic Co-operation and Development (OECD)-compliant assay, a bacterial reverse mutation assay was performed using 2 independent experiments (standard plate incorporation and pre-incubation) in *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 uvrA with and without metabolic activation (OECD 471 – *Bacterial Reverse Mutation Assay*). The test article was then tested at 31.6, 100, 316, 2,500, and 5,000 µg/plate. No precipitation or cytotoxic effects were observed in any of the experiments with and without S9 mix. In conclusion, Lacprodan® BLG was not mutagenic under the conditions tested.

An OECD-compliant *in vitro* micronucleus assay was used to assess the clastogenic potential of Lacprodan® BLG in in cultured human peripheral blood lymphocytes (OECD 487 – *In Vitro Mammalian Cell Micronucleus Test*). Concentrations ranging from 125 to 2,000 µg/mL of β-lactoglobulin were used, with and without metabolic activation, in 2 experiments (4-hour short-term exposure and 44-hour long-term exposure). No precipitation or cytotoxic effects were observed in any of the experiments, with or without metabolic activation. There were no statistically significant increases of cells with micronuclei after Lacprodan® BLG exposure, with the exception of the 1,500 µg/mL test concentration in the short-term experiment without S9 mix. This result was not considered biologically relevant, as there was no dose-dependent relationship, and the increase was within the historical range of negative controls. In conclusion, Lacprodan® BLG was not clastogenic in human lymphocytes under the conditions tested.

In a preliminary 14-day repeat dose rodent study, male and female Wistar rats were provided Lacprodan® BLG by oral gavage at dose levels of 0, 100, 300, and 1,000 mg/kg body weight per day at an application volume of 5 mL/kg body weight (OECD 407 – *Repeated Dose 28-day Oral Toxicity Study in Rodents*). No mortality or clinical signs were observed in the study. There were no test article–related changes in body weight and food consumption during the exposure period. Exposure to the test item had no toxicologically relevant effect on hematology or clinical biochemistry parameters. Exposure to Lacprodan® BLG up to 1,000 mg/kg body weight per day was well tolerated and did not result in any adverse effects.

An OECD-compliant 90-day subchronic toxicity study in 80 Wistar rats (40 males and 40 females) was performed in which groups of animals received 0, 100, 300, or 1,000 mg Lacprodan® BLG/kg body weight/day at an application volume of 5 mL/kg body weight by oral gavage (OECD 408 – *Repeated Dose 90-Day Oral Toxicity Study in Rodents*). Clinical condition, functional observations, body weight, food consumption, fertility, hematology, clinical biochemistry, urinalysis, pathology, organ weight, and histopathology were evaluated as part of this study. There was no effect on body weight or food intake between control and β-lactoglobulin–exposed rats. There were no mortalities, ophthalmoscopic, or functional changes observed during the study. There were no changes on male and female fertility parameters related to exposure of Lacprodan® BLG. There were no test article-related changes in hematology, coagulation, clinical chemistries, hormones, urinalysis, or absolute or relative organ weight. All inter-group differences from controls were minor, lacked a dose-related response, or were limited to 1 sex and were therefore attributed to normal biological variation. Some macroscopic findings such as abnormal content in urinary bladder (1 high-dose male), enlarged mesenterial lymph nodes (1 high-dose male), fluid-filled uterus (1 low-dose female), and white focus in lung (1 control) were observed but were not considered test article–related, as they were observations commonly seen in rats of same strain and age and only occurred randomly. There were no test article-related histopathological microscopic findings observed. It was concluded that the no-observed-adverse-effect level (NOAEL) under the study conditions was 1,000 mg/kg body weight/day, the highest dose tested.

In conclusion, a bovine β-lactoglobulin preparation (Arla Foods Inc., Lacprodan® BLG; total protein content ≥86%; β-lactoglobulin >90%) was not mutagenic or genotoxic, and was without evidence of toxicity in a 90-day subchronic rodent feeding study where a NOAEL value of 1,000 mg/kg body weight/day, the highest dose tested, was determined (Dybdahl *et al.*, 2021).

6.5 Allergenicity

Bovine milk has been identified as a major food allergen in the U.S. and around the world. β-Lactoglobulin has been recognized as a major allergic component of cow's milk with over 50% of milk allergic patients generating specific immunoglobulin E antibodies to β-lactoglobulin (Chatchatee *et al.*, 2001; Geiselhart *et al.*, 2021). Therefore, consumption of a product with Imagindairy's β-lactoglobulin may elicit a milk protein allergic response. All products containing Imagindairy's β-lactoglobulin will indicate and inform consumers that the product contains a milk allergen and will comply with all food allergen labeling requirements.

Imagindairy has provided a report by Dr. Richard Goodman and the Food Allergy and Resource Program at the University of Nebraska-Lincoln evaluating the potential additional allergenicity of its β-lactoglobulin produced by *A. oryzae* (see Appendix A). To confirm that the β-lactoglobulin does not contain residual amino acid sequences similar to known allergens that could potentially produce an allergenic response, Imagindairy produced 3 batches of the β-lactoglobulin product, and samples were evaluated using liquid chromatography with tandem mass spectrometry at the Weizmann Institute of Science (Rehovot, Israel). Dr. Goodman conducted a bioinformatic evaluation of the identified minor proteins using AllergenOnline database version 21 (available at http://www.allergenonline.org; updated 14 February 2021) and National Center for Biotechnology Information (NCBI) Protein database. Comparisons in AllergenOnline used a sliding 80–amino acid window looking for minimum identity matches of 35% or more. Additional searches were made with the NCBI Protein database using Protein Basic Local Alignment Search Tool (BLASTP) and looking for long alignments of >35% identity. The relative abundance of reported proteins considered possibly important was >1/1,000 of the total protein content. A literature search on the allergic potential of *A. oryzae* conducted by Dr. Goodman concluded that there was little evidence from the scientific literature that proteins from *Aspergillus* were responsible for food allergy. Bioinformatic evaluation resulted in no identity matches that were of high similarity to known allergens and of high abundance. It was concluded that the Imagindairy's β-lactoglobulin produced from *A. oryzae* does not pose a risk of food allergy due to residual *A. oryzae* proteins.

6.6 GRAS Conclusion

Imagindairy Ltd. has concluded that β-lactoglobulin produced by fermentation of *Aspergillus oryzae* is Generally Recognized as Safe (GRAS), on the basis of scientific procedures, for use in food and beverage products based on the following:

- Imagindairy's preparation contains highly purified β-lactoglobulin identical to bovine β-lactoglobulin.
- β-lactoglobulin will be manufactured under cGMP and consistently meets food-grade specifications that control for identity as well as microbial and heavy metal contaminants.
- *A. oryzae*, has consistently been concluded safe as a production organism in food manufacturing, with no evidence of pathogenic or toxigenic concerns. .
- The intended uses and use levels of β-lactoglobulin in conventional foods and beverages will be entirely substitutional with other bovine β-lactoglobulin preparations that have been concluded to be GRAS and received "no question" letters from the U.S. FDA (GRN 863 – U.S. FDA, 2020).
- β-lactoglobulin has many similarities to milk whey protein, which has been GRAS affirmed and is the subject of several GRAS Notices that have received "no question" letters from the U.S. FDA (GRN 37, 504, and 633 – U.S. FDA, 2000, 2014, 2016).
- Milk and milk protein have a long safe history of use as foods.
- Data from other β-lactoglobulin preparations support the safety of β-lactoglobulin. β-Lactoglobulin was not genotoxic, was well tolerated, and exhibited no adverse effects in a subchronic toxicity study (Dybdahl *et al.*, 2021).
- The evidence supporting Imagindairy's β-lactoglobulin produced from the fermentation of *A. oryzae* does not pose an increased allergenic risk.
- All products containing β-lactoglobulin will comply with all food allergen labeling requirements.

β-Lactoglobulin therefore can be marketed and sold for its intended purposes in the U.S. without the promulgation of a food additive regulation under 21 CFR §170.3 (U.S. FDA, 2022e).

PART 7 §170.255 LIST OF SUPPORTING DATA AND INFORMATION

Allwood JG, Wakeling LT, Bean DC (2021). Fermentation and the microbial community of Japanese *koji* and *miso*: A review. J Food Sci 86(6):2194-2207. DOI:10.1111/1750-3841.15773.

Amfep (2015). *List of Commercial Enzymes*. Brussels, Belgium: Association of Manufacturers & Formulators of Enzyme Products (Amfep). Available at: https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf [updated: May 2015].

Barbesgaard P, Heldt-Hansen HP, Diderichsen B (1992). On the safety of *Aspergillus oryzae*: a review. Appl Microbiol Biotechnol 36(5):569-572. DOI:10.1007/BF00183230.

Barbiroli A, Iametti S, Bonomi F (2022). Beta-lactoglobulin as a model food protein: how to promote, prevent, and exploit its unfolding processes. Molecules 27(3):1131 [17pp]. DOI:10.3390/molecules27031131.

CBD (1993). *Convention on Biological Diversity*. Rio de Janeiro, Brazil, June 5-14, 1992, June 15, 1992-June 4, 1993, New York. (entry into force: 29 December 1993). New York (NY): United Nations, Convention on Biological Diversity (CBD). Available at: https://www.cbd.int/doc/legal/cbd-en.pdf.

CBD (2011). *Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from Their Utilization To The Convention on Biological Diversity: Text and Annex*. Conference of the Parties to the Convention on Biological Diversity, Oct. 29, 2010, Nagoya, Japan. Montreal (QC): Secretariat of the Convention on Biological Diversity (CBD), United Nations Environmental Programme (UNEP). Available at: https://www.cbd.int/abs/; https://www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf.

CDC (2020a). *National Health and Nutrition Examination Survey (NHANES): 2015-2016*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2015 [Page last reviewed: 2/21/2020].

CDC (2020b). *National Health and Nutrition Examination Survey (NHANES): 2015-2016 – Dietary Data*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/search/datapage.aspx?Component=Dietary&CycleBeginYear=2015 [Page last reviewed: 2/21/2020].

CDC (2022a). *National Health and Nutrition Examination Survey (NHANES): 2017-2018*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2017 [NHANES home page last reviewed: March 31, 2022].

CDC (2022a). *National Health and Nutrition Examination Survey (NHANES): 2017-2018*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2017 [page last reviewed: March 31, 2022].

Chatchatee P, Järvinen K-M, Bardina L, Vila L, Beyer K, Sampson HA (2001). Identification of IgE and IgG binding epitopes on β- and κ-casein in cow's milk allergic patients. Clin Exp Allergy 31(8):1256-1262. DOI:10.1046/j.1365-2222.2001.01167.x.

Danish Veterinary and Food Administration (2016). *[Danish] List of Notified Microbial Cultures Applied in Food*. Glostrup, Denmark: Danish Veterinary and Food Administration [Administration [Milijø- og Føderaveministeriet, Fødevarestyrelsen]]. Available at: https://www.foedevarestyrelsen.dk/english/Food/Microbial_cultures/Pages/default.aspx [Last Modified: 12 December 2016].

Dybdahl M, Selesko DB, Mikkelsen UR (2021). Safety evaluation of whey derived beta-lactoglobulin, Lacprodan® BLG. Toxicol Rep 8:617-626. DOI:10.1016/j.toxrep.2021.03.012.

EFSA (2022). Scientific Opinion on the safety of Beta-lactoglobulin as a Novel food pursuant to Regulation (EU) 2015/2283. (EFSA Panel on Nutrition, Novel Foods and Food Allergens/NDA) (Question no: EFSA-Q-2020-00538, adopted: 28 February 2022 by European Food Safety Authority). EFSA J 20(4):7204 [24pp]. DOI:10.2093/j.efsa.2022.7204. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/7204.

EU (2017). Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. C/2017/8878. Off J Eur Union 60(L351):72-201. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1533914206967&uri=CELEX:32017R2470 [current consolidated version: 06/02/2023].

Farnfield MM, Trenerry C, Carey KA, Cameron-Smith D (2009). Plasma amino acid response after ingestion of different whey protein fractions. Int J Food Sci Nutr 60(6):476-486. DOI:10.1080/09637480701833465.

FSANZ (2022). Standard 1.3.3 — Processing aids (F2022C00981). In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2022C00981.

Geiselhart S, Podzhilkova A, Hoffmann-Sommergruber K (2021). Cow's milk processing-Friend or foe in food allergy? Foods 10(3):572 [17pp]. DOI:10.3390/foods10030572.

He B, Tu Y, Jiang C, Zhang Z, Li Y, Zeng B (2019). Functional genomics of *Aspergillus oryzae*: strategies and progress. Microorganisms 7(4):103 [13pp]. DOI:10.3390/microorganisms7040103.

Health Canada (2023). *List of Permitted Food Enzymes*. (Lists of Permitted Food Additives, No. 5). Ottawa (ON): Health Canada. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html [date modified: 2023-04-27].

JORF (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires NOR: ECOC0600115A [Article Annexe I C, enzymes]. J Off Repub Fr (Oct.). Available at: https://www.legifrance.gouv.fr/loda/id/LEGITEXT000020667468/2020-10-28/ [version consolidée au 20 Mars 2021].

Kontopidis G, Holt C, Sawyer L (2004). Invited review: β-lactoglobulin: binding properties, structure, and function. J Dairy Sci 87(4):785-796. DOI:10.3168/jds.S0022-0302(04)73222-1.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. Methods 25(4):402-408. DOI:10.1006/meth.2001.1262.

Mahé S, Benamouzig R, Gaudichon C, Huneau JF, De Cruz I, Rautureau J, *et al.* (1995). Nitrogen movements in the upper jejunum lumen in humans fed low amounts of casein or β-lactoglobulin. Gastroenterol Clin Biol 19(1):20-26.

Mahé S, Roos N, Benamouzig R, Davin L, Luengo C, Gagnon L, *et al.* (1996). Gastrojejunal kinetics and the digestion of [^{15N}]β-lactoglobulin and casein in humans: the influence of the nature and quantity of the protein. Am J Clin Nutr 63(4):546-552. DOI:10.1093/ajcn/63.4.546.

Murakami H, Okuyama K, Sugita S (1950). [Study on Kohji (I). Isolation and selection of Aspergilli]. Nippon Jozo Kyokaishi [J Brew Soc Jpn] 45(9):65-69 [Japanese, English abstract]. DOI:10.6013/jbrewsocjapan1915.45.9_65.

Remilk Ltd. (2022). *GRAS Notice for Non-Animal β-Lactoglobulin Whey Protein from Fermentation by Komagataella phaffi*. (GRN No. 1056). Submitted by Covington on behalf of Rehovot, Israel: Remilk Ltd. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=1053 [pending as of: Jul. 25, 2022].

Rieu I, Balage M, Sornet C, Debras E, Ripes S, Rochon-Bonhomme C, *et al.* (2007). Increased availability of leucine with leucine-rich whey proteins improves postprandial muscle protein synthesis in aging rats. Nutrition 23(4):323-331. DOI:10.1016/j.nut.2006.12.013.

Sanchón J, Fernández-Tomé S, Miralles B, Hernández-Ledesma B, Tomé D, Gaudichon C, *et al.* (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with *in vitro* gastrointestinal simulation. Food Chem 239:486-494. DOI:10.1016/j.foodchem.2017.06.134.

U.S. EPA (1997). *Aspergillus oryzae Final Risk Assessment: Attachment I--Final Risk Assessment of Aspergillus oryzae*. Washington (DC): U.S. Environmental Protection Agency (U.S. EPA), Biotechnology Program under the Toxic Substances Control Act (TSCA). Available at: http://www2.epa.gov/sites/production/files/2015-09/documents/fra007.pdf [last updated on September 27, 2012].

U.S. FDA (2000). *Agency Response Letter GRAS Notice No. GRN 37 [Whey protein isolate and dairy product solids, Chicago (IL): American Dairy Products Institute]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=037 [Apr. 21, 2000 - FDA response - no questions; Additional correspondence clarifying the term "dairy product solids": Jan. 30, 2001].

U.S. FDA (2014). *Agency Response Letter GRAS Notice No. GRN 504 [Concentrated milk proteins, Elmhurst (IL): American Dairy Products Institute / Arlington (VA): U.S. Dairy Export Council]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=504 [Nov. 21, 2014 - FDA response - no questions].

U.S. FDA (2016). *Agency Response Letter GRAS Notice No. GRN 633 [Concentrated milk protein with a ≥ 60:40 whey:casein ratio, Denver (CO): Leprino Foods Company]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=633 [Sep. 21, 2016 - FDA response - no questions].

U.S. FDA (2018). *Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List)*. U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list [content current as of: 01/04/2018].

U.S. FDA (2019). *Agency Response Letter GRAS Notice No. GRN 829 [Dried biomass of Aspergillus oryzae fermented with minerals*, Ames (IA): Cura Global Health, Inc.]. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=829 [Dec. 9, 2019; Addit. Correspond. May 18, 2020 - FDA response - no questions].

U.S. FDA (2020). *Agency Response Letter GRAS Notice No. GRN 863 [B-lactoglobulin produced by Trichoderma reesei, Berkley (CA): Perfect Day, Inc.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=863 [Mar. 25, 2020 - FDA response - no questions].

U.S. FDA (2022a). Part 170—Food additives. Section §170.30—Eligibility for classification as generally recognized as safe (GRAS). In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs (U.S. Food and Drug Administration)*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2022/title21.

U.S. FDA (2022b). *Agency Response Letter GRAS Notice No. GRN 1005 [β-Lactoglobulin from cow milk, Viby J, Denmark: Arla Foods Ingredients Group P/S]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=1005

U.S. FDA (2022c). *Agency Response Letter GRAS Notice No. GRN 1056, β-lactoglobulin produced by Komagataella phaffii strain "yRMK-66", Rehovot, Israel: Remilk Ltd.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=1056 [pending as of: Jul. 25, 2022].

U.S. FDA (2022d). Part 184—Direct food substances affirmed as generally recognized as safe. Section §184.1979—Whey. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs*. (U.S. Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2022/title21.

U.S. FDA (2022e). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2022/title21.

U.S. FDA (2023). *GRAS Notices [Inventory]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices [content current as of: 04/24/2023].

USDA (2019). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2015-2016.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: 10/17/2019].

USDA (2022). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2017-2018.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/wweianhanes-overview/#release [last modified: 08/09/2022].

APPENDIX A

Report by Dr. Richard Goodman and the Food Allergy and Resource Program at the University of Nebraska-Lincoln on the Potential Allergenicity of *beta*-Lactoglobulin Produced *via* Fermentation of *Aspergillus oryzae*

Study Title

Literature and bioinformatics comparisons for potential food allergy for Bovine Beta-Lactoglobulin in transformed *Aspergillus oryzae* based on LC-MS/MS peptides

Authors

Richard E. Goodman

Study Completed On

14 April 2023

Performed by

Richard E. Goodman
RE Goodman Consulting, LLC
8110 Dougan Circle
Lincoln, NE 68516

Client
ImaginDairy
Imagindairy Ltd.
3 HaBosem St.
Ashodod, Israel

Laboratory Project ID

Study Number: REG-ImaginDairy-2023

Summary

ImaginDairy has developed a recombinant food product using an Ascomycete fungal host, *Aspergillus oryzae,* which was transformed with the bovine beta-lactalbumin (BLG) gene. Bovine beta-lactoglobulin is recognized as a major milk allergen. The question under consideration here is whether proteins from the host organism are sufficiently identical to allergenic proteins and abundant enough to suspect potential allergic cross-reactivity for subjects with specific allergies.

The company produced three batches of their product and had the proteins digested with trypsin and evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Wiseman Institute of Science. They provided the peptide data to RE Goodman for evaluation of potential risks of allergy or allergic cross-reactivity using www.AllergenOnline.org and the NCBI Protein database. The LC-MS/MS peptides of bovine beta-lactoglobulin covered 100% of the length of the protein. The full-set of peptides reported from the analysis included a few that match human keratin, a common finding in LC-MS/MS samples due to contamination by human skin dust in the laboratory, and porcine trypsin which was used to digest the proteins. The other peptides were evaluated based on identities to proteins identified as allergens in the www.AllergenOnline.org database and compared to the NCBI Protein database to consider other protein sources matched to AllergenOnline.

The output included relative protein/peptide intensities for each aligned protein, which are important in considering risks of allergy since reactions are dose dependent. Many proteins in all eukaryotic organisms have identity matches to some evolutionarily conserved proteins from a wide variety of sources, a few of those proteins are called allergens due to published data of IgE binding from some humans with claimed allergy to the sources. Evolutionary conservation of common structural or biochemical proteins can be maintained due to conservation of metabolic pathways or structural features. Clinically important major allergens are usually abundant proteins, and most of the clinically relevant allergens do not share sequence identity matches across broadly spaced taxa. Instead, clinically important cross-reactivity is usually shared across two to a small number of taxonomically related species such as walnut and pecan tree nuts due to highly identical sequences of major seed storage proteins including vicillins, glycinins and 2S albumins. Yet, the standard criterion considered for possible risks of shared IgE binding is an identity match of >35% over 80 amino acids based on the CODEX Guideline for risks of genetically engineered crops (2003). That criterion was established for transgenic organisms that had from one, to five transferred proteins in historically consumed organisms like soybean or maize. The intent of CODEX was proteins with identities >35% identity over 80 amino acids would require specific serum IgE binding tests using sera of people allergic to the matched allergenic source. However, novel foods can have > 5,000 proteins including a number of evolutionarily conserved proteins and more complex evaluations are needed to understand potential risks as we demonstrated in 2021 (Abdelmoteleb et al., 2021). Some conserved proteins may share over 70% identities and yet they do not share clinical allergic cross-reactivity.

The LC-MS/MS data for this product lists 142 peptides including the intended protein, beta-lactoglobulin with 100% peptide sequence coverage from 60 unique peptides for lactoglobulin. The abundance of the BLG peptides account for relative intensity of $3.43 \times 10^{+10}$. The other identified proteins including the second most abundant protein identified was porcine trypsin are relatively low in abundance, and it is the digestive enzyme needed to perform mass spectrometry, since this is the enzyme used to cut the proteins for mass spec analysis, at 1.06×10^{9} or less than 1:20th the abundance of BLG. The abundance of other proteins ranged from $1x10^{+8}$ to less than $1x10^{+4}$ relative mass.

All proteins identified by sequence matches to the NCBI Protein database or UniProt were compared to AllergenOnline.org public database using the sliding window FASTA search method that has provide FASTA matches down to 35% identity over 80, and for proteins less than 80 amino acids, the percent identity is calculated to normalize to 80 amino acids. So, a sequence of BLG that is 70 amino acids long would be determined to be 87.5% identical to BLG even though it is 100% identical to the 70 AA segment of BLG. The data of the amino acid sequences of proteins identified by LC-MS/MS were located in NCBI Protein or UniProt and those sequences were searched vs. www.AllergenOnline.org. The data was captured in an Excel file, along with the number of unique peptides from the LC-MS/MS, the relative intensity and the information from AllergenOnline was entered based on protein matches in the AllergenOnline database. The data from AllergenOnline that was also reviewed included the protein(s) that matched the query sequence over 35% identity over 80 amino acids, relative abundance of the matched query protein by LS-MS/MS data and whether there were at least 2 unique peptides of at least 9 amino acids that match a documented protein sequence based on HUPO Guidelines (Human Proteome Project for identification of protein sequences). The intensity used here is >0.1 units relative to the BLG. Finally, potential risks were judged based on the information in publications listed in AllergenOnline, and in published literature from the NCBI PubMed database.

Importantly, the host species, *Aspergillus oryzae* has been accepted as Generally Recognized as Safe (GRAS) for Koji Fermented Mineral Products in 2018 and for production of fungal burgers (Rousta et al., 2021). An earlier GRAS was achieved for the same species in 2016 (Sewell et al., 2016). The species is closely related to a number of *Aspergillus* and *Penicillium* sp. that have been implicated as the cause of some allergic reactions. And the close evolutionary relationships mean that many genes and protein sequences share high identity matches by with homologous genes and proteins between *A. oryzae* and other species BLASTP or FASTA.

Evaluation of the matches identities to allergens, relative abundance of proteins in the LC-MS/MS analysis and whether the bioactivity assays were clear for purified protein was used to judge relevance. In the final analysis, it is unlikely that any of the proteins represent a clinically important risk of allergy to this novel food produced in *Aspergillus oryzae*.

Study Number: REG-Beta-Lactoglobulin-2022

Title: Literature and bioinformatics analysis of potential food allergy was conducted using the amino acid sequences from the *Aspergillus oryzae* produced by Imagindairy

Facility: RE Goodman Consulting LLC
8110 Dougan Circle
Lincoln, NE 68516
USA

Principle Investigator: Richard E. Goodman
Tel: +1 (402) 417-5549

Study Start Date: 5 February 2023

Study Completion Date: 14 April 2023

Records Retention: All study specific raw data and a copy of the final report will be retained by Richard E. Goodman.

14 April 2023

Principle Investigator: Richard E. Goodman Date

Table of Contents

Abbreviations and Definitions

AA	Amino acid
AC	Accession number from NCBI or UniProt
AOL v21	http://www.AllergenOnline.com/ database version 21
BLASTP	Algorithm used to find local high scoring alignments between a pair of protein sequences (using databases on Entrez)
Entrez NCBI	A public genetic database maintained by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health, Bethesda, MD. Protein entries in the Entrez search and retrieval system are maintained by the NCBI of the National Institutes of Health (U.S.A.)
FARRP	Food Allergy Research and Resource Program, University of Nebraska
FASTA3	Algorithm used to find local high scoring alignments between a pair or protein sequences (using the AllergenOnline database)
GI	A unique identification number assigned by NCBI to each sequence in the database
PubMed	A public information database of scientific journal articles and abstracts maintained by the National Library of Medicine, National Institutes of Health (U.S.A.)
80mer	Sliding window of 80 amino acids of query protein are compared to AOL v 21 by FASTA

1.0 Introduction.

ImaginDairy has developed a recombinant food product using an Ascomycete fungal host, *Aspergillus oryzae,* which was transformed with the bovine beta-lactalbumin (BLG) gene. Bovine beta-lactoglobulin is recognized as a major milk allergen and by law in the USA or EU, foods including that protein would have to be labeled as a milk. Therefore, the question under consideration here is whether proteins from the host organism are sufficiently identical to allergenic proteins in allergenic sources and would have to be labeled or tested for potential allergic cross-reactivity for subjects with specific allergies to *Aspergillus oryzae*.

The company produced three batches of their product. The proteins were digested with trypsin and evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Wiseman Institute of Science. The company provided the RAW peptide data to me for evaluation of potential risks of allergy or allergic cross-reactivity using www.AllergenOnline.org and the NCBI Protein database. Dr. Philip Johnson of FARRP used PEAKS to evaluate the LC-MS/MS peptides of bovine beta-lactoglobulin and proteins of *Aspergillus oryzae* (rice mold) to identify source proteins and put the data of protein identity and abundance into an Excel file for me to evaluate using bioinformatics with AllergenOnline.org and the NCBI Protein database and publications.

At the onset, I realized that both of the sequence in this species that WHO/IUIS Allergen Nomenclature had named as possible allergens, have been accepted as allergens in our database (www.AllergenOnline.org) based on peer reviewed published information. Importantly, there are 30 proteins from the taxonomically related *Aspergillus fumigatus* species and other closely relatived mold species. And that FASTA by these two sequences matched many proteins with moderate to high identities even through there are no publications showing clear cross-reactivity between these species. One of the proteins is an alkaline serine protease, Asp o 13, the other is a TAKA-amylase A, FASTA matches of those proteins to all of AllergenOnline show broad sequence matches down to 35%. identity over 80 amino acids. Asp o 21. cover 100% of the length of the protein.

This study design was to take the full-set of proteins identified following HUPO guidelines (two or more peptides) reported from the LC-MS/MS analysis and search our AllergenOnline.org database with them. Identity matches would be examined and the relative concentration of the proteins from the LC-MS/MS analysis would be used to consider potential risks. Since many allergens within the database are proteins that are conserved through evolution, it is important to compare the sequences to others that are common, including human proteins and others that are from species not known to cause human allergic disease, or at least rarely cause allergies.

Comparisons in www.AllergenOnline.org used a sliding 80 amino acid window looking for minimum Identity matches of 35% or more. Additional searches were made with the NCBI Protein database using BLASTP and looking for long alignments of > 35% identity. The relative abundance of reported proteins that was considered as possibly important was >1/1,000 of the total protein content. Results and evaluation are reported here.

2. Purpose. The purpose of this study is to perform an evaluation of potential food allergy risks that might arise for consumers with existing allergies. The risks are best judged by whether the source organism is a common food allergy source, or if the protein amino acid sequence matches the identity of known allergens from protein sequences of the host organism *Bos taurus* (bovine) for milk proteins, and the ascomycete fungus, (*Aspergillus oryzae*) for any residual proteins from the transgenic host organism.

3. Methods. The primary food protein is bovine milk protein beta-lactoglobulin, which is an important milk allergen. There is no need to perform additional literature searches or bioinformatics searches of that protein other than to confirm the protein full-length identity. Foods containing this material should be labeled as containing the milk allergen. Residual proteins from the host, *Aspergillus oryzae* do need to be evaluated based on literature searches for information about allergies from that species. The LC-MS/MS data of three production lots were analyzed using PEAKS software and compared to public information about the host proteins. The full-length proteins are being evaluated based on bioinformatics, sequence searches of full-length proteins that match peptides identified by mass spectrometry at the Wiseman Institute, to www.AllergenOnline.org by FASTA sliding 80mer window. Since we know that many of the proteins of this species will have evolutionary homologues with a wide variety of proteins from various sources, we also compared the sequences to all proteins in the NCBI Protein database by BLASTP. The importance of all matches were evaluated based on information in our AllergenOnline.org database and by literature in peer reviewed publications. The LC-MS/MS data also includes relative mass abundance of the proteins from the host organism and those values are being compared to the source total protein content and to consider possible risks based on abundance.

3.1 Scientific literature search strategies. The PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy. Allergy to bovine milk beta-lactoglobulin has been known for more than 30 years with the laboratory of Hugh Sampson performing many definitive tests on IgE antibodies to the proteins (Jarvinen et al., 2001). Allergy to the protein is not a question for this product, and all foods containing this product will have to be labeled clearly to warn milk allergic consumers of the presence of milk allergens. Questions of allergy to the recombinant host (*Aspergillus oryzae*) were investigated by searching in PubMed with *Aspergillus* AND allergy that identified 3,552 references. Adding *oryzae* after *Aspergillus*, including AND allergy reduced the number to 80 references including those related to Baker's asthma, alpha amylase and a protease. When Aspergillus oryzae AND food allergy were used as search terms, only 17 results were found, most relating to baker's asthma. When the species was *Aspergillus fumigatus* AND food allergy were searched, 43 publications were found. Publications were identified. That species, *A. fumigatus*, is known to be a common environmental allergen, mostly from spores.

3.2 Amino acid sequence queries for allergenicity. The 140 FASTA sequences of the proteins identified by LC-MS/MS from the three batches of product were compared to AllergenOnline.org by sliding 80mer window, sequence matches were identified and are

presented in Appendix 2. The characteristics of the proteins are shown, including the relative abundances compared to the primary protein in the production lots (beta-lactoglobulin), The percent identity of the protein sequence compared to the sequences and the information in our AllergenOnline.org database was considered along with relative abundance. The relative intensity of BLG is 3.43E+10 and the 8th row show a match of 52% Identity to Asp f 34 a protein reported as causing Baker's asthma. The relative abundance of that protein is 4.05E+07, which can be calculated as 4.05E+7/3.43E+10 = 0.00118 or 0.118% relative abundance.

There is not a lot of information about the abundance of Asp f 34 in that impacts bakers, but a protein that is at a concentration of 0.1% relative abundance is clearly less likely to cause a reaction in someone with allergies even if it was equally as potent in allergenicity. The results of bioinformatics can be seen in the PDF of the Excel file. Matches of very high identities are evaluate further as well be discussed.

4. Results.

4.1 Literature searches. There are many peer-reviewed publications about studies of allergenicity of bovine beta-lactoglobulin. Since the protein made in *Aspergillus oryzae* is identical in sequence to that made in bovine milk, it is expected to be as allergenic as if from the cow's milk. Proteins of the host, *Aspergillus oryzae* are expected to have the same characteristics as from the wild-type fungus. As shown in the literature search in PubMed, there are reports of allergy to this fungus, but only 80 in total and not all are related to this species. There are 17 publications of food allergy to this species. One important allergen noted from a few is the alpha-amylase that was named Asp o II, but it is now known as Asp o 21 in the WHO/IUIS Allergen Nomenclature database (Baur et al 1994). Those authors used enzyme allergosorbent tests (EAST) to demonstrate IgE binding and showed that 48 of 89 Bakers with work related asthma had IgE to this protein and that the IUIS Allergen Nomenclature committee agreed to call it an allergen. The study showed that 15 subjects were Skin-Prick test positive with the baking additive material and 13 had demonstrable IgE to the protein. They did not test bioactivity with the purified protein such as basophil or mast cell activation or SPT with purified protein. An alkaline serine proteinase was also recognized as an allergen by the WHO/IUIS committee (Shen et al., 1998). Their study showed some Taiwanese asthmatic subjects had IgE antibody binding to 13 proteins from extracts of this species of fungi including the 34 kDa major protein that was given the name Asp o 13 by the IUIS committee. They did not demonstrate bioactivity, but they did show the protein is highly similar to a protein from *Penicillium citrinum* and that IgE from those subjects bound to both proteins. Searches of PubMed for the genus, Aspergillus identified at least 43 publications related to food allergy. The WHO/IUIS Nomenclature database lists 30 proteins from *Aspergills fumigatus* as allergens. And our AllergenOnline database shows many airway allergens from this genus, but only two from this species, both as are airway allergens for some bakers (Shen et al., 1998; Shen et al. 1999; Baur et al, 1994; Baur and Czuppon, 1995). The two publications from Taiwan demonstrate that a number of asthmatic patients in Taiwan are reactive to various *Penicillium* and *Aspergillus* species and that they

have IgE binding primarily to alkaline or vacuolar serine proteinases. They developed a monoclonal antibody against one of the proteins from *Penicillium sp.* That also bound proteins in four Aspergillus species proteins of 33 or 34 kDa molecular weight. The proteins were identified by N-terminal sequencing and compared to predicted protein sequences from cDNA sequences in GenBank. Subjects were tested by immunoblots but there were no allergen challenges or basophil activity. And doses and sequences of the proteins were not reported. The reports of asthma related to alpha-amylase that were reported from Austria, Switzerland and Germany. They were for bakers with asthma who were exposed to high levels of particulate amylase as a wheat bread making ingredient. Protein identities, IgE binding and in some cases skin prick The nasal resistance was reported for that patient following oral food challenges with 100 g or white bread with 10 g alpha-amylase per 100 kg bread or without alpha-amylase. By my mathematical calculation, that is approximately 10 mg of alpha-amylase (Baur and Czuppon). Note that baker's asthma is a commonly reported allergic disease that is primarily caused by inhalation of wheat flour and other ingredients released as dust during mixing. Diagnosis can be confusing because some of the proteins are similar to grass pollen proteins. Yet most bakers can eat bread products without risk of reactions to the food, but have asthma during exposure to flour and dust at work (Cianferoni, 2016). IgE mediated food allergy to wheat is relatively common in young children, but rare in adults except for those with exercise induced allergy. Non-IgE mediated allergy is more common due to T helper 2 cell activation by wheat proteins that can lead to eosinophil reactivity. There is no published evidence that proteins from *Aspergillus sp.* or from the fermentation yeast *Saccharomyces cerevisiae* used to make sour dough bread cause food allergy. In summary, there is little evidence from the scientific literature that proteins from Aspergillus are responsible for food allergy.

4.2 Bioinformatics comparisons of proteins to allergens. Results of bioinformatics searches with proteins identified from the three-production lot LC-MS/MS peptides by the University of Nebraska Core facility were compiled and evaluated by Dr. Philip Johnson using PEAKS software. The output was entered into an Excel workbook and provided to Rick Goodman for review of potential meaning for allergenicity. The most abundant protein based on relative peptide abundance was bovine lactoglobulin. The next highest abundance protein at approximately one-thirtieth the abundance of BLG was porcine trypsin, the enzyme human used to cut the proteins into peptides that could be analyzed on the mass spectrometer. FASTA matches using the sliding 80mer window identified a number of other proteins with partial matches to proteins in www.AllergenOnline including two keratin proteins that are common contaminants in proteomics laboratories. Based on relative mass abundance, Aspergillus oryzae proteins including phosphocholine and peptidase proteins identified here were less than 1:100th the abundance of BLG.

The first moderate identity match to a putative allergen was from a cell wall protein with UniProt number Q2UIT6 at 5.26% identity to asp f 34 and Asp f 7, and 37.5% identity to grass pollen allergen Phl p 5. The abundance of this host protein was just over one-one thousandth as abundant as BLG. And the Asp f 34 protein is not known to be a clear biologically active allergen. The commonality of this type of protein was evaluated by

BLASTP vs the NCBI Protein database that showed 100 fungal sequences with 52% identity. Other protein matches that are shown in the PDF of Excel results are similar and with lower relative abundances. However, a few should be discussed as the identity matches were more significant. Glucan 1,4-alpha-glucosidase matched a *Schizophyllum commune* glycoside with 68.8% identity but was low in abundance. That protein is only noted as showing IgE binding from some subjects, but no biological activity. An aspergillopepsin (Q06902) had a 70% identity match to Asp f 10 and Asp f 10 although it only had IgE binding from a few people. There were 100 fungal proteins matched at similar identities to the NCBI protein database by BLASTP.

The three highest identity matches included a catalase B protein from the host with a 96% identity over 80 AA with a 77% identity to *Penicillin* catalase. The relative abundance of that protein was less than 1:1,000 of BLG. An alpha-amylase identity matched the noted alpha amylase but the relative abundance was 1:10,000 that of BLG. Importantly no major allergens of clinical significance were found to have matches to the host proteins that remained in the three production lots based on the data in this study.

5. CONCLUSIONS

My overall conclusion from this study is that the product of Bovine beta-lactoglobulin produced as a recombinant protein in *Aspergills oryzae* does not pose a risk of food allergy due to residual *Aspergillus spp.* proteins. There is a clear risk of food allergy for those consumers who are allergic to beta-lactoglobulin in cow's milk. Therefore, any food produced using this as a protein source must be labeled to ward consumers with cow's milk allergy to avoid consumption of the product.

6.0 References

Aalberse RC. (2000). Structural biology of allergens. *J Allergy Clin Immunol* 106:228-238.

Abdelmoteleb M, Zhang C., Furey B, Kozubal M, Griffiths H, Champeaud M, Goodman RE. (2021). Evaluating potential risks of food allergy of novel food sources based on comparison of proteins predicted from genomes and compared to www.AllergenOnline.org. *Food Chem Toxicol* 147:. Doi:10.1016/j.fct.2020.111888.

Baur X, Chen Z, Sander I. (1994). Isolation and denomination of an important allergen in baking additives: alpha-amylase from *Aspertillus oryzae* (Asp o II). *Clin Exp Allergy* 24(5):465-470.

Baur X, Czuppon AB. (1995). Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. *Allergy* 50(1):85-87.

Cianferoni A. (2016). Wheat allergy : diagnosis and management. *J Ashma Allergy* 9 :13-25.

Codex Alimentarius Commission. (2003). Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June-5 64: 321-331.July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity, pp. 47-60.

Deutsch EW, Lane L, Overall CM, Bandeira N, Baker MS, Bineau C, Moritz RL, Corrales F, Orchard S, van Eyk JE, Paik Y-K, Weintraub ST, Vandenbrouck Y, Omenn GS. (2019). Human proteome project mass spectrometry data interpretation guidelines 3.0. *J Proteome Research* 18"4108-4116.

Fukutomi Y, Taniguchi M. (2015). Sensitization to fungal allergens: Resolved and unresolved issues. *Allergology International*. 321-331.

Fukutomi Y, Tanimoto H, Yasueda H, Taniguchi M. (2016). Serological diagnosis of allergic bronchopulmonary mycosis: Progression and challenges. *Allergology International* 65.:30-36.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R. (2008). Allergenicity assessment of genetically modified crops—what makes sense? *Nat Biotechnol* 26(1):73-81.

Goodman RE. (2006). Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches. *Mol Nutr Food Res* 50:655-660.

Goodman RE. (2008). Performing IgE serum testing due to bioinformatics matches in the allergenicity assessment of GM crops. *Food Chem Toxicol* 46:S24-S34

Goodman RE, Ebisawa M, Ferreira F, Sampson HA, van Ree R, Vieths S, Baumert JL, Bohle B, Latlithambika S, Wise J, Taylor S. (2016). AllergenOnline: A peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity. *Mol Nutr Food Res* 60(5):1183-1198.

Glaser AG, Kirsch AI, Zeller S, Menz G, Rhyer C, Crameri R. (2009). Molecular and immunological characterization of Asp f 34, a novel major cell wall allergen of *Aspergillus fumigatus*. *Allergy* 64(8):1144-1151.

GRAS Notice No. 829. JHeimbach LLC, (21 November 2018). Generally Recognized as Safe (GRAS) Determination for the Intended Use of Koji Germented Mineral Products (Koji Mineral). Pg 1-38.

Iglesias R, Polito L, Bortolotti M, Pedrazzi M, Citores L, Ferreras JM, Bolognesi A. (2020). Primary sequence and 3D structure prediction of the plant toxin stenodactylin. *Toxins* 12, 538: doi:10.3390/toxins12090538.

Jarvinin KM, Chatchatee P, Bardina L, Bayer K, Sampson HA. (2001). IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int Arch Allergy Immunol* 126(2):111-118.

Jin Y, Goodman RE, Tetteh AO, Lu M, Tripathi L. (2017). Bioinformatics analysis to assess potential risks of allergenicity and toxicity of HRAP and PFLP proteins in genetically modified bananas resistant to Xanthomonas wilt disease. *Food Chem Toxicol* 109(Pt 1):81-89.

Kane PM, Holowka D, Baird B. (1988). Cross-linking of IgE-receptor complexes by rigid bivalent antigens >200 Å in length triggers cellular degranulation. *J Cell Biol* 107:969-980.

Kespohl S, Raulf M. (2014). Mould allergens: Where do we stand with Molecular allergy diagnostics. *Allergo J Int.* 23:120-125.

Knol EF. (2006). Requirements for effective IgE cross-linking on mast cells and basophils. *Mol Nutr Food Res* 50(7):620-624.

Rousta N, Hellwig C, Wainaina S, Lukitawwesa Lu, Agnihotrri S, Rousta K, Taherzadey MJ. (2021) Filamentous fungus *Aspergillus oryzae* for food: from submerged cultivation to fungal burgers and their sensory evaluation – a pilot study. *Foods,* MDPI. 10(11), 2774:https://doi.org/10.3390/foods10112774.

Sewalt V, Shanahan D, Gregg L, La Marta J, Carrillo R. (2016). Review: The generally recognized as safe (GRAS) process for industrial microbial enzymes. *Industrial Biotechnology*. October 2016. 12(5):295-302.

Shen HD, Lin WL, Tam MF, Wang SR, Tsai JJ, Chou H, Han SH. (1998). Alkaline serine proteinase: a major allergen of *Aspergillus oryzae* and its cross-reactivity with *Penicillium citrinum*. *Int Arch Allergy Immunol* 116(1):29-35.

Shen HD, Tam MF, Chou H, Han SH. (1999). The importance of serine proteinases as aeroallergens associated with asthma. *Int Arch Allergy Immunol*. 119(4):259-264.

Wright TR, Shan G, Walsh TA Lira JM, Cui C, Song P, Ahuang M, Aronold NL, Lin F, Yau K, Russell SM, Cicchillo RM, Peterson MA, Simpson DM, Zhou N, Ponsamuel J, Zhang Z. (2010). Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes. *Proc Natl Acad Sci USA*. 107(47) 20240-20245.

Appendix 1: Allergenonline.org database, version 21, 14 February 2021 (43 pages)

Appendix 2: FASTA of *Aspergillus oryzae* vs AllergenOnline v 21 (10 pages)