GRAS Notice (GRN) No. 1142 with amendments
https://www.fda.gov/food/generally-recognized-safe-gras/gras-notice-inventory

April 19, 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 Oubli Fruit Sweet Protein Derived from *Komagataella phaffii*

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Oobli, Inc. [202 Cousteau Place, Suite 210, Davis, CA, 95618 USA], 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 Oubli Fruit Sweet Protein, manufactured from a genetically engineered strain of *Komagataella phaffii*, is GRAS on the basis of scientific procedures, for use as a sweetening agent in conventional food and beverage products. These food uses of Oubli Fruit Sweet Protein are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Oobli's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

I certify that 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,

DocuSigned by:
[redacted]
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Jason Ryder, Ph.D.
Chief Technology Officer
Oobli, Inc.
jason.ryder@oobli.com

GRAS NOTICE FOR OUBLI FRUIT SWEET PROTEIN DERIVED FROM *KOMAGATAELLA PHAFFII*

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:
Oobli, Inc.
202 Cousteau Place, Suite 210
Davis, CA
95618 USA

DATE:
18 April 2023

GRAS Notice for Oubli Fruit Sweet Protein Derived from *Komagataella phaffii*

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GRAS Notice for Oubli Fruit Sweet Protein Derived from *Komagataella phaffii*

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, Oobli, Inc. ("Oobli") hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of its Oubli Fruit Sweet Protein Product, derived from *Komagataella phaffii* and containing ≥35% brazzein by weight (hereinafter "Oubli Fruit Sweet Protein," or "OFSP"). The intended uses of OFSP as a sweetener 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 Oobli's view that these notified uses of OFSP are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Oobli, 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 as described in Section 6.1. Oobli considered all unfavorable as well as favorable information that is publicly available and/or known to Oobli and that is pertinent to the evaluation of the safety and GRAS status of OFSP as a food ingredient for addition to conventional food and beverage products, as described herein.

Signed,

DocuSigned by:
[redacted]
D6AD848049D24FB...

4/18/2023

Jason Ryder, Ph.D.
Chief Technology Officer
Oobli, Inc.
jason.ryder@oobli.com

Date

1.1 Name and Address of Notifier

Oobli, Inc.
202 Cousteau Place, Suite 210
Davis, CA
95618 USA

1.2 Common Name of Notified Substance

Oubli fruit sweet protein (OFSP)

Oubli fruit sweet protein

Oubli fruit sweet protein product

Oubli fruit sweet protein ingredient

1.3 Conditions of Use

Oobli's OFSP is intended for use as a sweetening agent in various conventional foods and beverages. A summary of the food categories and use levels in which OFSP is intended for use is provided in Table 1.3-1 below. Food uses are organized according to 21 CFR §170.3 (U.S. FDA, 2022a).

OFSP is not intended for use in infant formula or infant food products, and the proposed food categories do not include food uses that are subject to the oversight by the United States Department of Agriculture (USDA) and the USDA Food Safety Inspection Service.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for OFSP in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2022a)	Food Uses[a]	OFSP Use Levels (mg/100 g)
Beverages, alcoholic	Cocktail drinks (pre-packaged)	7
Beverages and Beverages Bases, non-alcoholic	Packaged water-based beverages	22
	Non-milk-based meal replacement beverages and protein drinks	2
Chewing Gum	Chewing gum	99
Coffee and Tea	Ready-to-drink coffee beverages	7
	Ready-to-drink tea beverages	9
Dairy Product Analogs	Milk analogs	4
	Non-dairy yogurts	13
Frozen Dairy Desserts and Mixes	Ice cream	23
	Frozen yogurt	23
	Frozen milk desserts and bars	24
Fruit and Water Ices	Edible ices	24
	Sherbet	30
	Sorbet	31
Grain Products and Pastas	Cereal bars, granola bars, energy, protein, and meal replacement bars	30
	Granola	27
Milk Products	Packaged milk-based beverages	6
	Yogurt	12
	Yogurt drinks	9
Processed Fruits and Fruit Juices	Packaged fruit drinks, nectar, and fruit-based smoothies	16
Snack Foods	Fruit-based bars (without granola)	36
Soft Candy	Truffles	47
	Gummy bear	62

CFR = *Code of Federal Regulations*; OFSP = Oubli Fruit Sweet Protein.
[a] OFSP is intended for use in unstandardized products and products with standards of identity, as established under 21 CFR §130 to 169, do permit its addition.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) (U.S. FDA, 2022b), Oobli has concluded that the intended uses of OFSP 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:

Oobli, Inc.
202 Cousteau Place, Suite 210
Davis, CA
95618 USA

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

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

It is Oobli'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*, 5 U.S.C. 552.

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

2.1 Identity of the Ingredient

2.1.1 Oubli Fruit Sweet Protein

OFSP is produced through a strain of *K. phaffii* that has been genetically engineered to express a gene encoding for brazzein-53; brazzein is the component of OFSP that provides the ingredient's sweetening properties and is discussed in greater detail under Section 2.1.2 below. OFSP is comprised of primarily protein (*ca.* 80%) which contains the active constituent brazzein (*ca.* 40%) with remaining balance of host cell proteins carried over from the fermentation process of *K. phaffii* (*ca.* 40%), followed by ash (*ca.* 5%), moisture (*ca.* 5%), carbohydrates (*ca.* 10%), and fat (*ca.* 0.3%).

A proteomics assessment of OFSP was performed to investigate the identity of the host cell proteins originated from the production organism. Protein solutions (1 mg/mL) were prepared from three production batches of OFSP (Lot Nos. OFSPB53-304, OFSPB53-215, and OFSPB53-538), digested by trypsin, and analyzed using liquid chromatography with tandem mass spectrometry (LC- MS/MS), where peptides were then reassembled into the complete protein sequence. The database search tool MSFragger was used for peptide identification using the brazzein 53-amino acid sequence and the *K. phaffii* proteome data for brazzein protein identification and host cell protein identification, respectively. The results were returned using Scaffold Proteome Software. Data were reported on the basis of total spectral count, defined as the total number of spectra identified for a protein, yielding a semiquantitative measure of protein abundance in proteomic studies. An approximate 1% spectra count cut-off criteria was used for identifying host cell proteins in the final product. As shown in Table 2.1.1-1, five host cell proteins were identified that met this 1% cut-off value in the proteomics analysis, accounting for up to 13.2% of the total protein fraction of OFSP. The remaining total protein fraction are comprised of residual host cell proteins that were below the 1% cut-off value. Of the total protein in OFSP, the average brazzein content was reported to be 46.78% of the total spectrum, with the balance of 53.22% to be residual host cell proteins derived from the *K. phaffii* production strain. The complete list of residual host cell proteins and their average values in the three production batches of OFSP is provided in Appendix A.

Table 2.1.1-1 Summary of the Abundance of Residual *Komagataella* Proteins in OFSP

Identified Protein	Accession Number	Protein Length (amino acids)	Manufacturing Lot No.			Average % of total spectrum)
			OFSPB53-304 (% of total spectrum)	OFSPB53-215 (% of total spectrum)	OFSPB53-538 (% of total spectrum)	
Brazzein protein	P56552	53	50.04	39.02	51.29	46.78
Fusion protein, identical to Rpl40Bp OS=Komagataella phaffii (strain GS115 / ATCC 20864) OX=644223 GN=PAS_chr2-1_0486 PE=4 SV=1	C4R0U2	128	6.06	5.87	6.78	6.23
Transaldolase OS=Komagataella phaffii (strain GS115 / ATCC 20864) OX=644223 GN=PAS_chr2-2_0337 PE=3 SV=1	C4R245	324	2.86	3.25	3.34	3.15

Table 2.1.1-1 Summary of the Abundance of Residual *Komagataella* Proteins in OFSP

Identified Protein	Accession Number	Protein Length (amino acids)	Manufacturing Lot No.			Average % of total spectrum)
			OFSPB53-304 (% of total spectrum)	OFSPB53-215 (% of total spectrum)	OFSPB53-538 (% of total spectrum)	
Vacuolar aspartyl protease (Proteinase A) OS=Komagataella phaffii (strain GS115 / ATCC 20864) OX=644223 GN=PAS_chr3_1087 PE=3 SV=1	C4R6G8	410	1.77	1.19	1.8	1.59
Peptide hydrolase OS=Komagataella phaffii (strain GS115 / ATCC 20864) OX=644223 GN=PAS_chr1-4_0611 PE=3 SV=1	C4QYZ6	509	1.18	0.95	1.54	1.22
S-adenosylmethionine synthase OS=Komagataella phaffii (strain GS115 / ATCC 20864) OX=644223 GN=PAS_chr3_0876 PE=3 SV=1	C4R5U7	384	0.42	1.67	0.94	1.01

OFSP = Oubli Fruit Sweet Protein.

2.1.2 Brazzein

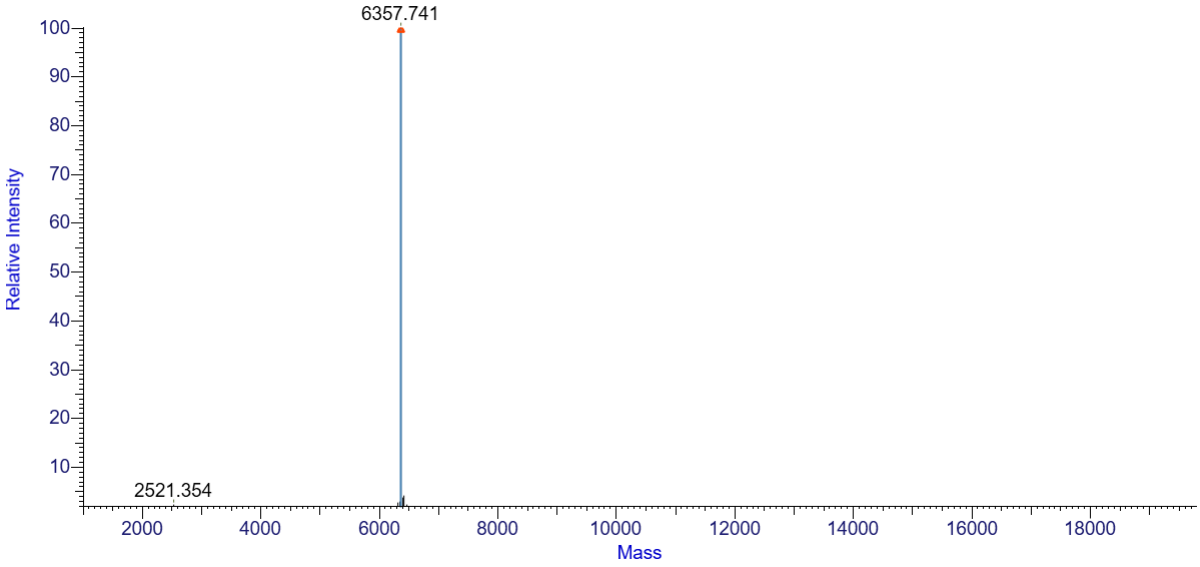
Brazzein protein is the characterizing component of OFSP. Brazzein is naturally present in the fruit of the West African plant *Pentadiplandra brazzeana* Baillon, commonly referred to as oubli fruit (Caldwell *et al*., 1998). Natural brazzein has been found to occur in the oubli fruit in 2 isoforms: major isoform with 54 amino acids (~80%) and minor isoform with 53 amino acids (~20%) (Neiers *et al*., 2021). The amino acid sequence of the major isoform (referred to as "brazzein-54") is listed in the UniProt database under Accession No. P56552. Sensory analyses have revealed that the minor isoform (referred to as "brazzein-53") is sweeter than the major form (*i.e.*, brazzein-54) (Poirer *et al*., 2012). The brazzein characterizing the OFSP is the minor isoform (brazzein-53), which has a monoisotopic mass of 6,357.734 Da in its native conformation. The amino acid sequence of Oobli's brazzein-53 is shown below.

DKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY

The identity of the active brazzein constituent of OFSP has been demonstrated *via* tandem mass spectrometry (MS/MS) peptide mapping and intact protein mass spectrometry (IPMS). As shown in Figure 2.1.2-1, the peptide map of Oobli's brazzein-53, obtained by tryptic digest peptide mapping, demonstrates a high degree of sequence coverage (~96%) with 15 exclusive unique peptides matching the amino acid sequence to native, plant-based brazzein-53 sequence. The IPMS-measured mass of Oobli's brazzein-53 is 6,357.741 Da and can be matched to the calculated mass of 6,357.734 Da for brazzein-53 in its native conformation (see Figure 2.1.2-2). These data indicate that brazzein-53 produced by Oobli is substantially equivalent to native brazzein.

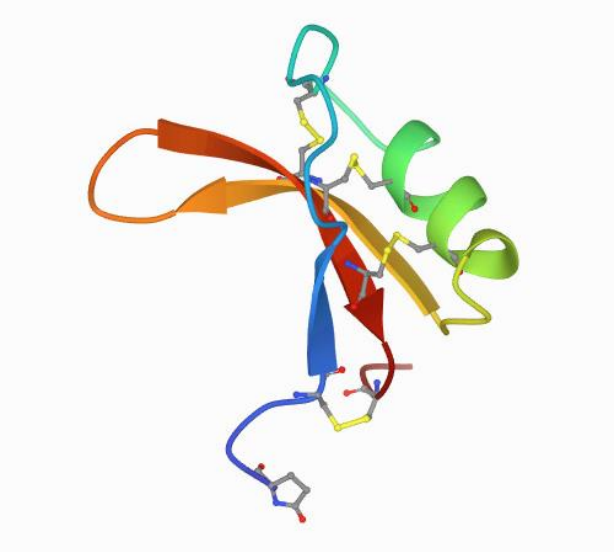
Figure 2.1.2-1 Sequence Coverage from Tandem Mass Spectrometry Peptide Mapping Analysis of Oobli's Brazzein-53

Figure 2.1.2-2 Deconvoluted MS1 Spectrum of Oobli's Brazzein-53



The three-dimensional (3D) structure of brazzein in solution was determined by proton nuclear magnetic resonance spectroscopy at pH 5.2 and 22°C (Caldwell *et al*., 1998). The 3D structure of brazzein is shown in Figure 2.1.2-3 (Caldwell *et al*., 1998). The protein contains 1 α-helix, 3 strands of antiparallel β-sheet, and 4 disulfide bonds, which confer stability to the chemical structure. The protein structure of brazzein is unique such that it does not share any similarity to other sweet-tasting proteins with known structures, including monellin and thaumatin (Caldwell *et al*., 1998; Picone and Temussi, 2012).

Figure 2.1.2-3 Three-Dimensional Structure of Brazzein



2.2 Manufacturing Process

2.2.1 Description of the Manufacturing Process

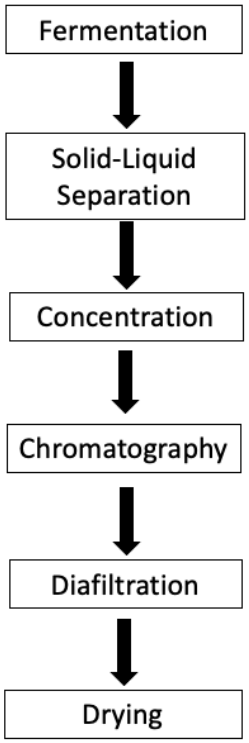
The manufacturing process of OFSP complies with current Good Manufacturing Practice (cGMP) and Hazard Analysis and Critical Control Points (HACCP) principles. A schematic of the production process is provided in Figure 2.2.1-1 and is described below.

The manufacturing process for OFSP uses precision fermentation to produce the naturally sweet brazzein protein. As a first step, inoculum is prepared from working cell banks using multiple seed propagation steps. Next, the resulting bolus of cells is used to inoculate the production fermentation process. Both the seed propagation and production fermentation steps are monitored for cell growth, culture purity, protein titer, glucose, nitrogen, phosphate, and ethanol. Process variables including pH, temperature, and dissolved oxygen are monitored and controlled throughout the precision fermentation process.

Upon reaching the end of fermentation, the whole cell broth is processed to recover, separate, and purify OFSP. First, a solid–liquid separation process separates the wet biomass from the fermentation supernatant containing brazzein. This supernatant is then concentrated, pH-adjusted, and further purified using a sequence of filtration, chromatography, and diafiltration steps. The resulting concentrated OFSP solution is then dried into a powder consisting of at least 35% (w/w) brazzein in addition to sodium salts used as stabilizing agents from the purification process.

All raw materials and processing aids, filtration aids, and pH adjusters used in the fermentation and recovery processes for OFSP are safe and suitable standard ingredients that meet predefined quality standards used in the food/enzyme industry. The raw materials conform to either the specifications set out in the *Food Chemicals Codex* (11th edition) or to other applicable regulatory standards. The raw materials are food-grade, of high purity and quality (Aunstrup *et al*., 1979), and suitable for their intended use, *i.e.*, they are either food-grade and GRAS or of high-quality chemical or pharmaceutical grades (*United States Pharmacopeia* [USP], *National Formulary* [NF], or American Chemical Society [ACS] grades) from approved suppliers.

Figure 2.2.1-1 Manufacturing Process for OFSP



OFSP = Oubli Fruit Sweet Protein.

2.2.2 Information on the Production Microorganism

2.2.2.1 Parental (Host) Organism

The parental (host) organism of OFSP is *K. phaffii*. Current laboratory strains of *K. phaffii* are from lineages isolated from oak and chestnut trees and have been deposited in the culture collection at the Northern Regional Research Laboratories (NRRL). The genome of *K. phaffii* was sequenced in 2009 (De Schutter *et al*., 2009) and has been extensively employed in the production of food ingredients and pharmaceutical products (Ahmad *et al*., 2014). It is estimated that approximately 17% of total recombinant products produced in 2009 were obtained from *K. phaffii* production organisms. The available evidence demonstrates that *K. phaffii* is a well characterized microorganism with an established history of safe use in food production. A summary of GRAS Notices pertaining to food ingredients obtained through fermentation of *K. phaffii* that were filed without objection from the U.S. FDA is provided in Table 2.2.2.1-1 below.

Table 2.2.2.1-1 Summary of GRAS Notices for Food Ingredients Produced Through Fermentation of *Komagataella phaffii*

GRN No.	Substance	Intended Use
737	Soy leghemoglobin preparation from a strain of *Pichia pastoris*	For use at levels up to 0.8% soybean leghemoglobin protein to optimize flavor in ground beef analogue products intended to be cooked.
967	Soluble egg-white protein produced by *Komagataella phaffii* strain GSD-1209	Intended for use as a substitute for egg-white protein in foods containing eggs; and as a source of protein in nutritional powders and drinks; bars; and certain snack foods at levels in accordance with current good manufacturing practices (excluding infant formula, or in any products under the jurisdiction of the United States Department of Agriculture).
1001	Myoglobin preparation from a strain of *Pichia pastoris* expressing the myoglobin gene from *Bos taurus*	Intended to impart flavor and aroma at levels up to 2% myoglobin in ground meat and poultry analogue products.

The safe strain lineage of *K. phaffii* NRRL Y-11430 is largely based on the recognized non-pathogenic and non-toxigenic nature of this strain and the fact that the species itself has not been implicated with any known adverse effects throughout its extensive use in food production. In addition, proteins originating from a production strain derived from *K. phaffii* NRRL Y-11430 have been demonstrated to lack allergenic and toxigenic potential (Jin *et al*., 2018; Reyes *et al*., 2021). Based on the Joint FAO/WHO Expert Committee on Food Additives (JECFA) criteria for safe strain lineage, *K. phaffii* NRRL Y-11430 has been considered to be a safe strain lineage suitable to serve as a host organism for food production (FAO/WHO, 2020). As summarized in Table 2.2.2.1-1, a number of food ingredients, including soy leghemoglobin, bovine myoglobin, and hen egg ovomucoid, produced using *K. phaffii* NRRL Y-11430 as a host organism currently have GRAS status under GRAS Notice (GRN) 737, 1001, and 967, respectively (U.S. FDA 2018, 2021a,b).

OFSP is produced by precision fermentation using a strain of *K. phaffii* that has been genetically engineered to express genes encoding for the biosynthesis of brazzein. The production strain is derived from *K. phaffii* BG10, which originates from the well-characterized host organism *K. phaffii* NRRL Y-11430. The taxonomic identity of *K. phaffii* is shown in Table 2.2.2.1-2 below. The host organism, *K. phaffii* BG10, has been demonstrated to be genomically similar to *K. phaffii* NRRL Y-11430 and does not contain any native plasmids or antibiotic resistance genes.

Table 2.2.2.1-2 Taxonomic Identity of *Komagataella phaffii*

Kingdom	Fungi
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Phaffomycetaceae
Genus	*Komagataella*
Species	*phaffii*

2.2.2.2 Construction of the Production Organism

The production strain for OFSP was derived from *K. phaffii* BG10 through a series of transformations with different expression constructs to enable the biosynthesis of brazzein. The promoter and terminator within the expression cassette are native sequences of *K. phaffii*. The gene encoding for brazzein was synthesized *de novo* and codon-optimized from *Pentadiplandra brazzeana* (UniProtKB P56552). Brazzein expression cassettes were stably integrated into the genome, where no plasmids or antibiotic resistance genes were present in the production strain. Therefore, no plasmids or antibiotic resistance genes were expected to be transferred to non-related microorganisms or the final product. All gene sequences were assembled into a cloning plasmid, where the expression cassette was transformed into *K. phaffii*. The plasmid was selected using zeocin, geneticin, or hygromycin. The plasmid was cured by re-streaking the colony 3 times and was confirmed by negative selection on both selection and non-selection plates, as well as validated by colony polymerase chain reaction (PCR). A glycerol stock of the production strain and wild-type strain was streaked on yeast extract peptone dextrose (YPD) agar plates with the antibiotics hygromycin, zeocin, and geneticin, with YPD agar plates without antibiotics used as a positive control. On the plates with the selection markers, there was no indication of growth of the production strain or wild-type strain (see Figure 2.2.2.2-1), indicating that the strain does not contain any plasmids with selective genes. The absence of plasmids was also confirmed with colony PCR using primers to amplify the selective genes used for plasmid selection (*i.e.*, encoding for resistance to hygromycin, geneticin, or zeocin (see Figure 2.2.2.2-2).

Furthermore, as the introduced genes were obtained *de novo* by DNA synthesis and were not derived directly from the source organism, there was no risk of introducing unintended or undesirable genes from the source organism into the production strain. The genetic stability of the production strain was confirmed by Sanger sequencing at the beginning and end of the fermentation process. Additionally, bioinformatics searches conducted using the amino acid sequence of brazzein encoded by the inserted gene confirm that there are no significant sequence homology to known protein toxins or to known allergens (see Section 6.3.4 for further details).

Figure 2.2.2.2-1 Streaked Yeast Extract Peptone Dextrose Plates with Hygromycin, Zeocin, or Geneticin Selection Markers of the Production and Wild-type *Komagataella phaffii* Strain

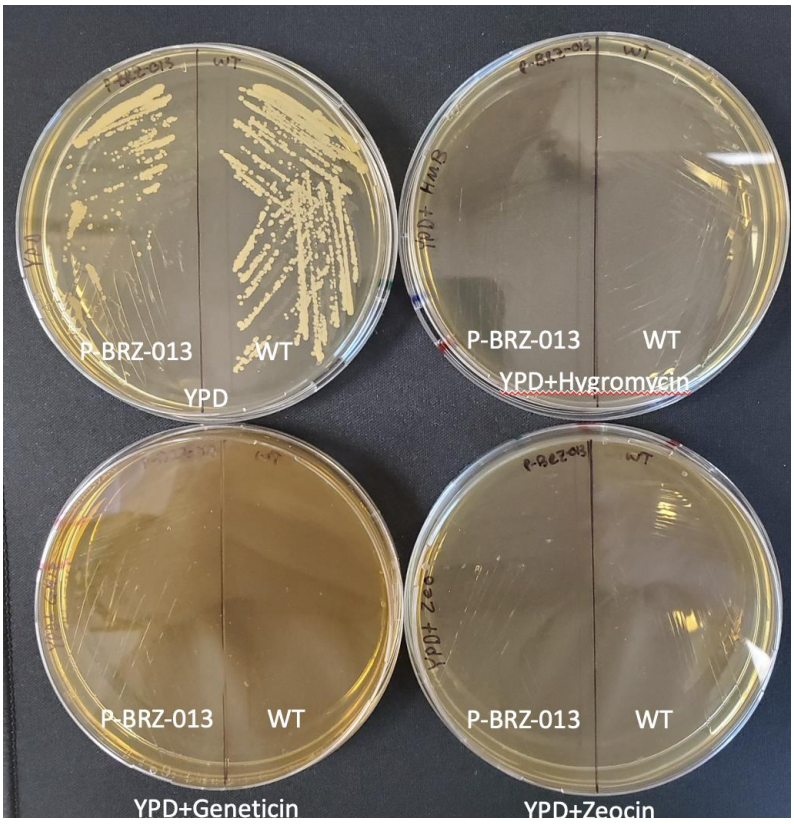
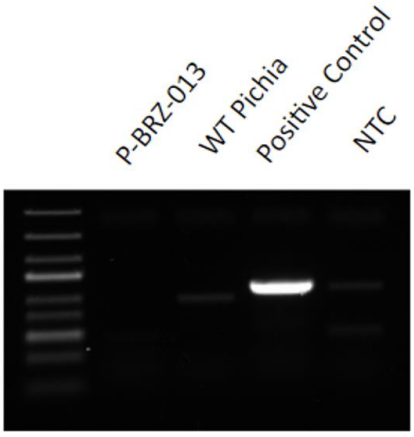


Figure 2.2.2.2-2 Colony Polymerase Chain Reaction Results of the Production Strain



2.3 Product Specifications and Batch Analyses

2.3.1 Product Specifications

Food-grade product specifications have been established for OFSP. Specification limits have been established for the proximate parameters (*e.g.*, moisture, total protein, fat, ash, and carbohydrates), as well as for brazzein as a percentage of the total mass of the ingredient as well as the percentage of the total protein, heavy metals (arsenic, cadmium, lead, mercury), and microbiological contaminants. The methods of analysis for each parameter follow internationally recognized methods (*e.g.*, Association of Official Analytical Collaboration [AOAC]). The brazzein content is measured using a validated internal method based on high-performance liquid chromatography. See Table 2.3.1-1 for details.

Table 2.3.1-1 Product Specifications for OFSP

Specification Parameter	Specification Limit	Method of Analysis
Moisture (% w/w)	<10%	AOAC 950.46
Total protein (% w/w)	>70%	AOAC 950.23
Brazzein purity (% of total protein)	>40%	Calculation (brazzein mass/total protein)
Total brazzein (% total mass w/w)	>35%	Internal method (HPLC)
Fat by fatty acid profile (%)	<1%	AOAC 996.06
Ash[a] (% w/w)	<10%	AOAC 945.46
Carbohydrates (% w/w)	<15%	Calculated
Heavy Metals		
Arsenic	<0.5 ppm	AOAC 2015.01
Cadmium	<0.5 ppm	AOAC 2015.01
Lead	<0.5 ppm	AOAC 2015.01
Mercury	<0.5 ppm	AOAC 2015.01
Microbiological Contaminants		
Aerobic plate count	<10,000 CFU/g	AOAC OMA
Total coliforms	<10 CFU/g	AOAC OMA
Escherichia coli	<10 CFU/g	AOAC OMA
Yeast	<10 CFU/g	AOAC OMA
Mold	<10 CFU/g	AOAC OMA
Salmonella spp.	Not detected in 10 g	AOAC OMA
Listeria	Not detected in 10 g	AOAC OMA

AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; HPLC = high-performance liquid chromatography; OFSP = Oubli Fruit Sweet Protein; OMA = Official Methods of Analysis; ppm = parts per million.
[a] The ash content of the ingredient is composed of primarily sodium and lesser amounts of other minerals.

2.3.2 Batch Analysis

Analysis of three non-consecutive production batches of OFSP demonstrates that the manufacturing process as described in Section 2.2 produces a consistent product that meets the established specification limits (see Table 2.3.2-1).

Table 2.3.2-1 Summary of the Batch Analysis for 3 Production Batches of OFSP

Specification Parameter	Specification Limit	Manufacturing Lot		
		OFSPB53-304	OFSPB53-231	OFSPB53-061
Moisture (% w/w)	<10%	2.82	2.29	2.53
Total protein (% w/w)	>70%	87.47	88.43	88.5
Brazzein Purity (% of total protein)	>40%	57.2	44.8	48.2
Total Brazzein (% total mass w/w)	>35%	50.1	39.7	42.7
Fat by fatty acid profile (%)	<1%	0.12	0.03	0.76
Ash (% w/w)	<10%	7.25	4.73	4.79
Carbohydrates (% w/w)	<15%	2.34	4.52	3.42
Heavy Metals				
Arsenic (ppm)	<0.5 ppm	0.03	0.10	0.11
Cadmium (ppm)	<0.5 ppm	0.02	0.006	<0.001
Lead (ppm)	<0.5 ppm	0.05	0.01	<0.01
Mercury (ppm)	<0.5 ppm	0.047	0.041	<0.005
Microbiological Contaminants				
Aerobic plate count (CFU/g)	<10,000 CFU/g	190	330	40
Coliforms (CFU/g)	<10 CFU/g	<10	<10	<10
Escherichia coli (CFU/g)	<10 CFU/g	<10	<10	<10
Yeast (CFU/g)	<10 CFU/g	<10	<10	<10
Mold (CFU/g)	<10 CFU/g	<10	<10	<10
Salmonella spp.	Not detected in 10 g	ND	ND	ND
Listeria	Not detected in 10 g	ND	ND	ND

CFU = colony-forming units; ND = not detected; OFSP = Oubli Fruit Sweet Protein; ppm = parts per million.
Note: <10 = less than the reporting limit as noted.

2.3.3 Mineral Profile

The mineral profile of three batches of OFSP (Lot Nos. OFSPB53-304, OFSPB53-231, and OFSPB53-061) was determined using AOAC 2015.01. OFSP contains, on average, approximately 17,700 parts per million (ppm) sodium, 2,408 ppm magnesium, 151 ppm manganese, 151 ppm iron, 1,560 ppm calcium, 108 ppm zinc, 73 ppm copper, 1 ppm molybdenum, 0.6 ppm nickel, 83 ppm phosphorus, and 10 ppm potassium. Based on the proposed food uses of the OFSP, the resulting dietary exposures to these minerals in final consumers are not expected to pose a safety concern (see Section 3.1.3).

2.4 Stability

The shelf-life stability of OFSP was investigated under ambient temperature at 23.5°C and 40% relative humidity. OFSP powder (Lot No. OFSPB53-101) was heat-sealed in mylar foil bags at room temperature and stored for up to 12 months. Samples were taken at 0, 4, 6, 9, 12, and 15 months for analysis of aerobic plate count, coliforms, *Escherichia coli*, yeast, mold, *Salmonella* spp., *Listeria*, as well as moisture, total protein, fat by fatty acid, ash, and carbohydrates. As shown in Table 2.4-1, brazzein content of Oobli's ingredient remained stable over the storage periods, with no significant changes in any other measured parameter, including the proximate and microbiological profile, indicating that the shelf-life of OFSP is at least 15 months. There were no significant changes in the brazzein content of the ingredient over the 15-month storage period.

Table 2.4-1 Summary of the Analysis for Proximates and Microbiological Parameters of OFSP Following Storage for 15 Months at Ambient Conditions (Temperature: 23.5°C, Relative Humidity: 40%)

Parameter	Specification Limit	Timepoint					
		0 Months	4 Months	6 Months	9 Months	12 Months	15 Months
Moisture (% w/w)	<10%	7.33	7.74	8.28	8.94	8.45	7.21
Total protein (% w/w)	>70%	91.3	88.47	87.74	87.16	88.00	90.63
Brazzein purity (% of total protein)	>40%	60.2	NM	NM	63.1	NM	61.7
Total brazzein (% total mass w/w)	>35%	55	NM	NM	55	NM	56
Fat by fatty acid profile (g/100 g)	<1%	0.09	0.1	0.02	0.02	0.02	0.03
Ash (% w/w)	<10%	3.09	3.1	3.06	3.27	3.34	3.59
Carbohydrates (% w/w)	<15%	<0.1	0.59	0.9	0.61	0.19	<0.1
Aerobic plate count (CFU/g)	<10,000 CFU/g	5,400	4,700	2,500	8,500	1,900	1,600
Total coliforms (CFU/g)	<10 CFU/g	<10	<10	<10	<10	<10	<10
Escherichia coli (CFU/g)	<10 CFU/g	<10	<10	<10	<10	<10	<10
Yeast (CFU/g)	<10 CFU/g	<10	50	<10	<10	<10	<10
Mold (CFU/g)	<10 CFU/g	<10	<10	290	<10	<10	<10
Salmonella spp. (/10 g)	Not detected in 10 g	ND	ND	ND	ND	ND	ND
Listeria (/10 g)	Not detected in 10 g	ND	ND	ND	ND	ND	ND

CFU = colony-forming units; ND = not detected; NM = not measured; OFSP = Oubli Fruit Sweet Protein.

2.5 Technical Effect

2.5.1 Mechanism of Action of Brazzein

The protein characterizing the OFSP is the 53-amino acid brazzein, which is comprised of 1 α-helix (21–29), 3 strands of antiparallel β-sheet (strand I: 5–7; strand II: 44–50; strand III: 34–39), and 4 disulfide bonds (see Figure 2.1.2-3). It has been previously reported that brazzein binds 536–545 residues of T1R3 (Jiang *et al*., 2004). However, recent studies using *in silico* docking modeling have demonstrated that the primary binding of brazzein is to the G protein-coupled receptor (GPCR), T1R2, and stabilized by T1R3 (Walters and Hellekant, 2006; Belloir *et al*., 2017). These taste receptors are composed of the Venus flytrap motif (VFTM), cysteine-rich domain, and transmembrane domain, and are present in both oral and extraoral tissues, such as in the gastrointestinal tissue (Walters and Hellekant, 2006; Laffitte *et al*., 2014; Belloir *et al.*, 2017; Kim *et*

al., 2022). Binding to GPCR yield a taste signal through the GPCR signal transmission mechanism (Kim *et al*., 2022). Using high-resolution structure data and models, Kim *et al*. (2022) demonstrated that brazzein binds between the cysteine-rich domain of T1R2 and T1R3 to form a heterocomplex to elicit a sweet taste response.

The binding mechanism of brazzein to T1R2 and T1R3 at the cysteine-rich domain is similar to other known sweet proteins such as thaumatin and monellin, despite the differences in their protein structure (Caldwell *et al*., 1998; Kim *et al*., 2022). Other commonly consumed small molecule sweetening agents, such as sucrose, aspartame, neotame, saccharin, cyclamate, and sucralose, also bind to the T1R2 and T1R3 receptors, although on different regions, such as the pocket cavity of the VFTM of T1R2 and T1R3 (sucrose), amino terminal domain of T1R2 (aspartame, neotame, saccharin), transmembrane region of T1R3 (cyclamate), and amino terminal domain of both T1R2/T1R3 (sucralose) (Kim *et al*., 2022). Laffitte *et al*. (2014) reported that the sweet taste receptors, T1R2 and T1R3, expressed on enteroendocrine cells in the gastrointestinal (GI) tract, especially in the small intestine and colon (Kojima and Nakagawa, 2011), are involved in glucose sensing, expression of glucose transporters, and maintenance of glucose homeostasis. However, as discussed in Sections 6.3.1 and 6.3.3, brazzein is expected to denature under the highly acidic conditions of the stomach, thereby losing both its characteristic 3D protein structure as well as its ability to bind to the T1R2 and T1R3 receptors encountered within the GI tract. In addition, as discussed in Section 6.3.1.2 of *in silico* digestibility, the primary protein sequence is expected to be readily broken down into smaller peptides of less than 11 amino acids in length, which would even further reduce or eliminate its ability to bind to or stimulate these receptors. In summary, while brazzein is able to maintain its characteristic 3D structure and elicit a sweet response in the more favorable conditions within the mouth, it is likely both denatured and degraded under the much less favorable conditions within the GI tract, thus rendering both protein and resulting peptides unable to bind or elicit a biological response. Furthermore, when compared against sugars and other small molecule sweeteners which continue to stimulate T1R2 and T1R3 receptors while traveling through the GI tract, the consumption of brazzein as a sweetening agent represents a potential net reduction in overall stimulation of these receptors and any associated negative feedback loops.

2.5.2 Sensory Studies on Oubli Fruit Sweet Protein

Oobli intends to market OFSP, as a sweetening agent in conventional food and beverage products. As reported in the scientific literature, brazzein has a sweetness potency between 500 and 2,000 times that of a 10% or 2% sucrose solution, respectively (Izawa *et al.*, 1996). The sweet taste of brazzein remains stable over pH 2.5 to 8 and upon heating up to 80°C for 4.5 hours or 98°C for 2 hours (Stone and Oliver, 1969). Oobli has determined the relative sweetness intensity of OFSP against a 5% sucrose solution, which has a designated sweetness intensity of 100. Five concentrations of OFSP (brazzein purity: 79%), *i.e.*, 0.001, 0.003, 0.005, 0.007, and 0.01%, were prepared and given to a trained panel (N=8). Based on the results of this study, Oobli's OFSP was determined to have a sweetness intensity of 750 times that of a 5% sucrose solution.

PART 3. §170.235 DIETARY EXPOSURE

3.1 Estimated Dietary Consumption of Oubli Fruit Sweet Protein

3.1.1 Methodology

An assessment of the anticipated intake of OFSP as an ingredient under the intended conditions of use was conducted using data available in the 2017-2018 cycle of the U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) (CDC, 2022a,b; USDA, 2022). Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of OFSP by the U.S. population. Estimates for the daily intake of OFSP represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018; these average amounts comprised the distribution from which mean and percentile intake estimates were determined. Mean and percentile estimates were generated incorporating survey weights in order to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of OFSP averaged over all individuals surveyed, regardless of whether they consumed food products in which OFSP is proposed for use, and therefore includes individuals with "zero" intakes (*i.e*., those who reported no intake of food products containing OFSP during the 2 survey days). "Consumer-only" intake refers to the estimated intake of OFSP by those individuals who reported consuming food products in which the use of OFSP is currently under consideration. Individuals were considered "consumers" if they reported consumption of 1 or more food products in which OFSP is proposed for use on either Day 1 or Day 2 of the survey.

The estimates for the intake of OFSP were generated using the maximum use level indicated for each intended food use, as presented in Table 1.3-1, together with food consumption data available from the 2017-2018 NHANES datasets. The results for these assessments are presented in Section 3.1.2.

3.1.2 Intake Estimates for Oubli Fruit Sweet Protein

A summary of the estimated daily intake of OFSP from proposed food uses is provided in Table 3.1.2-1 on an absolute basis (mg/person/day) and in Table 3.1.2-2 on a body weight basis (mg/kg body weight/day).

The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 83.8% of the population groups consisted of consumers of food products in which OFSP is currently proposed for use (see Table 3.1.2-1). Children 6 to 11 years old had the greatest proportion of consumers, at 95.7%. The consumer-only estimates are more relevant to risk assessments, as they represent exposures in the target population. Only the consumer-only intake results are discussed herein.

Among the total population (2 years and older), the mean and 90th percentile consumer-only intakes of OFSP were determined to be 89 and 199 mg/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of OFSP on an absolute basis, at 119 and 260 mg/person/day, respectively, while children 2 to 5 years old had the lowest mean and 90th percentile consumer-only intakes, at 35 and 76 mg/person/day, respectively (see Table 3.1.2-1).

Table 3.1.2-1 Summary of the Estimated Daily Intake of OFSP from Proposed Food Uses in the United States by Population Group (2017-2018 NHANES Data)

Population Group	Age Group (Years)	*Per Capita* Intake (mg/day)		Consumer-Only Intake (mg/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Children	2 to 5	32	70	92.0	412	35	76
Children	6 to 11	54	115	95.7	642	56	117
Female teenagers	12 to 19	64	137	91.4	403	71	143
Male teenagers	12 to 19	88	174	91.8	394	95	183
Female adults	20 and older	65	162	83.8	1,793	78	172
Male adults	20 and older	102	252	85.2	1,660	119	260
Total population	2 and older	77	181	86.5	5,304	89	199

n = sample size; NHANES = National Health and Nutrition Examination Survey; OFSP = Oubli Fruit Sweet Protein.

On a body weight basis, the total population (2 years and older) mean and 90th percentile consumer-only intakes of OFSP were determined to be 1.30 and 2.88 mg/kg body weight/day, respectively. Among the individual population groups, children 2 to 5 years old were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, at 2.03 and 4.25 mg/kg body weight/day, respectively. Female adults had the lowest mean and 90th percentile consumer-only intakes, at 1.05 and 2.21 mg/kg body weight/day, respectively (see Table 3.1.2-2).

Table 3.1.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of OFSP from Proposed Food Uses in the United States by Population Group (2017-2018 NHANES Data)

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Children	2 to 5	1.87	4.20	92.4	408	2.03	4.25
Children	6 to 11	1.64	3.41	95.7	640	1.72	3.43
Female teenagers	12 to 19	1.06	2.18	91.5	397	1.16	2.25
Male teenagers	12 to 19	1.37	2.72	91.7	391	1.50	2.96
Female adults	20 and older	0.88	2.09	83.8	1,779	1.05	2.21
Male adults	20 and older	1.13	2.82	85.1	1,647	1.33	3.11
Total population	2 and older	1.12	2.63	86.5	5,262	1.30	2.88

bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; OFSP = Oubli Fruit Sweet Protein.

3.1.3 Dietary Intake Estimates of Minerals from Oubli Fruit Sweet Protein

As discussed in Section 2.3.3, OFSP contains measurable amounts of minerals such as sodium, magnesium, manganese, iron, calcium, and zinc. The dietary intakes of the minerals present in OFSP were estimated based on the highest estimated intake of 260 mg/day of OFSP by male adults (90th percentile). As shown in Table 3.1.3-1 below, the estimated intakes of minerals present in the OFSP based on its proposed conditions of use are well below the respective upper limit as established by the National Academies of Sciences, Engineering, and Medicine (NASEM) [formerly Institute of Medicine (IOM)]. For sodium, the proposed uses of OFSP contributes up to 5 mg/day to the American diet, which is well below the chronic disease risk reduction intake established by the NASEM, which sets the limit for sodium for individuals 14 years and older at 2,300 mg/day. For the other minerals, the contribution of these minerals to the diet based on the proposed food uses of OFSP are negligible and therefore, it is not anticipated that dietary consumption of the minerals present in OFSP would pose any safety concern.

Table 3.1.3-1 Comparison of Mineral Intake from OFSP and Corresponding Tolerable Upper Limit Values as Established by NASEM

Mineral	Average Mineral Composition of OFSP (mg/kg)	Mineral Intake from OFSP (mg/day)[a]	UL (mg/day)[b]
Calcium	1,560	0.41	2,500
Copper	73	0.02	10,000
Iron	151	0.04	45
Magnesium	2,408	0.63	350
Manganese	151	0.039	11
Molybdenum	1.1	0.0003	2,000
Nickel	0.59	0.0002	1.0
Phosphorus	83	0.02	4,000
Potassium	10	0.003	ND
Sodium	17,700	4.6	ND
Zinc	108	0.03	40

NASEM = National Academies of Sciences, Engineering, and Medicine; ND = not detected; OFSP = Oubli Fruit Sweet Protein; UL = tolerable upper limit intake.

[a] Calculated based on consumption of 260 mg/day of OFSP by male adults (90th percentile).

[b] Value represent the tolerable upper intake level for male adults (age ≥18 years) as established by NASEM (2019).

3.1.4 Summary and Conclusions

Consumption data and information pertaining to the intended food uses of OFSP were used to estimate the *per capita* and consumer-only intakes of this ingredient for specific demographic groups and for the total U.S. population. There were a number of assumptions included in the assessment which render exposure estimates suitably conservative. For example, it has been assumed in this exposure assessment that all food products within a food category contain OFSP at the maximum specified level of use. In reality, the levels added to specific foods will vary depending on the nature of the food product and it is unlikely that OFSP will have 100% market penetration in all identified food categories.

PART 4. §170.240 SELF-LIMITING LEVELS OF USE

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

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 subject of this GRAS Notice is OFSP, a novel protein-based sweetener manufactured by Oobli through microbial fermentation of a bioengineered strain of *K. phaffii.* The production strain was derived from the safe strain lineage *K. phaffii* NRRL Y-11430, a non-pathogenic and non-toxigenic species, and was genetically engineered to express the gene encoding for brazzein. The gene encoding for brazzein was identified from the naturally occurring plant source, *Pentadiplandra brazzeana*, within a gene databank and was synthesized *de novo* prior to introduction into the host organism through standard biotechnology techniques. The production strain underwent fermentation and the resulting expressed brazzein was concentrated, purified, and processed into a powder consisting of ≥70% total protein (w/w) and ≥35% brazzein (w/w). OFSP is demonstrated to be absent of chemical (*e.g.*, heavy metals) and microbiological impurities that would raise a safety concern. Therefore, the safety assessment of OFSP is focused on 2 aspects: the safety of the production organism and the safety of the ingredient itself. The safety assessment of OFSP followed the principles described by Pariza and Johnson (2001) and Sewalt *et al.* (2016) that are commonly employed in the evaluation of microbially-derived enzyme preparations. Under this safety paradigm, elements such as characterization of the production organism and the genetic modification steps to obtain this organism were evaluated, as well as any available toxicological studies on the resulting ingredient (*i.e.*, OFSP), as well as bioinformatics evaluation of the characterizing protein, brazzein, within the OFSP. These elements are discussed in further detail in the sections that follow.

To facilitate the safety assessment of OFSP, a comprehensive search of the scientific literature was conducted through March 2023 to identify publications on the safety of brazzein and the plant source (*Pentadiplandra brazzeana*). The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile®. One publication was identified in which mice were provided a 3M-brazzein solution in the drinking water over 15 weeks and obesity-related endpoints were observed (Kim *et al.*, 2020). No significant effects on adiposity hypertrophy, glucose homeostasis, insulin resistance, or inflammation were observed throughout the study period. However, this study did not evaluate standard toxicological endpoints such as those described in Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 and did not report on the purity of brazzein. This study was therefore not considered to be relevant to the safety discussion of OFSP. A second study was identified which discussed a series of toxicological studies investigating whether OFSP containing its active constituent, brazzein, has any genotoxic or systemic toxicity potential (Lynch *et al.*, 2023). These studies included a bacterial reverse mutation test (OECD Test Guideline 471), an *in vitro* mammalian micronucleus test (OECD Test Guideline 487), and a 90-day repeated-dose oral toxicity study in rats (OECD Test Guideline 408) (OECD, 2016, 2018, 2020). All studies were conducted in accordance with Good Laboratory Practices (GLP) and appropriate OECD Test Guidelines with OFSP meeting specifications of Section 2.3. These studies are discussed in Section 6.3. The studies described by Lynch *et al.* (2023) served as the pivotal evidence of safety to support OFSP as described herein.

6.2 Safety of the Production Strain

The safety of Oobli's production strain used in the production of OFSP was assessed using the principles commonly employed in the safety evaluation of microbially-derived enzyme preparations (Pariza and Johnson, 2001; Sewalt *et al.*, 2016; FAO/WHO, 2020). This approach included an evaluation of the pathogenicity, toxigenicity, and antimicrobial resistance of the production strain, as well as the genetic modification steps to generate the production strain. These elements are discussed in further detail herein.

The production strain, *K. phaffii* P-BRZ-013, was derived from the host organism *K. phaffii* strain BG10, which is a derivative of *K. phaffii NRRL* Y-11430, a Biosafety Level 1 organism. Strain BG10 and strain NRRL Y-11430 are genomically similar (see Section 2.2.2.1 for further information on the history of these strains) and have been well documented in GRNs 737, 967, 1001, and 1056 (U.S. FDA, 2018, 2021a,b, 2022c). *K. phaffii* (formerly referred to as *Pichia pastoris*) has an established history of safe use in food production, particularly in the production of food enzymes and other food ingredients. In the U.S., *P. pastoris* is permitted for use as a source of protein in broiler feed at levels up to 10% (21 CFR §573.750 – U.S. FDA, 2022d). In the European Union, *K. phaffii* (known as *Komagataella pastoris*) has qualified presumption of safety status for use in food production on the basis that it is incapable of producing toxic metabolites under standard conditions of food processing. *K. phaffii* (formerly referred to as *P. pastoris*) is not listed as a pathogen by the European Commission, National Institute of Allergy and Infectious Diseases, or the U.S. FDA (EC, 2000; NIAID, 2018; U.S. FDA, 2022e). A search of the PubMed database indicates that there have not been any pathogenic or toxigenic reports associated with this species in the scientific literature. The totality of evidence indicates that *K. phaffii* (formerly referred to as *P. pastoris*) is a non-pathogenic and non-toxigenic species, a viewpoint that is generally recognized by the scientific community given its extensive use as a source organism for the production of food ingredients. Therefore, the publicly available information on *K. phaffii* strains BG10 and NRRL Y-11430, as documented in previous GRAS Notices for various food ingredients and food enzymes that received "no questions" from the FDA, demonstrates that these species are non-pathogenic, non-toxigenic, and have an extensive history of safe use in food production; these species would therefore serve as a safe and suitable source organism for the production of brazzein.

As described in Section 2.2.2.2, Oobli's production strain has been bioengineered to express the gene encoding for brazzein using standard biotechnology techniques. The production strain has been demonstrated to be absent of antibiotic resistance genes, and it does not contain any remaining expression plasmids. The synthetic gene encoding for brazzein is the only exogenous genetic material introduced to *K. phaffii* BG10. This gene is well characterized and has been demonstrated through bioinformatics means to express the protein (brazzein) and does not confer any pathogenic or toxigenic factor into the host organism. Oobli has demonstrated the production strain to be genetically stable after fermentation. Therefore, it is concluded that Oobli's production strain, *K. phaffii* P-BRZ-013, meets the criteria for a safe and suitable source organism as described by Pariza and Johnson (2001).

6.3 Safety of Oubli Fruit Sweet Protein

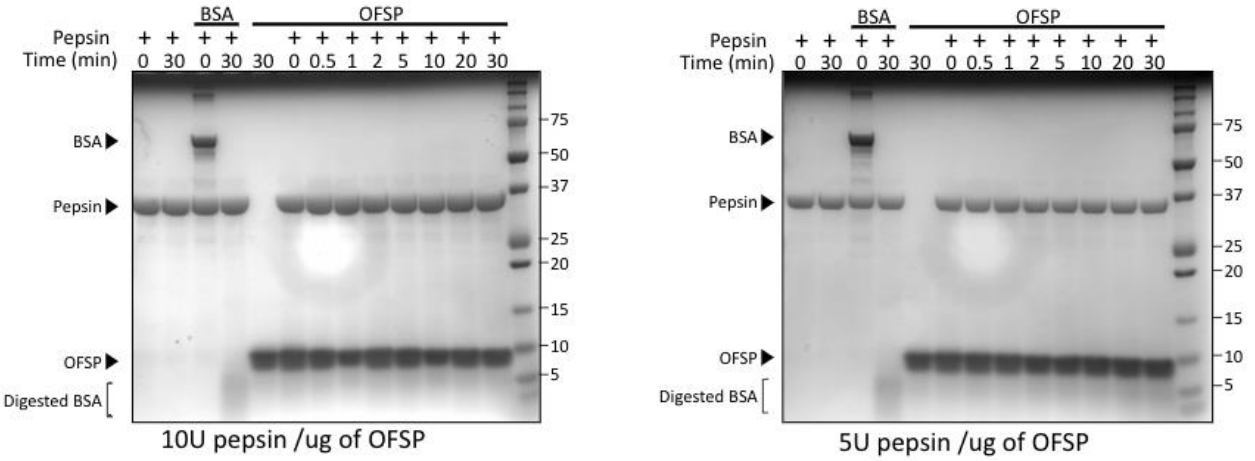
6.3.1 Digestibility of OFSP and Brazzein

6.3.1.1 In Vitro Digestibility of OFSP

Two *in vitro* digestibility studies have been conducted using OFSP containing brazzein. In the first study, the methodology described by Thomas *et al.* (2004) was employed. The digestion assay was performed with simulated gastric fluid (SGF) at pH 2 with pepsin/OFSP ratios of 5 and 10 U/µg of OFSP. The digestion of the sample was run for 0.5, 1, 2, 5, 10, 20, and 30 minutes. An aliquot from each digestion time point was then run on a sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. As shown in Figure 6.3.1.1-1 below, OFSP was not digested at any tested pepsin concentration at up to 30 minutes of incubation.

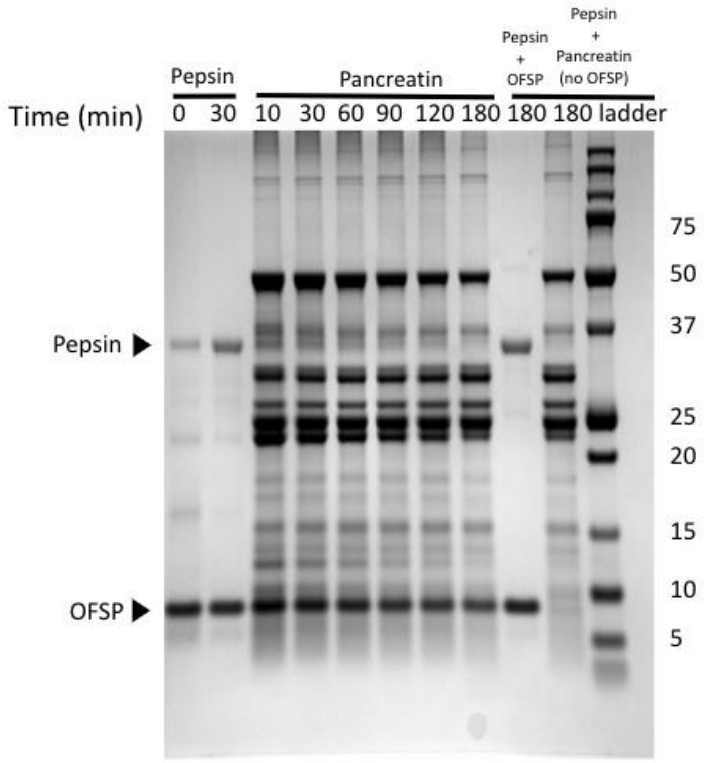
A second 2-step *in vitro* digestibility study was conducted using a methodology described by Brodkorb *et al.* (2019) with SGF at pH 2 with pepsin at 10 U/µg of OFSP and sequentially with simulated intestinal fluid (SIF) at pH 7 with a mixture of pancreatic enzymes (pancreatin), including trypsin, chymotrypsin, amylase, lipase, and colipase, at enzyme concentrations of 0.5 U/µg of OFSP for up to 180 minutes. Samples were digested in SGF with pepsin for 30 minutes, pH adjusted to pH 7 and introduced into SIF with pancreatin. These samples then were obtained after 10, 30, 60, 90, 120, and 180 minutes. As shown in Figure 6.3.1.1-2, OFSP was partially digested after 180 minutes. Based on a semiquantitative analysis from the SDS-PAGE gel, after 180 minutes, approximately 70% of OFSP was digested by pancreatin. The findings of these studies suggest that OFSP containing brazzein is partially digested under simulated gastric followed by intestinal conditions.

Figure 6.3.1.1-1 Results of *In Vitro* Digestibility Study with OFSP with Simulated Gastric Fluid



BSA = bovine serum albumin; min = minutes; OFSP = Oubli Fruit Sweet Protein.

Figure 6.3.1.1-2 Results of *In Vitro* Digestibility Study with OFSP with Pepsin in Simulated Gastric Fluid and with Pancreatin in Simulated Intestinal Fluid



min = minutes; OFSP = Oubli Fruit Sweet Protein.

6.3.1.2 In Silico Digestibility of Brazzein

The digestibility of brazzein, the active protein within OFSP, was investigated using an *in silico* model, PeptideCutter, under 2 conditions with pepsin (pH 1.3 and >2.0). A summary of the model results is provided in Table 6.3.1.2-1. The PeptideCutter predictions suggest that brazzein is broken down by pepsin into peptides that are up to 11 amino acids in length; the majority of the peptide digests are less than 11 amino acids in length, suggesting that brazzein is readily digested.

Following modeling of protein digestion using pepsin, the digests obtained from each condition were evaluated with PeptideCutter using trypsin to further simulate digestion conditions within the GI tract. Peptides that were less than 5 amino acids in length were not predicted to be digested by trypsin. The remaining digests (DKCKKVYENY, PVSKCQL, DKHARSGEC, and DEKRNLQCICD) that were 10, 7, 9, and 11 amino acids in length, respectively, were predicted to be readily digested by trypsin as the digests were less than 5 amino acids in length (see Table 6.3.1.2-2). Considering that small peptides <5 amino acids in length were identified following trypsin digestion, these peptides were not further considered for bioinformatics evaluation for allergenicity and/or toxigenicity potential. As discussed in Section 6.3.4, bioinformatics assessment of the full-length sequence of brazzein did not identify any concern for allergenicity and/or toxigenicity. Overall, the PeptideCutter modeling results suggest that brazzein is digested under GI conditions and intact protein would not be present in the intestinal tract.

Table 6.3.1.2-1 Summary of *In Silico* Digestion Using PeptideCutter with Pepsin (pH 1.3 and >2.0)

Position of Cleavage Site	Cleaving Enzyme	Resulting Peptide Sequence	Peptide Length (amino acids)	Peptide Mass (Da)
10	Pepsin (pH>2)	DKCKKVYENY	10	1289.469
17	Pepsin (pH1.3) Pepsin (pH>2)	PVSKCQL	7	773.946
22	Pepsin (pH>2)	ANQCN	5	548.571
23	Pepsin (pH>2)	Y	1	181.191
26	Pepsin (pH1.3) Pepsin (pH>2)	DCK	3	364.417
27	Pepsin (pH1.3) Pepsin (pH>2)	L	1	131.175
36	Pepsin (pH1.3) Pepsin (pH>2)	DKHARSGEC	9	1002.070
37	Pepsin (pH1.3) Pepsin (pH>2)	F	1	165.192
38	Pepsin (pH>2)	Y	1	181.191
49	Pepsin (pH>2)	DEKRNLQCICD	11	1336.500
50	Pepsin (pH>2)	Y	1	181.191
52	Pepsin (pH>2)	CE	2	250.270
53	**end of sequence**	Y	1	181.191

Table 6.3.1.2-2 Summary of *In Silico* Digestion Using PeptideCutter with Trypsin

Position of Cleavage Site	Cleaving Enzyme	Resulting Peptide Sequence	Peptide Length (amino acids)	Peptide Mass (Da)
DKCKKVYENY				
2	Trypsin	DK	2	261.278
4	Trypsin	CK	2	249.328
5	Trypsin	K	1	146.189
10	**end of sequence**	VYENY	5	686.719
PVSKCQL				
4	Trypsin	PVSK	4	429.517
7	**end of sequence**	CQL	3	362.444
DKHARSGEC				
2	Trypsin	DK	2	261.278
5	Trypsin	HAR	3	382.423
9	**end of sequence**	SGEC	4	394.400
DEKRNLQCICD				
2	Trypsin	DK	2	261.278
5	Trypsin	HAR	3	382.423
9	**end of sequence**	SGEC	4	394.400

It is generally recognized that the normal fate of dietary proteins is digestion into small peptides and individual amino acids that are absorbed into the systemic circulation *via* transcellular or paracellular routes (EFSA, 2021). Proteins that are not fully digested or are partially digested travel to the large intestine where they are ultimately fermented by the gut microbiota (Portune *et al*., 2016; Joye, 2019). In the large intestine, where the microbiota concentration is much higher and the transit time is longer than in the small intestine, the remaining protein is broken down to peptides and amino acids *via* extracellular bacterial proteases and peptidases (Macfarlane *et al*, 1986). As part of the weight-of-evidence for the safety discussion of a protein, the resistance of a protein to partial or complete digestion is relevant, as it suggests that the protein could have the potential to elicit localized toxic effects by microbial fermentation (*i.e*., in the gut) or could be absorbed as an intact protein that may elicit toxic effects in the systemic circulation and/or allergenic effects (EFSA, 2021). It is noted that only a small number of food proteins have been demonstrated to be capable of causing adverse effects with respect to allergy or toxicity when consumed (Markell *et al*., 2017). It should be noted that dietary proteins are denatured at low pH (1.5 to 3.5) that occur in the stomach, thus unfolding their 3D structure to yield the polypeptide chain, thereby impairing the protein's function (Callahan *et al*., 2022). Therefore, it would be expected that brazzein within OFSP, following consumption, would not be present in an active form in the stomach and would be digested under gastric and/or intestinal conditions.

As discussed in Sections 6.3.3 and 6.3.4, the potential systemic toxicity of OFSP was investigated in a 90-day dietary toxicity study conducted in accordance with OECD Test Guideline 408 (OECD, 2018), and a bioinformatics assessment was conducted to investigate the allergenicity potential of brazzein. The results of the 90-day dietary toxicity study conducted with OFSP demonstrate that the intact brazzein protein and any potential peptide digests do not pose any safety concern to final consumers based on the absence of adverse effects in any toxicologically relevant test endpoint at the highest tested dose of 1,000 mg/kg body weight/day (see Section 6.3.3 for further details). Additionally, OFSP was demonstrated to have a low potential for allergenicity based on the results of an *in silico* investigation following internationally recognized *in silico* guidelines established by FAO/WHO (2001) and Codex Alimentarius (2009) (see Section 6.3.4 for further details).

6.3.2 Mutagenicity/Genotoxicity Studies on Oubli Fruit Sweet Protein

Bacterial Reverse Mutation Test

The potential mutagenicity of OFSP was investigated in a bacterial reverse mutation assay with *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2*uvr*A in the presence and absence of metabolic activation (Lynch *et al*., 2023). The study was conducted in accordance with the OECD Test Guideline 471 (OECD, 2020) and the Principles of GLP (OECD, 1998). The main study was conducted using the plate incorporation method. OFSP was tested in triplicate at concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1,580, and 5,000 µg/plate with and without metabolic activation. A confirmatory test using the pre-incubation method was performed using the same strains and concentrations as the main study. All strains received distilled water as the negative control. The compounds used as positive controls in the assays conducted in the absence of metabolic activation included 2-nitrofluroene (2-NF), sodium azide (NaN_3), 9-aminoanthracene (9-AA), or 4-nitroquinoline 1-oxide (4-NQO). Assays conducted in the presence of metabolic activation used 2-aminoanthracene (2-AA) or benzo(a)pyrene as the positive control. The number of revertant colonies in the positive controls were greater than 2 times (or greater than 3 times in the case of strains TA1535 and TA1537) those reported in the negative controls.

OFSP did not induce any biologically relevant, concentration-related, or statistically significant increases in revertant colony numbers compared with vehicle control counts in either the presence or absence of metabolic activation. The mean number of revertant colonies of the vehicle controls were all within the historical vehicle control ranges. Based on the results of the bacterial reverse mutation assay, OFSP is concluded to have no mutagenic potential.

In Vitro Mammalian Cell Micronucleus Test

The genotoxic potential of OFSP was investigated in an *in vitro* mammalian cell micronucleus test (Lynch *et al*., 2023). The study was conducted in accordance with OECD Test Guideline 487 (OECD, 2016). Human peripheral blood lymphocytes were collected from whole blood samples *via* venous puncture from healthy, non-smoking donors. An initial test for cytotoxicity of OFSP was conducted with and without metabolic activation at concentrations of 15.6, 31.3, 62.5, 125, 250, 500, 750, 1,000, 2,500, and 5,000 µg/mL. Cytotoxicity was assessed *via* cytokinesis block proliferation index, which was used to determine the proportion of cytostasis (the inhibition of cell growth of treated cultures in comparison to control cultures). The short-term experiment was conducted in duplicate at concentrations of 62.5, 125, 250, 500, 1,000, and 2,000 µg/mL with and without metabolic activation for 4 hours. The long-term experiment was conducted in duplicate with the same concentrations of OFSP without metabolic activation for 24 hours. The compounds used as positive controls in the assays conducted in the absence of metabolic activation included methylmethanesulfonate (MMS; clastogenic control) and colchicine (aneugenic control). Assays conducted in the presence of metabolic activation used cyclophosphamide as the positive clastogenic control. The S9 liver microsomal fraction was obtained from male Sprague-Dawley rats induced with phenobarbital/ β-naphthoflavone. The positive controls induced biologically relevant and statistically significant increases in the percentage of micronucleated cells compared with vehicle controls, with mean values within historical positive control ranges.

In the initial cytotoxicity test, OFSP did not induce excessive cytotoxicity (≤30% cytostasis); hence, the maximum concentration tested in the short-term and long-term experiments was 2,000 µg/mL, the highest recommended by OECD Test Guideline 487 (OECD, 2016). In the short-term experiment, a statistically significant increase in the percentage of micronucleated cells was observed at the highest concentration of 2,000 µg/mL with metabolic activation when compared with vehicle controls. However, the number of micronucleated cells was within the range of the historical negative control; therefore, this increase was regarded as not biologically relevant. In the long-term experiment, no biologically relevant increases in the percentage of micronucleated cells were reported at any of the concentrations analyzed. Based on the results of this study, OFSP was concluded to have no clastogenic or aneugenic potential in human peripheral blood lymphocytes, as there were no biologically relevant increases in the percentage of micronucleated cells at any of the concentrations analyzed compared with vehicle controls.

6.3.3 Studies in Animals

6.3.3.1 Systemic Toxicity Studies with Oubli Fruit Sweet Protein

A 90-day oral toxicity study was conducted with in accordance with OECD Test Guideline 408 (OECD, 2018) and the Principles of GLP (OECD, 1998) (Lynch *et al*., 2023). Adult CRL: Sprague-Dawley® CD® IGS rats (n=10/sex/group) were provided OFSP in the diet at concentrations of 0 (control diet), 250 (low-dose), 500 (mid-dose), and 1,000 (high-dose) mg/kg body weight/day for 90 days. The animals in all study groups were provided access to water and food *ad libitum*. The doses were selected based on the results of 14-day oral repeated-dose study that served as a dose range–finding study in which no treatment-related toxicological effects were reported at the highest dose tested (nominally 1,000 mg/kg body weight/day).

The dietary concentrations of OFSP achieved the targeted nominal intake values of 250, 500, and 1,000 mg/kg body weight/day (actual values: 0, 245, 490, and 978 mg/kg body weight/day and 0, 245, 493, and 985 mg/kg body weight/day in males and females, respectively). The general condition of the animals was assessed 3 times a day. Weekly measurements were conducted in all animals for body weight and food consumption. Ophthalmological examinations were conducted before the study and at Week 13. In the final week of the dose administration period, urinalysis was conducted. At the end of the study period, hematology, blood chemistry, and pathological examinations (*i.e*., organ weight measurement, macroscopic and histopathological examinations) were conducted. The incidence of alopecia was well dispersed throughout the control and treatment groups of both sexes. This finding was due to the type of feeding equipment used per the guidelines in the protocol. There were no treatment-related toxicological effects on body weights, body weight gain, food consumption, functional observation battery (*i.e*., sensorimotor function, grip strength, and locomotor activity), ophthalmological findings, hematological and blood chemistry parameters, urinalysis findings, organ weights, macroscopic, and histopathological findings. No mortality was reported.

Functional observational battery (FOB) tests and motor activity (MA) tests were conducted prior to dose administration and monthly thereafter. A statistically significant interaction with time, but not treatment, was reported for the FOB tests; however, this was an expected finding as the animals are more active in the initial sessions relative to the later ones.

Hematological evaluations revealed the following statistically significant findings: decreased mean cell hemoglobin (MCH) and platelet counts in the top 2 dose group females, a slight decrease in lymphocyte counts in high-dose females, and a slight increase in activated partial thromboplastin time (APTT) time in top dose females only. There were no statistically significant changes in any of the male dose groups. The decrease in MCH was not of toxicological significance since all values remained within the normal historical control range. The decrease in platelet count was considered of no toxicological significance as in each group, including the controls, the values reported were above the historical control range and no decreases were noted in males. Likewise, the slight increase in APTT time in high-dose females was slight, not seen in males, and not accompanied by any changes in prothrombin time (PT).

Statistically significant changes in clinical chemistry parameters included increased aspartate aminotransferase (AST) in high-dose females, slightly increased total protein in low- and high-dose females, and increased total cholesterol and low-density lipoprotein in mid-dose males. The AST values in the high-dose females remained within the historical control range and were not accompanied by any statistically significant increases in other enzymes associated with liver function (*i.e*., alanine transaminase, alkaline phosphatase, SDH, and gamma-glutamyl transferase). In addition, there was no evidence of any increase in AST in males or changes in liver histopathology indicative of an adverse effect of treatment in either sex.

The weights of the epididymides were slightly decreased in the treated males, with relative-to-body weight values achieving statistical significance in the low- and high-dose groups and relative-to-brain weight values achieving statistical significance in the top 2 dose groups. There were no statistically significant effects on the absolute weights of the epididymides. In addition, pituitary gland weights (absolute and relative-to-body weight) were slightly increased in mid-dose males, prostate gland weights (absolute and relative-to-body) slightly increased in the low- and high-dose groups, and the thyroid parathyroid weights minimally increased in low-dose males. None of these changes were observed in females.

The few statistically significant changes reported from the FOB, hematology, clinical chemistry, and organ weight analyses did not show a dose-dependent increase and did not correlate with any adverse histopathological findings.

Under the conditions of this 90-day dietary toxicity study conducted in compliance with appropriate test guidelines (OECD Test Guideline 408) and GLP, in the absence of test article–related adverse effects in both male and female rats at any tested dose, the no-observed-adverse-effect level (NOAEL) for OFSP was concluded to be 978 mg/kg body weight/day in males and 985 mg/kg body weight/day in females, the highest tested dose (OECD, 1998, 2018). Based on the reported NOAEL and the highest estimated dietary intake of 4.25 mg/kg body weight/day (90^{th} percentile in children 2 to 5 years of age), an approximately 230-fold margin of exposure (MOE) can be estimated. This suggests that there are no safety concerns of OFSP under its proposed conditions of use as described in Section 1.3.

6.3.3.2 Other Brazzein Studies

Kim *et al.* (2020) investigated the effects of exposure to brazzein on obesity, metabolic disorder, and inflammation markers in 7-week old C57BL/6J mice (N=20). Animals were provided a 3M-brazzein solution in the drinking water for 15 weeks. The 3M-brazzein solution was produced by *Kluyveroyces lactis* and was reported to be a 6.5 kDa protein that is 22,500 times sweeter than a 1% sucrose solution, and approximately 6,000 times sweeter than a 10% sucrose solution on a weight basis. The food and water intake were monitored daily, and body weights were measured weekly. A glucose tolerance test and insulin tolerance test were performed after fasting for 12 hours. Blood samples were collected from the tail vein at 0, 15, 30, 60, and 120 minutes after glucose or insulin injection, and blood glucose was measured. All animals were terminated at the end of the study and blood samples were collected for biochemical analysis. Inguinal and epididymal white adipose tissue and liver were collected for analysis.

In addition, animals were administered 3M-brazzein by intraperitoneal (i.p.) injection, and blood glucose and insulin levels were measured and compared to animals administered glucose by i.p. injection after 12 hours of fasting. The authors reported that blood glucose levels increased in the animals administered glucose, but not in animals administered 3M-brazzein. No insulin response was reported in animals receiving 3M-brazzein.

Following consumption of 3M-brazzein in the drinking water for 15 weeks, no significant changes in body weight, food intake, water intake, total calorie intake, body fat accumulation, glucose homeostasis, insulin response, or inflammation biomarker (interleukin [IL]-1β, IL-6, tumor necrosis factor [TNF]-α) were reported compared to the control group receiving a normal chow diet. The levels of insulin and c-peptide were higher in the animals receiving sucrose than 3M-brazzein, which was not significantly higher compared to the control. No effects on insulin resistance, or insulin signaling (as measured by mRNA expression in the liver insulin pathway of IRS2 and GLUT4) were reported in animals receiving 3M-brazzein. The findings of this study suggest that brazzein, following consumption, does not impact any obesity-related endpoint, and does not elicit an insulin response.

6.3.4 Bioinformatics Assessment of Brazzein and Residual Host Cell Proteins within Oubli Fruit Sweet Protein

6.3.4.1 Allergenicity

The Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) and *Codex Alimentarius* have recommended a stepwise approach to the allergenicity evaluation of proteins in which the source of the protein of interest is first evaluated then the protein itself is assessed for sequence homology to known allergenic proteins (FAO/WHO, 2001; Codex Alimentarius, 2009). The protein of interest is considered to share significant sequence homology if a set of 80-amino acid length sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length amino acid sequence shares more than 35% identity with a known allergen, or an exact match of 6 contiguous amino acids, in which case possible cross-reactivity between the proteins may exist (FAO/WHO, 2001; Codex Alimentarius, 2009). The sliding window of 80 amino acids correspond with a typical domain size of a protein, such that a single protein domain may contain epitopes that mediate antibody binding. In cases where sequence homology is not identified, the protein of interest is not considered to contain potential allergenicity and unlikely to be cross-reactive to known allergens (Codex Alimentarius, 2009). In theory, 6 to 8 contiguous amino acids may represent a minimum length of linear immunoglobulin E (IgE) binding epitopes and T cell epitopes from peanut allergens (Ara h 1 and Ara h 2) (Herman *et al*., 2009; Ladics, 2019; Abdelmoteleb *et al*., 2021); however, its usefulness in predicting potential allergenicity is unclear, as these matches have been known to produce false positives (Ladics, 2019). The allergenicity potential of brazzein and five residual host cell proteins in OFSP was investigated using the step-wise approach described by FAO/WHO (2001) and Codex Alimentarius (2009). Sequence homology searches were conducted against the curated databases of AllergenOnline and Allermatch. In addition, a support vector machine (SVM) search with AlgPred was conducted with the same brazzein and the five residual host cell proteins. The results of the searches are summarized below.

To assess whether the brazzein (*i.e*., brazzein-53) within OFSP shares amino acid sequence homology to putative allergens that would suggest potential for allergenic cross-reactivity, a search of the AllergenOnline database (Version 21) maintained by the Food Allergy Research and Resource Program of the University of Nebraska-Lincoln was conducted using the approaches described by FAO/WHO (2001) and Codex Alimentarius (2009). The 80-amino acid alignment searches were conducted using default settings (percent identity >35%) and the FASTA36 algorithm. No matches between brazzein and putative allergens were identified sharing greater than 35% identity, indicating that brazzein would be unlikely to have any allergenic cross-reactivity. Despite the potential for false positives, a search for exact 8-amino acid matches was performed with the amino acid sequence of brazzein against the AllergenOnline database. No 8-amino acid exact matches were identified.

A full-length sequence homology search was also conducted with brazzein against the AllergenOnline database using default settings. One match to a putative allergen from peach (*Prunus persica*) with >50% identity was identified. The corresponding E-value was 0.76 and bit-score was 24.6. It has been reported in the scientific literature that allergenic cross-reactivity between proteins sharing less than 50% similarity is rare and typically requires >70% identity (Aalberse, 2000). In addition, the biological relevance of identified matches in *in silico* allergenicity assessment is typically inferred through the identity score, E-value, and bit-score of the match. Pearson (2013) reported that an E-value of less than 0.001 and bit-scores greater than 40 can be reliably used to infer homology; matches meeting these criteria may be considered significant. In a recent bioinformatics publication on a comprehensive allergenicity assessment of the proteomes of novel microorganisms, Abdelmoteleb *et al.* (2021) demonstrated that an E-value less than 10^{-7} reflects a functional similarity between two proteins and likely suggests a biologically relevant similarity for allergenic cross-reactivity potential. Therefore, considering that the identified match in the

full-length search was less than 70% identity with an E-value and bit-score of 0.76 and 24.6, respectively, this finding was not considered to be significant and is not likely to be reflective of allergenic cross-reactivity of brazzein.

Additional *in silico* allergenicity searches were performed using Allermatch, which is maintained by a research group at Wageningen University. The Allermatch database is composed of known allergenic proteins from the UniProtKB allergen database,[1] the WHO and International Union of Immunological Societies list of allergen nomenclature,[2] and the Comprehensive Protein Allergen Resource database.[3] The 80-amino acid alignment searches were conducted using default settings (percent identity >35%) and the FASTA algorithm. Similar to the search with AllergenOnline, no matches sharing greater than 35% identity were identified with brazzein and putative allergens from the Allermatch database. An 8-amino acid exact match search was also performed, and no 8-amino acid exact matches were identified. A full-length search of the amino acid sequence of brazzein was also conducted using default settings against the Allermatch database.

The results of this search indicated that brazzein shares >50% identity with 20 sequences from the Allermatch database (see Table 6.3.4.1-1). Despite these putative allergens sharing >50% identity, the statistical significance of the sequence homology is very low based on the E-values and bit-scores of the matches. Given that the all of the E-values of the significant hits from the full-length search of the Allermatch database were above 0.001 (the lowest E-value was 1.1) and that all of the bit-scores were below 40 (the highest bit-score was 24.1) these alignments with greater than 50% identity are not considered significant sequence alignments.

[1] https://www.uniprot.org/docs/allergen.
[2] http://www.allergen.org/.
[3] https://comparedatabase.org/.

Table 6.3.4.1-1 Summary of the Allermatch Results of Brazzein within OFSP (Full-Length Sequence Alignment)

Protein	Species	% Identity[a]	% Similarity[a]	E-value	Bit-score	Amino Acid Overlap	Amino Acid Length	Accession No.
Basic form of pathogenesis related protein 1	*Prunus persica*	60.0	73.3	1.1	24.1	15	161	GenBank XP_007199020
PREDICTED: lysozyme C, milk isozyme	*Equus asinus*	53.3	73.3	17	20.0	15	148	GenBank XP_014705584
Phospholipase A1 2	*Vespa affinis*	72.7	72.7	28	20.3	11	301	UniProt PA12_VESAF
Venom allergen 5.02	*Vespa crabro*	58.3	75.0	31	19.6	12	202	UniProt VA52_VESCR
Venom allergen 5.01	*Vespa crabro*	58.3	75.0	31	19.6	12	202	UniProt VA51_VESCR
Alcohol dehydrogenase	*Cochliobolus lunatus*	54.5	81.8	38	20.1	11	352	UniProt Q2HYZ7_COCLU
Unnamed protein product, partial	*Dermatophagoides pteronyssinus*	53.8	69.2	50	18.2	13	129	GenBank CAD38378
Unnamed protein product, partial	*Dermatophagoides pteronyssinus*	53.8	69.2	50	18.2	13	129	GenBank CAD38381
Unnamed protein product, partial	*Dermatophagoides pteronyssinus*	53.8	69.2	50	18.2	13	129	GenBank CAD38383
Unnamed protein product, partial	*Dermatophagoides pteronyssinus*	53.8	69.2	50	18.2	13	129	GenBank CAD38382
Unnamed protein product, partial	*Dermatophagoides pteronyssinus*	53.8	69.2	50	18.2	13	129	GenBank CAD38379
36 kda allergen {peptide 143-115}	*Blattella germanica*	57.1	71.4	74	15.3	7	25	GenBank AAB29345
Phospholipase A1 1	*Dolichovespula maculate*	63.6	72.7	76	18.8	11	300	UniProt PA11_DOLMA
Phospholipase A1 1	*Vespa affinis*	63.6	72.7	76	18.8	11	301	UniProt PA11_VESAF
Phospholipase A1	*Vespa crabro*	63.6	72.7	76	18.8	11	301	UniProt PA1_VESCR
Phospholipase A1 2	*Dolichovespula maculate*	63.6	72.7	76	18.8	11	303	UniProt PA12_DOLMA
Phospholipase A1	*Vespa velutina*	63.6	72.7	77	18.8	11	304	UniProt PA1_VESVE
PREDICTED: parvalbumin *beta*	*Crocodylus porosus*	50.0	83.3	80	17.3	12	109	GenBank XP_019397705
Procalin	*Triatoma protracta*	55.6	88.9	83	17.7	9	151	UniProt PRCLN_TRIPT
Hydrophobic seed protein	*Glycine max*	60.0	70.0	95	16.6	10	80	UniProt HPSE_SOYBN

OFSP = Oubli Fruit Sweet Protein.

[a] Percent identity refers to the ratio of the number of matching residues to the total length of the alignment. Percent similarity counts “similar” residues (usually amino acids) in addition to the identical ones.

Brazzein was co-purified with other proteins from its *K. phaffii* host cell organism in OFSP. Using methods described in Section 2.1.1, five host cell proteins were identified to be co-purified with brazzein. The allergenicity potential of the five host cell proteins was investigated using the stepwise approach described above with AllergenOnline. Of the five host cell proteins, two of the proteins—transaldolase (UniProt: C4R245) and vacuolar aspartyl protease (UniProt: C4R6G8)—shared a number of matches to putative allergens in the 80-amino acid sliding window search (see Table 6.3.4.1-2). No matches were identified for the other three proteins. The identified matches suggest significant sequence homology to allergenic proteins from fungal species such as *Fusarium proliferatum, Pencillium chrysogenum, Cladosporium cladosporioides, Rhizopus oryzae,* and *Aspergillus fumigatus*, as well as mosquito (*Aedes aegypti*) and wild boar (*Sus scrofa*). The matches to the proteins from fungal sources are likely matches to respiratory allergenic proteins, while the matches to proteins from mosquito and wild boar are likely dermal and oral allergens, respectively. No matches were identified to major food allergens that are of relevance to final consumers. While allergenic reactions to mosquitoes have been documented, mosquito allergy is quite rare and it is expected that the prevalence of mosquito allergy is rare in the global population (Arias-Cruz *et al.*, 2006; González Diaz *et al.*, 2010). In the U.S., the prevalence of mosquito allergy is estimated to be in the range of 1 to 10 per 10 million people (Kausar, 2018). Similarly, respiratory allergy from fungal sources is rare and is estimated to impact up to 6% of the general population (Horner *et al.*, 1995). Therefore, although the identified matches may be suggestive of a significant sequence homology with potential for allergenic cross-reactivity, under the proposed conditions of use of OFSP, the allergenic risk would be low. Furthermore, the allergenic potential of residual host cell proteins from production strains derived from *K. phaffii* NRRL Y-11430 and BG10 have been previously discussed (Jin *et al.*, 2018; Reyes *et al.*, 2021) as part of the GRAS determination of soy leghemoglobin and bovine myoglobin derived from a genetically engineered strain of *K. phaffii* (see GRNs 737 and 1001).

No proteins native to *Komagataella* have been identified as allergenic concerns. Considering that Oobli's production strain is derived from *K. phaffii* BG10, and the genetic modifications to the host organism are well characterized and do not pose any allergenic concern, it is not expected that any native residual proteins from the host organism would pose a risk for allergenicity.

Table 6.3.4.1-2 Summary of the AllergenOnline (Version 21) Results of Residual *Komagataella* Proteins in OFSP

Protein	Species	Best % Identity	# Hits >35%	Full Alignment		
				E-value	% Identity	Length
Transaldolase						
Transaldolase	*Fusarium proliferatum*	81.30%	245/245	1.5e-098	72.40%	322
Transaldolase	*Penicillium chrysogenum*	75.00%	245/245	1e-090	66.00%	324
Transaldolase	*Cladosporium cladosporioides*	73.80%	245/245	2.6e-090	66.30%	323
Vacuolar Aspartyl Protease (Proteinase A)						
Aspartyl endopeptidase	*Rhizopus oryzae*	63.70%	307/331	2.3e-088	48.50%	410
Lysosomal aspartic protease	*Aedes aegypti*	61.70%	276/331	1.3e-066	47.40%	340
Pepsin A	*Sus scrofa*	53.80%	152/331	4.5e-054	39.50%	324
Aspergillopepsin i	*Aspergillus fumigatus*	36.27%	25/331	7.1e-022	29.10%	357

OFSP = Oubli Fruit Sweet Protein.

The allergenicity potential of brazzein and the five residual host cell proteins was also considered through a search using AllerTOP (version 2.0),[4] a bioinformatics tool for prediction of allergenicity. The method is based on auto cross covariance transformation of protein sequences into uniform equal-length vectors as developed by Wold *et al.* (1993). AllerTOP predicted brazzein to be a "probable allergen," with the nearest protein being the antigen five precursor from *Glossina morsitans* (Accession No. ADD19985.1). With the exception of vacuolar aspartyl protease (UniProt No. C4R6G8) and transaldolase (UniProt No. C4R245), AllerTOP predicted the other three residual host cell proteins to be "probable non-allergens." Vacuolar aspartyl protease was predicted to be a "probable allergen" with the nearest protein to be transaldolase from *Homo sapiens* (UniProt No. P37837), while transaldolase was predicted to be a "probable allergen" with the nearest protein to be pollen allergen Sec c 4 from *Secale cereale* (Accession No. CAH92627.1).

In addition to the *in silico* searches against AllergenOnline and Allermatch, the allergenicity potential of brazzein and the five residual host cell proteins was investigated using a SVM analysis from AlgPred.[5] Information on the sensitivity, specificity, and error rate of AlgPred, as well as the results of the analysis for brazzein, are summarized in Table 6.3.4.1-3. The results for the five residual host cell proteins are presented in Table 6.3.4.1-4. The results of AlgPred identified mixed results: the brazzein protein was predicted to be a non-allergen based on algorithms for IgE epitopes, the Motif Alignment and Search Tool, and allergen representative peptides, and was predicted to be a potential allergen based on SVM analysis of the amino acid composition and dipeptide composition. Similar results were identified for the five residual host cell proteins. AlgPred has been used previously for the allergenicity assessment of soy leghemoglobin obtained from a genetically engineered strain of *P. pastoris*, which has GRAS status for use in meat-analogue products as described in GRN 737 (U.S. FDA, 2018). The notifier reported that the allergenicity assessment using AllergenOnline,[6] similar as described above, was "*more than adequate to demonstrate that both soy leghemoglobin and the Pichia proteins within LegH Prep have little or no allergenic potential*." Furthermore, the notifier stated that SVM-based analysis is controversial, as the reliability of this method is questionable. AlgPred predicted the soy leghemoglobin to be a potential allergen; however, it was noted that this methodology has a high false positive rate. The applicant stated that AlgPred identified 46% of all proteins in the SwissProt to be potential allergens, even after all known allergens and related proteins were removed (Saha and Raghana, 2006; Impossible Foods Inc., 2018).

[4] https://www.ddg-pharmfac.net/AllerTOP/index.html.
[5] http://crdd.osdd.net/raghava/algpred/index.html.
[6] http://www.allergenonline.org/.

Table 6.3.4.1-3 Assessment of the Allergenicity Potential of Brazzein within OFSP Using AlgPred

Algorithm	Result	Sensitivity (True Allergen)	Specificity (True Non-Allergen)	Error Rate (False Allergen)	Analysis Type
IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope	10.84%	98.25%	1.75%	Sequence motif
Motif Alignment and Search Tool (MAST)	Non-allergen	22.05%	86.68%	13.32%	Sequence motif
Allergen representative peptides (ARP)	Non-allergen	66.56%	97.97%	2.03%	Sequence motif
Support vector machine (SVM) amino acid composition	Potential allergen	84.21%	56.07%	43.93%	Amino acid composition
Support vector machine (SVM) dipeptide composition	Potential allergen	84.83%	61.09%	38.91%	Amino acid Composition

IgE = immunoglobulin E; OFSP = Oubli Fruit Sweet Protein.

Table 6.3.4.1-4 Assessment of the Allergenicity Potential of Residual Host Cell Proteins from *Komagataella phaffii* Using AlgPred

Protein	Algorithm	Result
Peptide hydrolase (UniProt No. C4QYZ6)	IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope.
	MAST	Non-allergen
	ARP	Non-allergen
	SVM amino acid composition	Allergen
	SVM dipeptide composition	Allergen
Fusion protein (UniProt No. C4R0U2)	IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope.
	MAST	Non-allergen
	ARP	Non-allergen
	SVM amino acid composition	Potential allergen
	SVM dipeptide composition	Potential allergen
S-adenosylmethionine synthase (UniProt No. C4R5U7)	IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope.
	MAST	Non-allergen
	ARP	Non-allergen
	SVM amino acid composition	Non-allergen
	SVM dipeptide composition	Non-allergen
Vacuolar aspartyl protease (UniProt No. C4R6G8)	IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope.
	MAST	Non-allergen
	ARP	Non-allergen
	SVM amino acid composition	Allergen
	SVM dipeptide composition	Allergen
Transaldolase (UniProt No. C4R245)	IgE epitopes	The protein sequence does not contain experimentally proven IgE epitope.
	MAST	Non-allergen

Table 6.3.4.1-4 Assessment of the Allergenicity Potential of Residual Host Cell Proteins from *Komagataella phaffii* Using AlgPred

Protein	Algorithm	Result
	ARP	Non-allergen
	SVM amino acid composition	Allergen
	SVM dipeptide composition	Allergen

ARP = Allergen Representative Peptides; IgE = immunoglobulin E; MAST = Motif Alignment and Search Tool; SVM = support vector machine.

The results of the above-described *in silico* allergenicity assessment has been published by Lynch *et al.* (2023). The publicly available information sufficiently demonstrates that the brazzein protein within OFSP and presence of residual *Komagataella* proteins do not contain any inherent allergenicity potential and would pose a low risk for allergenicity in final consumers.

6.3.4.2 Toxigenicity

To determine whether brazzein within OFSP shares significant sequence homology with known protein toxins, sequence homology searches were conducted using the Basic Local Alignment Search Tool (BLAST) with the amino acid sequence of brazzein-53 and the residual *Komagataella* proteins (from Table 2.1.1-1) against protein sequences obtained from a curated databases of 7,683 animal venom proteins and toxins[7] maintained by UniProt. The BLAST searches were conducted using algorithm (word size of 6 and expect threshold of 0.001) and scoring parameters (BLOSUM62 scoring matrix with default gap costs and composition adjustments). Sequences were considered to share structural homology/similarity based on the criteria described by Pearson (2013). Pearson (2013) reported that "*homologous sequences that share more than 40% identity are very likely to share functional similarity*," and that an E-value of <0.001 can reliably be used to infer sequence homology (Pearson, 2013). Alternatively, the bit-score can be used to infer homology and is considered to be more reliable indicator of significant sequence homology. A bit-score of 50 is "*almost always significant*," while a bit-score of 40 is only significant (E-value <0.001) in searches of protein databases with less than 7,000 entries (Pearson, 2013).

Based on the above criteria, no significant similarity to any of the venom proteins and toxins were identified (E-values >0.0015) in the homology searches with brazzein and the residual *Komagataella* proteins. These findings suggest that brazzein and the presence of any residual *Komagataella* protein within OFSP does not harbor any toxigenic potential based on its amino acid sequence.

6.4 GRAS Panel Evaluation

Oobli has concluded that OFSP is GRAS for use in conventional food and beverage products, 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 OFSP, as discussed herein, 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: Professor Joseph Baumert (University of Nebraska-Lincoln); Professor Emeritus George C. Fahey, Jr. (University of Illinois); and Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison).

The GRAS Panel, convened by Oobli, independently and critically evaluated all data and information presented herein, and also concluded that OFSP is GRAS for use in conventional food and beverage products

[7] UniProt release Oct, 2017; available at: https://www.uniprot.org/program/Toxins.

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 of OFSP, is presented in Appendix B.

6.5 Conclusion

Oobli intends to manufacture the Oubli Fruit Sweet Protein for use as a sweetener in various food and beverage products. Oobli produces brazzein through precision fermentation using a genetically engineered host strain of *Komagataella phaffii*. The production strain has been derived from the safe strain lineage *K. phaffii* NRRL Y-11430, a non-pathogenic and non-toxigenic species, that has been genetically engineered to express the gene encoding for brazzein. The gene encoding for brazzein was identified from the naturally occurring plant source, *Pentadiplandra brazzeana*, synthesized *de novo*, and introduced to the host organism through standard biotechnology techniques. OFSP is manufactured in accordance with cGMP and HACCP using appropriate food-grade raw materials and processing aids. The final product consists of ~35% total brazzein (w/w), ~80% total protein (w/w), ~5% moisture (w/w), ~5% ash (w/w), and ~10% carbohydrates (w/w) and does not contain levels of heavy metals and microbiological contaminants that could pose a safety concern.

Oobli has conducted a series of *in silico*, *in vitro*, and *in vivo* studies to address safety concerns of allergenicity and toxicity of brazzein within OFSP and residual host cell proteins, as well as *in vitro* and *in silico* digestibility assays, which have indicated that the majority of the protein is digested in simulated intestinal conditions. Additionally, *in silico* analyses for potential allergenicity and toxigenicity of brazzein and residual proteins from *K. phaffii* in the final ingredient have been assessed. The results of these studies, collectively, demonstrate that brazzein within OFSP and any residual proteins from the production strain lack allergenicity potential. The OFSP has also been subject to the standard battery of toxicology studies to investigate its mutagenic/genotoxic profile and systemic toxicity. These studies included a bacterial reverse mutation test (OECD Test Guideline 471), *in vitro* micronucleus test (OECD Test Guideline 487), and a 90-day dietary toxicity study in rats (OECD Test Guideline 408). All studies were conducted in accordance with appropriate OECD Test Guidelines and GLP (OECD, 1998, 2016, 2018, 2020). Further, the toxigenicity potential of brazzein within OFSP investigated through an *in silico* approach against a curated database of animal venom proteins and toxins suggest the proteins also lack toxigenicity potential, which is consistent with the results of the 90-day dietary toxicity study in rats.

The use levels of OFSP range between 2 and 99 mg/100 g in the different food categories in the U.S. Using information within the NHANES database, the resulting mean and 90th percentile intakes is approximately 1.30 mg/kg body weight/day and 2.88 mg/kg body weight/day, respectively. The findings from these studies demonstrate OFSP to be non-mutagenic and non-genotoxic, and the NOAEL from the 90-day study at the highest dose tested was approximately 978 mg/kg body weight/day in males and 985 mg/kg body weight/day in females. Based on the highest estimated dietary intake of 4.25 mg/kg body weight/day (90th percentile in children 2 to 5 years of age), the MOE is estimated to be 230-fold, suggesting that OFSP, under its proposed conditions of use, does not pose any safety concerns to end consumers.

Based on the technical and scientific data and information presented herein, Oobli has concluded that the OFSP produced from a genetically engineered strain of *K. phaffii* is GRAS for use in conventional food and beverage products on the basis of scientific procedures. OFSP 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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Protein ID	Organism	Description	Average percentage of 3 lots of OFSP (OFSPB53-304, OFSPB53-215, OFSPB53-538)
P56552		Brazzein	46.78
C4R0U2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Fusion protein, identical to Rpl40Bp	6.23
C4R245	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Transaldolase	3.15
C4R6G8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Vacuolar aspartyl protease (Proteinase A)	1.59
C4QYZ6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Peptide hydrolase	1.22
C4R5U7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	S-adenosylmethionine synthase	1.01
C4R2P5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Long chronological lifespan protein 2	0.97
C4R7E5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Thioredoxin	0.91
C4R7F9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Golgi-localized protein with homology to gamma-adaptin	0.91
C4QW42	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Chitin deacetylase, together with Cda1p involved in the biosynthesis ascospore wall component	0.83
C4QWG6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, identical to Rpl2Bp	0.72
C4R3H3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.68
C4R8X7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Superoxide dismutase [Cu-Zn]	0.61
C4R2G3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	FK506-binding protein	0.56
C4R537	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein that binds to cruciform DNA structures	0.5
C4QY12	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Suppressor protein STM1	0.47
C4QZY8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	RNAse	0.47
C4R9F6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.47
C4QVG3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.41
C4R312	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Dihydrolipoyl dehydrogenase	0.42
C4QW56	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.39
C4QYU3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Phosphotransferase	0.39
C4QZU2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cobalamin-independent methionine synthase, involved in amino acid biosynthesis	0.39
C4R6V3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear protein required for transcription of MXR1	0.39
C4R708	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Tetradecameric mitochondrial chaperonin	0.38
C4R1K7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein that binds tRNA and methionyl-and glutamyl-tRNA synthetases (Mes1p and Gus1p)	0.33
C4R1R2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Ribosomal protein 51 (Rp51) of the small (40s) subunit	0.33
C4R5R5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.33
P52710	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Carboxypeptidase Y	0.31
C4QYT0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Vacuolar proteinase B (YscB), a serine protease of the subtilisin family	0.31
C4QYX3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) subunit, essential for control of translational accuracy	0.3
C4R074	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA	0.3
C4R2Z2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein chaperone involved in regulation of the HSP90 and HSP70 functions	0.3
C4R3J1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear protein that binds to RNA and to Mex67p, required for export of poly(A)+ mRNA from the nucle	0.3
C4R3X5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Primary component of eisosomes	0.3
C4R5M4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial intermembrane space cysteine motif protein	0.3
C4R5P6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Succinate--CoA ligase [ADP-forming] subunit alpha, mitochondrial	0.3
C4R686	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutathione reductase	0.3
C4R282	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein with seven cysteine-rich CCHC zinc-finger motifs, similar to human CNBP	0.28
C4R887	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATPase involved in protein folding and nuclear localization signal (NLS)-directed nuclear transport	0.27
C4QW48	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.25
C4QZ51	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.24
C4R2U6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.25
C4R3T2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Major of three pyruvate decarboxylase isozymes	0.25
C4R430	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	6-phosphogluconate dehydrogenase, decarboxylating	0.25
C4R7U6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytochrome c oxidase subunit	0.25
C4R9E0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Alcohol dehydrogenase	0.25
C4QVU1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Thiol oxidase required for oxidative protein folding in the endoplasmic reticulum	0.25
C4QVY8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Translation initiation factor eIF4G, subunit of the mRNA cap-binding protein complex (EIF4F)	0.22
C4QW09	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis	0.22
C4QWA6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.22
C4QWH9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Isocitrate dehydrogenase [NADP]	0.22
C4QYE8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	40S small ribosomal subunit	0.22
C4QYV1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Primary component of eisosomes	0.22
C4QYY3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutamyl-tRNA synthetase, cytoplasmic	0.22
C4R1P7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Acetyl-coenzyme A synthetase	0.22
C4R1R9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Long chain fatty acyl-CoA synthetase with a preference for C12:0-C16:0 fatty acids	0.22
C4R2R2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.22
C4R736	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nucleolar protein that binds nuclear localization sequences	0.22
C4QW46	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutamate dehydrogenase	0.19
C4QY91	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.19
C4R0Q7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Major exo-1,3-beta-glucanase of the cell wall, involved in cell wall beta-glucan assembly	0.2
C4R184	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.19
C4R715	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.19
C4R8Z2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Coatomer subunit beta	0.19
C4R1C9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Ribosomal protein L15	0.19
C4QV89	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70)	0.2
C4R2P3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Methionine aminopeptidase 2	0.17
C4QV16	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl4Ap	0.17
C4QY72	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.16
C4QZ89	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.16
C4R0B4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Calnexin	0.17
C4R0P1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glyceraldehyde-3-phosphate dehydrogenase	0.16
C4R3H8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Enolase I, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosph	0.16
C4R4Y8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATP synthase subunit alpha	0.17
C4R5F9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytochrome c oxidase assembly protein/Cu2+ chaperone	0.16
C4R5T3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein with a potential role in actin cytoskeletal organization	0.17
C4R5X1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Homoserine kinase, conserved protein required for threonine biosynthesis	0.16
C4R6D0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Translation initiation factor eIF-4B, has RNA annealing activity	0.17
C4R6L9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytochrome c, isoform 1	0.17
C4R6U5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Putative transcription factor involved in regulating the response to osmotic stress	0.16
C4R8S2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Tyrosine--tRNA ligase	0.17
C4R8P5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic isoleucine-tRNA synthetase, target of the G1-specific inhibitor reveromycin A	0.16
C4R1X8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	0.17

C4QZC2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Phosphatidylglycerol/phosphatidylinositol transfer protein	0.16
C4R1C2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Key endocytic protein involved in a network of interactions with other endocytic proteins	0.16
C4R8L5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	GTP-binding protein	0.17
Q92448	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATP-dependent 6-phosphofructokinase subunit alpha	0.14
C4QVL4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	1,3-beta-glucanosyltransferase	0.14
C4QWH2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Vacuolar proteinase B (YscB), a serine protease of the subtilisin family	0.14
C4QY74	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit of cleavage factor I	0.14
C4QYQ8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit of the heterodimeric FACT complex (Spt16p-Pob3p)	0.14
C4R0W4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.14
C4R201	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Aminopeptidase	0.13
C4R262	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Actin-binding protein of the cortical actin cytoskeleton	0.14
C4R461	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Constituent of the mitochondrial inner membrane presequence translocase (TIM23 complex)	0.14
C4R4H3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Ornithine aminotransferase	0.14
C4R4V8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	cAMP-dependent protein kinase regulatory subunit	0.14
C4R5W4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.14
C4R6N7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein kinase involved in the response to oxidative and osmotic stress	0.14
C4R7R0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	One of six ATPases of the 19S regulatory particle of the 26S proteasome	0.14
C4QXJ0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Beta subunit of the translation initiation factor eIF2	0.14
C4R885	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.14
C4QVB5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Probable di-and tri-peptidase	0.14
C4QVX0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Delta subunit of the coatomer complex (COPI), which coats Golgi-derived transport vesicles	0.14
C4R300	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nucleoside diphosphate kinase	0.14
C4R646	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Transcription factor involved in cell-type-specific transcription and pheromone response	0.14
C4QXR4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of the holoenzyme form of RNA polymerase transcription factor TFIIH	0.14
C4QV80	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Dihydrolipoyl transsuccinylase, component of the mitochondrial alpha-ketoglutarate dehydrogenase	0.11
C4QW50	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein involved in negative regulation of transcription of iron regulon	0.11
C4QXW3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4QYF5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of mRNP complexes associated with polyribosomes	0.11
C4QZB0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Elongation factor 1-alpha	0.11
C4QZE7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl7Ap and has similarit	0.11
C4QZS3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATPase involved in protein import into the ER, also acts as a chaperone to mediate protein folding i	0.11
C4R0N8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R1S5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Presumed helicase required for RNA polymerase II transcription termination and processing of RNAs	0.11
C4R2S0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	0.11
C4R2X4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial import inner membrane translocase subunit TIM50	0.11
C4R5A8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Part of actin cytoskeleton-regulatory complex Pan1p-Sla1p-End3p	0.11
C4R5K2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic glyoxalase II	0.11
C4R6F1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R6Z0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit	0.11
C4R7F0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein interacting with poly(A)-binding protein Pab1p	0.11
C4R7I8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R7T7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl13Bp	0.11
C4R7T1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R9C0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.11
C4R5T7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R650	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic mRNA cap binding protein	0.11
C4R6Y3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit	0.11
C4R901	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	NADPH--cytochrome P450 reductase	0.11
C4R8L6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	GTPase-activating protein (RhoGAP) for Rho3p and Rho4p	0.11
C4QVA4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R8S3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	One of six ATPases of the 19S regulatory particle of the 26S proteasome	0.11
C4QVQ4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Peptidyl-prolyl cis-trans isomerase	0.11
C4QXM0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.11
C4R0E2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Methionyl-tRNA synthetase, forms a complex with glutamyl-tRNA synthetase (Gus1p) and Arc1p	0.11
C4R2S5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Peroxidase	0.11
C4R4M5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Alanine--tRNA ligase	0.11
C4R7L8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein that interacts with Spt6p and copurifies with Spt5p and RNA polymerase II	0.11
C4R8X6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutamine synthetase	0.11
C4QWN4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.11
C4QZ92	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	60S ribosomal protein L36	0.11
Q9P4D1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Actin	0.08
C4QXU3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Essential phosphoprotein component (P150) of the COPII coat of secretory pathway vesicles	0.08
C4QXU4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.09
C4QZ09	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Actin assembly factor, activates the Arp2/3 protein complex that nucleates branched actin filaments	0.08
C4R2Q1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p	0.08
C4R348	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	One of several homologs of bacterial chaperone DnaJ, located in the ER lumen	0.08
C4R3T7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, identical to Rpl42Ap and has similarity to r	0.08
C4R3X8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATPase involved in protein folding and the response to stress	0.08
C4R4K7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Putative dihydrokaempferol 4-reductase	0.08
C4R796	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear type II J heat shock protein of the E. coli dnaJ family	0.08
C4R938	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein disulfide isomerase, multifunctional protein resident in the endoplasmic reticulum lumen	0.08
C4R4X4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Elongation factor Tu	0.08
C4QZ47	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4QZA7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Vacuolar protein sorting-associated protein 27	0.08
C4R1H5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4QY71	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit	0.08
C4R760	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Major ADP/ATP carrier of the mitochondrial inner membrane	0.08
C4R5L9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4R4X1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ADP-ribosylation factor GTPase activating protein (ARF GAP)	0.08
C4R339	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Pyruvate carboxylase	0.08
C4R2D3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.09
C4R7G7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4R547	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Tryptophan synthase	0.08
C4R6C8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4R7R8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytosolic leucyl tRNA synthetase, ligates leucine to the appropriate tRNA	0.08
C4QWQ9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Rho GDP dissociation inhibitor involved in the localization and regulation of Cdc42p	0.08
C4R0T7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Ribosomal protein 59 of small subunit, required for ribosome assembly and 20S pre-rRNA processing	0.08
C4R1A7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Chorismate synthase	0.08

C4R3C8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cysteine desulfurase involved in iron-sulfur cluster (Fe/S) biogenesis	0.08
C4R5Z3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4R641	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutamate decarboxylase	0.08
C4R6N9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Translational elongation factor 3, stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes	0.08
C4R3E7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	60S ribosomal protein L20	0.08
C4R0V9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Peroxiredoxin	0.08
C4R1T4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit of the HIR complex, a nucleosome assembly complex	0.09
C4QX18	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear protein involved in asymmetric localization of ASH1 mRNA	0.08
C4R376	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.08
C4R707	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Histone H2A	0.08
C4R0M7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Histone H2B	0.08
C4QWE4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Aspartate aminotransferase	0.05
C4QZS2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial ribosomal protein of the small subunit	0.06
C4R1D1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Enzyme of 'de novo' purine biosynthesis	0.05
C4R2H4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Guanylate kinase, converts GMP to GDP	0.05
C4R6J5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear envelope protein, interacts with GDP-bound Gsp1p and with proteins of the nuclear pore	0.06
C4QVA2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.06
C4QVT8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Fatty acid synthase subunit beta	0.05
C4QZT4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.06
C4QZU0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Anthranilate synthase, catalyzes the initial step of tryptophan biosynthesis, forms multifunctional	0.05
C4R306	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Phosphomannomutase	0.05
C4R570	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Aconitate hydratase, mitochondrial	0.05
C4QVA8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.05
C4QWS5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Diadenosine 5',5''-P1,P4-tetraphosphate phosphorylase II (AP4A phosphorylase)	0.06
C4R099	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glucose-6-phosphate 1-dehydrogenase	0.05
C4QXV1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl34Bp	0.06
C4QXZ1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway	0.05
C4R4A2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Non-specific serine/threonine protein kinase	0.05
C4QW14	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Regulatory subunit of type 1 protein phosphatase Glc7p	0.05
C4R4J6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	5'-3' exoribonuclease 1	0.06
C4QXF2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic aspartyl-tRNA synthetase, homodimeric enzyme	0.05
C4R259	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Co-chaperone that stimulates the ATPase activity of the HSP70 protein Ssc1p	0.05
C4R2H3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Thioredoxin peroxidase, acts as both a ribosome-associated and free cytoplasmic antioxidant	0.05
C4R3R8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Arginine biosynthesis bifunctional protein ArgJ, mitochondrial	0.05
C4QW19	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	RNA-binding protein that carries poly(A)+ mRNA from the nucleus into the cytoplasm	0.06
C4QW75	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.06
C4QWX1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein that interacts with Cdc48p and Npl4p, involved in recognition of polyubiquitinated proteins	0.05
C4QXG9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.06
C4QYF6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase)	0.05
C4QZG8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Hsp70 (Ssa1p) nucleotide exchange factor, cytosolic homolog of Sil1p, which is the nucleotide exchan	0.05
C4QZL6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step	0.06
C4R0T4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	dUTPase, catalyzes hydrolysis of dUTP to dUMP and PPi	0.06
C4R2M5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.06
C4R3G3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Carnitine acetyl-CoA transferase present in both mitochondria and peroxisomes, transfers activated a	0.06
C4R410	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.05
C4R4E1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial intermembrane space protein	0.05
C4R526	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit Va of cytochrome c oxidase	0.05
C4R546	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Carboxypeptidase	0.06
C4R8G8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.05
C4R3I6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl19Ap and has similari	0.05
C4QXH9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.05
C4R2U4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit	0.05
C4R2N9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Adenosine kinase, required for the utilization of S-adenosylmethionine (AdoMet)	0.05
C4QWM6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	6-phosphogluconolactonase-like protein	0.06
C4QXL0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Alpha aminoadipate reductase	0.06
C4R3P1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glucose-repressible protein kinase involved in signal transduction during cell proliferation in resp	0.06
C4R8H9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Acetylornithine aminotransferase	0.05
C4R577	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.05
C4QYG2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.06
C4R566	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Phosphatidylinositol/phosphatidylcholine transfer protein	0.06
C4QV38	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	rRNA-processing protein	0.05
C4R0S8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial alcohol dehydrogenase isozyme III	0.05
C4R666	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nexin-1 homolog required for localizing membrane proteins	0.06
C4R3Z9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of the chromatin assembly complex (With Rlf2p and Msi1p)	0.05
C4R6X3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit beta of the cytosolic chaperonin Cct ring complex, related to Tcp1p	0.05
C4QXS0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	RNA helicase in the DEAH-box family involved in the second catalytic step of splicing, exhibits ATP	0.03
C4R0G3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoskeletal protein binding protein required for assembly of the cortical actin cytoskeleton	0.03
C4QXW1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Acetyl-CoA carboxylase, biotin containing enzyme	0.03
C4R1M6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Allantoicase, converts allantoate to urea and ureidoglycolate	0.03
C4R0F5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R444	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.03
C4R4W6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R7J7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Methionine and cysteine synthase (O-acetyl homoserine-O-acetyl serine sulfhydrylase)	0.03
C4QZ37	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Mitochondrial respiratory chain complexes assembly protein RCA1	0.03
C4R4T7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Essential component of the nuclear pore complex	0.03
C4R722	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	E3 ubiquitin protein ligase	0.03
C4R0G5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	3-isopropylmalate dehydratase	0.03
C4QZQ0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	0.03
C4QVD6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	High osmolarity signaling protein SHO1	0.03
C4QVD4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein implicated in polar growth, functionally redundant with Boi1p	0.03
C4QWG1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QWI0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QWK1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	GTPase	0.03
C4QWU8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the large (60S) ribosomal subunit	0.03
C4QYN4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	40S ribosomal protein subunit	0.03
C4QZE9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03

C4R007	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nicotinate phosphoribosyltransferase	0.03
C4R048	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R0N1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R101	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Putative benzil reductase	0.03
C4R1A2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles	0.03
C4R1H2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R2Z5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p) that may participate in DNA replication	0.03
C4R453	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein required for proper cell fusion and cell morphology	0.03
C4R5D9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytidine deaminase	0.03
C4R5R4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic response regulator, part of a two-component signal transducer that mediates osmosensing	0.03
C4R7U9	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nucleolar GTP-binding protein 2	0.03
C4R7Z0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R8R1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Thioredoxin	0.03
C4QZ61	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Small nuclear ribonucleoprotein G	0.03
C4QWN1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Xylulokinase, converts D-xylulose and ATP to xylulose 5-phosphate and ADP	0.03
C4QZV6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Alpha-1,4 glucan phosphorylase	0.03
C4R5F1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Transcription initiation factor IIE subunit beta	0.03
C4R382	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QZ53	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R5Z8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QWV4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R781	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Copper-binding protein of the mitochondrial inner membrane	0.03
C4R1K3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Putative transporter, member of the sugar porter family	0.03
C4R4N5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R922	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Myo-inositol transporter with strong similarity to the minor myo-inositol transporter Itr2p	0.03
C4QZF4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Putative kinase, suppressor of GTPase mutant, similar to bovine rhodopsin kinase	0.03
C4R7M5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Adenylate cyclase, required for cAMP production and cAMP-dependent protein kinase signaling	0.03
C4QVL2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R1V5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Subunit 2 of the ubiquinol cytochrome-c reductase complex	0.03
C4R8Z0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Nuclear protein related to mammalian high mobility group (HMG) proteins	0.03
C4QVK2	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Deoxyhypusine synthase, catalyzes formation of deoxyhypusine	0.03
C4QV10	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Cytoplasmic inorganic pyrophosphatase (PPase)	0.03
C4QZP0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Fructose-2,6-bisphosphatase, required for glucose metabolism	0.03
C4R0C5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Probable cochaperone, regulates activity of Cyr1p (Adenylyl cyclase)	0.03
C4R107	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Bisphosphate-3'-nucleotidase, involved in salt tolerance and methionine biogenesis	0.03
C4R1W5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Vacuolar membrane protein involved in vacuolar polyphosphate accumulation	0.03
C4R1J7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R4N4	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Aspartic beta semi-aldehyde dehydrogenase	0.03
C4R2S1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Catalase	0.03
C4R6K6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QWZ3	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Argininosuccinate lyase, catalyzes the final step in the arginine biosynthesis pathway	0.03
C4R194	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Glutamine-fructose-6-phosphate amidotransferase	0.03
C4QZA6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein ROT1	0.03
C4R0D6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QW98	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R130	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein involved in error-free postreplication DNA repair	0.03
C4R5L7	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Large subunit of carbamoyl phosphate synthetase	0.03
C4R498	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R3Q6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Arginyl-tRNA synthetase	0.03
C4R5B6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R5R6	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p)	0.03
C4QXA5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	ATP-dependent 6-phosphofructokinase subunit beta	0.03
C4R241	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Metalloprotease subunit of the 19S regulatory particle of the 26S proteasome lid	0.03
C4R310	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of the RSC chromatin remodeling complex	0.03
C4QWZ5	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R162	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4QY47	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Ribosomal RNA-processing protein 8	0.03
C4R389	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03
C4R8A0	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Phosphatidylinositol transfer protein (PITP)	0.03
C4R7T8	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Protein component of the small (40S) ribosomal subunit	0.03
C4R024	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Malate dehydrogenase	0.03
C4R4Q1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Kynureninase	0.03
C4R476	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Component of the commitment complex	0.03
C4R7E1	*Komagataella phaffii* (strain GS115 / ATCC 20864) OX=644223	Uncharacterized protein	0.03

GRAS Panel Evaluation of Oubli Fruit Sweet Protein (Brazzein) for Uses in Conventional Food and Beverage Products

6 December 2022

INTRODUCTION

Oobli, Inc. convened a panel of independent scientists (the GRAS Panel), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of data and information pertinent to the safety of the company's Oubli fruit sweet protein (hereinafter referred to as "brazzein") and to determine whether the intended uses of brazzein in conventional food and beverage products as a sweetener as described in Table A-1 would be Generally Recognized as Safe (GRAS) based on scientific procedures.

A GRAS Panel was selected and convened in accordance with the U.S. Food and Drug Administration (FDA)'s draft guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S. FDA, 2017). Oobli confirms that prior to convening the GRAS Panel, all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in appropriate scientific disciplines deemed necessary for the safety evaluation of brazzein, and efforts were placed on identifying conflicts of interest or relevant appearance issues that would potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance of conflicts were identified. The GRAS Panel received reasonable honoraria as compensation for its time, and honoraria provided to the GRAS Panel were not contingent upon the outcome of the GRAS Panel's deliberations. The GRAS Panel consisted of the below-signed qualified scientific experts: Professor Joseph Baumert (University of Nebraska-Lincoln); Professor Emeritus George C. Fahey, Jr. (University of Illinois); and Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison).

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of publicly available scientific data and information compiled from the literature and summarized in a dossier titled "*Documentation Supporting the GRAS Use of Oubli Fruit Sweet Protein (Brazzein) in Conventional Food and Beverage Products*" (dated 11 November 2022), which included an evaluation of available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food and beverage uses of brazzein. This information was prepared in part from a comprehensive search of the scientific literature conducted through December 2022 and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, and the safety of brazzein. A summary of the information critically evaluated by the GRAS Panel is presented below.

SUMMARY AND BASIS FOR GRAS

Brazzein is a naturally occurring sweet protein present in the fruit of the West African Oubli plant (*Pentadiplandra brazzeana*). The sweet taste of brazzein remains stable over pH 2.5 to 8 and upon heating up to 80°C for 4.5 hours or 98°C for 2 hours (Stone and Oliver, 1969). Brazzein binds to the taste receptors T1R2 and T1R3, with the binding mechanism similar to other sweet proteins such as thaumatin (Walters and Hellekant *et al.,* 2006; Belloir *et al.,* 2017; Kim *et al.,* 2022). Two isoforms of brazzein exist in nature: major isoform with 54 amino acids (~80%) and minor isoform with 53 amino acids (~20%) (Neiers *et al.,* 2021).

Oobli manufactures the brazzein 53-amino acid isoform through precision fermentation of a genetically modified (GM) strain of *Komagataella phaffi* (formerly *Pichia pastoris*) that has been codon-optimized to improve protein expression using standard biotechnology practices. The identity of the active constituent of Oobli's brazzein has been demonstrated to be the 53-amino acid isoform and substantially equivalent to the naturally occurring protein *via* MS/MS peptide mapping and intact protein mass spectrometry. The protein has a sweetness potency of approximately 750 times that of a 5% sucrose solution on a weight-weight basis as determined by Oobli in a sensory panel.

The GRAS Panel critically evaluated the manufacturing process for brazzein. Oobli's brazzein is produced through precision fermentation of a GM strain of *K. phaffi* and follows standard fermentation processes that are closely controlled and monitored. At the end of fermentation, the whole cell broth is processed to recover, separate, and purify the brazzein ingredient. These downstream processing steps include solid-liquid separation steps to separate the wet biomass from the fermentation supernatant containing brazzein. This supernatant is then concentrated and further purified using a sequence of filtration, chromatography, and diafiltration steps. The resulting concentrated brazzein solution is then dried into a powder consisting of at least 30% (w/w) brazzein. The manufacturing process complies with current Good Manufacturing Practice (cGMP) and Hazard Analysis and Critical Control Points (HACCP) principles. All materials used in the manufacturing process are food-grade and of high purity and quality, and have been concluded to be GRAS and suitable for their intended purpose.

The GRAS Panel noted that the production strain is derived from *K. phaffii* BG10, which originates from the well-characterized host organism, *K. phaffii* NRRL Y-11430. *K. phaffii* NRRL Y-11430 is recognized as a non-pathogenic and non-toxigenic microorganism that has not been implicated with any known adverse effects. *K. phaffi* NRRL Y-11430 is suitable for use in food production as a production organism. The GRAS Panel noted that proteins originating from this strain has been demonstrated to lack allergenic and toxigenic potential (Jin *et al*., 2018; Reyes *et al*., 2021). Oobli's production strain derived from *K. phaffii* BG10 is genetically modified to express copies of brazzein-encoding genes under the presence of promoter and terminator genes from *K. phaffii*. The gene encoding for brazzein was identified from a publicly available protein sequence database and synthesized *de novo*. The brazzein expression cassettes were stably integrated into the *K. phaffii* genome and demonstrated that no plasmids or antibiotic resistance genes were present in the production strain by plate streaking and colony polymerase chain reaction (PCR). The genetic stability of the production strain was confirmed by Sanger sequencing before and after the fermentation process.

Food-grade specifications have been established for brazzein. These specifications include limits for the proximate profile, brazzein content, heavy metals, and microbial contaminants. All analytical methods are validated and fit-for-purpose. The GRAS Panel reviewed the results of 4 production batches of brazzein and concluded that the manufacturing process yields a consistent product that conforms to the established product specifications. The mineral profile of the ingredient is well characterized and based on the proposed food uses of the brazzein ingredient, the resulting dietary exposures to these minerals in final consumers were well below the corresponding Institute of Medicine's upper limit for each mineral, as available. Therefore, the mineral content of brazzein would not pose a safety concern to the final consumer under the conditions of intended use.

The GRAS Panel reviewed the data related to the shelf-life stability of Oobli's brazzein, which indicate no significant change in the brazzein content or proximates and microbiological profile when the ingredient is stored at 23.5°C and 40% relative humidity for up to 9 months.

Brazzein is intended for use as a food ingredient in a variety of conventional foods and beverages as a sweetener (see Table A-1). The GRAS Panel reviewed data related to the estimated dietary intakes of brazzein in the U.S. population under the described conditions of use. The dietary intake of the ingredient was estimated using consumption data from the 2017-2018 cycle of the National Health and Nutrition Examination Survey (NHANES) combined with the maximum use level of the ingredient for each intended use. Among the total population (2 years and older), the mean and 90th percentile consumer-only intakes of brazzein were determined to be 89 and 199 mg/person/day, respectively. On a body weight basis, the total population (2 years and older) mean and 90th percentile consumer-only intakes of brazzein were determined to be 1.30 and 2.88 mg/kg body weight/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of brazzein on an absolute basis, at 119 and 260 mg/person/day, respectively, equivalent to 1.33 and 3.11 mg/kg body weight/day, respectively. Children 2 to 5 years old had the lowest mean and 90th percentile consumer-only intakes of 35 and 76 mg/person/day, respectively, equivalent to 2.03 and 4.25 mg/kg body weight/day, respectively.

The GRAS Panel reviewed scientific information supporting the safety of brazzein and the production organism, *K. phaffi* strain NRRL Y-11430 BG10, using the principles described by Pariza and Johnson (2001) and Sewalt *et al.* (2016). This included information on characterization of the production organism and the genetic modification steps to obtain the production organism, as well as *in vitro* digestibility studies on brazzein, *in silico* assessments of allergenicity and toxigenicity potential of the protein, as well as published product-specific pivotal toxicology studies (Lynch *et al.,* 2023). These elements are discussed as follows.

The GRAS Panel reviewed information on the production strain demonstrating that the organism is derived from the host organism *K. phaffii* strain BG10, a derivative of *K. phaffii NRRL* Y-11430, a Biosafety Level 1 organism. This host organism is a non-pathogenic and non-toxigenic species with an established history of safe use in food production, and therefore, considered to be a safe and suitable source organism for the production of brazzein. The GRAS Panel noted that the genetic modification steps to obtain the production strain is well characterized and does not introduce any exogenous factors into the production organism that would pose a safety concern. The introduced genes encode for brazzein and does not encode for any genetic element that would pose a pathogenic, allergenic, or toxigenic risk. The GRAS Panel concluded that there are no safety concerns with the production organism used to produce Oobli's brazzein.

The GRAS Panel reviewed the results of two *in vitro* digestibility studies conducted with Oobli's brazzein. The first study followed the methods described by Thomas *et al.* (2004), and the results indicate that brazzein was not digested at any tested pepsin concentration up to 30 minutes of incubation. The second digestibility study was conducted using the methodology described by Brodkorb *et al.* (2019) and Atallah *et al.* (2020) at enzyme concentrations of 0.05, 0.25, and 0.5 U/µg of brazzein for up to 120 minutes. Brazzein was partially digested after 10 minutes at concentrations of 0.05, 0.25, and 0.5 U/µg. Based on a semiquantitative analysis from the SDS-PAGE gel, after 120 minutes, approximately 26%, 46%, and 67% of brazzein was digested by pancreatin concentrations of 0.05, 0.25, and 0.5 U/µg, respectively. The GRAS Panel concluded that brazzein is partially digested under simulated intestinal conditions.

A comprehensive search of the scientific literature was conducted through December 2022 to identify publications on the safety of brazzein and the plant source (*Pentadiplandra brazzeana*). The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile®. Two publications were identified (Kim *et al.,* 2020; Lynch *et al.,* 2023).

In the first study, mice were provided a 3M-brazzein solution in the drinking water over 15 weeks and obesity-related endpoints were observed (Kim *et al.*, 2020). No significant effects on adiposity hypertrophy, glucose homeostasis, insulin resistance, or inflammation were observed throughout the study period. The GRAS Panel noted that this study did not evaluate standard toxicological endpoints such as those described in Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 and did not report on the purity of brazzein or its source. Therefore, the GRAS Panel did not consider this study to be relevant to the safety discussion of Oobli's brazzein.

In the second study by Lynch *et al.* (2023) described the results of a bacterial reverse mutation test, an *in vitro* micronucleus test, and a 90-day oral (dietary) toxicity study in rats that were conducted with Oobli's brazzein. The studies were conducted in accordance with appropriate OECD Test Guidelines and Principles of Good Laboratory Practice (GLP).

In the bacterial reverse mutation test, brazzein did not induce any biologically relevant, concentration-related, or statistically significant increases in revertant colony numbers at test concentrations up to 5,000 µg/plate compared with vehicle control counts in either the presence or absence of metabolic activation. In the *in vitro* micronucleus test, a statistically significant increase in the percentage of micronucleated cells was observed at the highest concentration of 2,000 µg/mL with metabolic activation when compared with vehicle controls. However, the number of micronucleated cells was within the range of the historical negative control; therefore, this increase was regarded as not biologically relevant. In the long-term experiment, no biologically relevant increases in the percentage of micronucleated cells were reported at any of the concentrations analyzed. Based on the results of these two *in vitro* studies, the GRAS Panel concluded that brazzein does not have any mutagenic or clastogenic or aneugenic potential.

In the 90-day oral (dietary) toxicity study, adult Sprague-Dawley rats (10/sex/group) were provided brazzein in the diet at concentrations of 0 (control diet), 250 (low-dose), 500 (mid-dose), and 1,000 (high-dose) mg/kg body weight/day for 90 days. The dietary concentrations of brazzein achieved the targeted nominal intake values of 250, 500, and 1,000 mg/kg body weight/day (actual values: 0, 245, 490, and 978 mg/kg body weight/day and 0, 245, 493, and 985 mg/kg body weight/day in males and females, respectively). With the exception of statistically significant changes in hematology, clinical chemistry, and organ weight biomarkers, no treatment-related toxicological effects on body weights, body weight gain, food consumption, functional observation battery (*i.e.,* sensorimotor function, grip strength, and locomotor activity), ophthalmological findings, urinalysis, macroscopic, and histopathological findings. No mortality was reported in any test group. The statistically significant changes in hematology, clinical chemistry, and organ weight parameters were within the historical control range for the testing laboratory, or they occurred in a non-dose-related manner, or were not associated with any adverse histopathological findings. Therefore, the GRAS Panel concluded that these findings were not toxicologically relevant. The NOAEL was concluded to be 978 mg/kg body weight/day in males and 985 mg/kg body weight/day in females, the highest tested dose. Based on the reported NOAEL and the highest estimated dietary intake of 4.25 mg/kg body weight/day (90^{th} percentile in children 2 to 5 years of age), an approximately 230-fold margin of exposure (MOE) can be estimated.

The GRAS Panel reviewed a proteomics assessment of the final brazzein ingredient using LC-MS/MS, which indicated that the ingredient contains 6 host cell proteins each at levels above 1%, accounting for up to 5.6% of the final ingredient. The amino acid sequence of brazzein and the residual host cell proteins were investigated for their allergenicity potential using the stepwise approach described by FAO/WHO (2001) and Codex Alimentarius (2009), as well as their toxigenicity potential using the Animal Toxin Annotation Project maintained in the UniProtKB database. The allergenicity searches were conducted against the curated databases of AllergenOnline and Allermatch, and a support-vector machine (SVM) search was conducted

with AlgPred. No matches between brazzein and putative allergens were identified sharing greater than 35% identity or 8-amino acid exact matches, indicating that brazzein would be unlikely to have any allergenic cross-reactivity. In the full-length amino acid searches, brazzein shared >50% identity with a number of sequences from the AllergenOnline and Allermatch databases. However, the GRAS Panel noted that the corresponding E-values and bit-scores suggest the findings to not be statistically significant, and may not be suggestive of allergenic cross-reactivity. In the -amino acid sliding window searches with the residual host cell proteins, two proteins shared a number of matches to putative allergens from fungal species such as *Fusarium proliferatum, Pencillium chrysogenum, Cladosporium cladosporioides, Rhizopus oryzae,* and *Aspergillus fumigatus*, as well as mosquito (*Aedes aegypti*) and wild boar (*Sus scrofa*). The identified matches were to respiratory fungal allergens, or dermal allergens (from mosquitoes), or oral allergens (from wild boars). The GRAS Panel noted that no matches were identified to major food allergens that are of relevance to final consumers, and the allergenic potential of residual host cell proteins from production strains derived from *K. phaffii* NRRL Y-11430 and BG10 have been previously discussed in the scientific literature (Jin *et al.*, 2018; Reyes *et al.*, 2021). Considering that Oobli's production strain is derived from *K. phaffii* BG10, and the genetic modifications to the host organism is well characterized and do not pose any allergenic concern, the GRAS Panel concluded that any native residual proteins from the host organism would pose a low risk for allergenicity. The SVM searches using AlgPred predicted mixed results for brazzein and the residual host cell proteins. The GRAS Panel noted that AlgPred has been used previously for the allergenicity assessment of soy leghemoglobin obtained from a genetically modified strain of *P. pastoris* as described in GRN 737 (U.S. FDA, 2018), and concluded that the reliability of the SVM-based analysis is controversial due to reliability of the method to accurately predict the allergenicity potential of a protein. Based on the totality of evidence, the GRAS Panel concluded that brazzein and the residual host cell proteins in the final ingredient pose a low risk for allergenicity to final consumers. Collectively, the results of the *in silico* assessment of allergenicity along with the absence of any adverse effects in the 90-day dietary toxicity study demonstrate that the intact protein and any potential undigested peptides of Oobli's brazzein do not pose any safety or allergenicity concern to final consumers.

In addition to the allergenicity searches, brazzein and the residual host cell proteins were investigated for significant sequence homology with known protein toxins using BLAST. A curated database of known animal venom proteins and toxins maintained by UniProt was searched against the amino acid sequence of brazzein and the residual host cell proteins using default parameters. Sequences were considered to share structural homology/similarity based on the criteria described by Pearson (2013). No significant similarity to any of the venom proteins and toxins were identified (E-values >0.0015) in the homology searches with brazzein and the residual *Komagataella* proteins. These findings suggest that brazzein and any residual *Komagataella* protein does not harbor any toxigenic potential based on its amino acid sequence.

Based on the technical and scientific data and information presented herein, Oobli, Inc. has concluded that the Oubli fruit sweet protein (brazzein) produced from a genetically modified strain of *Komagataella phaffii* is GRAS for use in conventional food and beverage products on the basis of scientific procedures. Brazzein 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*.

CONCLUSIONS OF THE GRAS PANEL

We, the GRAS Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the proposed uses of brazzein as an ingredient in conventional foods and beverages, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice, are Generally Recognized as Safe (GRAS) based on scientific procedures.

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

[redacted]

Professor Joseph Baumert, Ph.D.
University of Nebraska-Lincoln

[redacted]

Date

[redacted]

Professor Emeritus George C. Fahey, Jr., Ph.D.
University of Illinois

[redacted]

Date

[redacted]

Professor Emeritus Michael W. Pariza, Ph.D.
University of Wisconsin-Madison

January 23 2023

Date

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Table A-1 Summary of the Individual Proposed Food Uses and Use Levels for Brazzein in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2021b)	Food Uses[a]	Purified Brazzein Use Levels (mg/100 g) Based on 750x Sweetness Potency
Beverages, alcoholic	Cocktail drinks (pre-packaged)	7
Beverages and Beverages Bases, non-alcoholic	Packaged water-based beverages	22
	Non-milk-based meal replacement beverages and protein drinks	2
Chewing Gum	Chewing gum	99
Coffee and Tea	Ready-to-drink coffee beverages	7
	Ready-to-drink tea beverages	9
Dairy Product Analogs	Milk analogs	4
	Non-dairy yogurts	13
Frozen Dairy Desserts and Mixes	Ice cream	23
	Frozen yogurt	23
	Frozen milk desserts and bars	24
Fruit and Water Ices	Edible ices	24
	Sherbet	30
	Sorbet	31
Grain Products and Pastas	Cereal bars, granola bars, energy, protein, and meal replacement bars	30
	Granola	27
Milk Products	Packaged milk-based beverages	6
	Yogurt	12
	Yogurt drinks	9
Processed Fruits and Fruit Juices	Packaged fruit drinks, nectar, and fruit-based smoothies	16
Snack Foods	Fruit-based bars (without granola)	36
Soft Candy	Truffles	47
	Gummy bear	62

CFR = *Code of Federal Regulations*.

[a] Brazzein is intended for use in unstandardized products and products with standards of identity, as established under 21 CFR §130 to 169, do permit its addition.

From:	Jason Ryder
To:	Kampmeyer, Christopher
Cc:	Ali Wing; Naomi Sachs; Tina Wang
Subject:	[EXTERNAL] Re: Questions for GRN 001142
Date:	Wednesday, December 13, 2023 12:15:31 PM
Attachments:	image013.png image014.png image015.png image016.png image017.png image018.png GRAS Notice No. 001142_Technical Review Questions and Responses_121323-1.pdf

Dear Mr. Kampmeyer,

Attached please find Oobli, Inc.'s responses to the technical questions from the FDA's review of GRAS Notice No. 001142 shared on December 4, 2023. Note that we've set forth the FDA team's questions, followed by our responses.

Wishing you and the team a joyous and restful holiday season. We look forward to hearing from you again soon.

Best,

Jason

--
Jason Ryder
CTO & Co-Founder

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Oobli.com

Engage with us!

On Mon, Dec 4, 2023 at 1:52 PM Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Dear Dr. Ryder,

We noted some questions during our review of GRAS Notice No. 001142—please see the attachment in this email.

We respectfully request a response within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses. Thank you in advance for your attention to our comments.

Best regards,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

December 4, 2023

Dear Dr. Ryder:

During our review of GRAS notice (GRN) 001142, submitted by Oobli, Inc. (Oobli; "the notifier"), regarding the intended use of brazzein produced by *K. phaffii*, we noted the following questions. We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that timeframe or have questions, please contact me to discuss via email at Christopher.kampmeyer@fda.hhs.gov.

Chemistry Questions

1. Please state whether any of the raw materials used in the fermentation are major allergens or are derived from major allergens. If any of the raw materials used are major allergens or are derived from major allergens, please discuss why these materials do not pose a safety concern.

2. On p. 11 of notice, the final brazzein product is described to contain sodium salts that are used as stabilizing agents. Please identify the sodium compounds used in the production of brazzein. In addition, please provide a statement that all materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

3. The specifications on p. 16 of the notice include a limit of <0.5 mg/kg for arsenic, cadmium, lead, and mercury. We note that the results of the analyses (p. 17) of three batches of the notified substance for these heavy metals are well below the specified limit. We note that specifications help to ensure that the ingredient is being manufactured in accordance with good manufacturing practices. In addition, FDA's recent "Closer to Zero" initiative focuses on reducing dietary exposure to heavy metals from food. We request that specifications for heavy metals be as low as possible and consistent with the methods used and the results obtained from the batch analyses.

4. The structure depicted in Figure 2.1.2-3 (p. 11) appears to be the 54-amino acid isoform of brazzein that includes a N-terminal pyroglutamic acid. Please confirm whether the structure presented in the notice corresponds to the subject of the notice, *des*-pyroglutamic acid brazzein, or provide an amended figure with the correct structure.

Toxicology Questions

1. Is the sweetening effect of brazzein transient, similar to polysaccharide-based sweeteners, or are effects on taste receptors sustained for a significant duration? Please note that such activity has been reported in sensory studies of other protein-based sweeteners, such as miraculin.

Background information in the GRN that provides the basis for the following questions: A BLASTp of the brazzein sequence (PDB ID: 7W8E_A) shows that it has 100% identity with defensin-like protein.

Figure 6.3.1.1.-1 shows that the OFSP band is persistent throughout the entire period of incubation, indicating that brazzein is resistant to pepsin digestion at pH 2.0. Additionally, Figure 6.3.1.1.-2 shows that only about OFSP is not fully digested by pancreatin at pH 7.0 after a significant duration (180 min). Since brazzein has four disulfide bonds and disulfide bonds are difficult to hydrolyze even in acidic pH, it is understandable why brazzein may be resistant to digestion by pepsin in acidic pH. Brazzein from *Pentadiplandra brazzeana* is apparently also thermostable (PMID 7957951).

Since you state that the OFSP has >35% brazzein by weight, implying that there are significant amounts of other proteins, such as *P. pastoris* proteins, it is not clear what the digestion percentage represents, that of brazzein or other proteins in OFSP.

FDA notes that pepsin preferentially cleaves certain peptide bonds and not all. Hence, it is not clear that the *in silico* prediction of digestibility by PeptideCutter can be accurately applied to brazzein, which is resistant to pepsin digestion and nearly resistant to pancreatin digestion. PeptideCutter should more accurately predict pepsin digestibility for proteins that are denatured into a linearized/nearly linearized form following consumption with all/most of the peptide bonds exposed to pepsin action.

2. Since brazzein has 100% amino acid identity with defensin-like protein, does brazzein have potential "defensin-like" function that could be cause for a safety concern to the consumers, especially because brazzein is not digested in the GI tract following oral consumption? Please provide an explanation.

3. The stability of brazzein raises some questions that need to be addressed. In the literature, there are reports that resistance of proteins to gastric digestion and thermal stability may be correlative indicators for potential allergenic risk (e.g., PMID: 9631091, 30134536, FAO/WHO (2001)). However, other recent publications conclude that protein stability is relevant for allergenicity of some proteins, but not for all (e.g., PMIDs: 31063834, 33473251, 21906650). Please provide a scientific narrative that addresses why the stability of brazzein (digestion and thermal) is not a cause for a safety concern when consumed orally.

December 13, 2023

Chris Kampmeyer
Regulatory Review Scientist
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

Re: GRAS Notice No. 001142 - Technical Review Questions & Answers

Dear Chris:

We are writing to respond to the technical questions from the FDA's review of GRAS Notice No. 001142 attached to your email on December 4, 2023. Below please find Oobli, Inc.'s responses to the review team's questions. Note that we've set forth the FDA team's questions, followed by our responses.

Yours sincerely,

Jason

Jason Ryder, Ph.D.
CTO & Founder, Oobli, Inc.
510.684.5610
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CC: Ali M. Wing, CEO, Oobli
Naomi Sachs, CFO, Oobli
Tina Wang, Oobli

Chemistry Questions

QUESTION 1: Please state whether any of the raw materials used in the fermentation are major allergens or are derived from major allergens. If any of the raw materials used are major allergens or are derived from major allergens, please discuss why these materials do not pose a safety concern.

RESPONSE 1: The raw materials used in the fermentation media are not major allergens or derived from major allergens. Oobli obtains a Certificate of Analysis for each incoming raw material to ensure that they are absent of major allergens prior to use in the production process.

QUESTION 2: On p. 11 of notice, the final brazzein product is described to contain sodium salts that are used as stabilizing agents. Please identify the sodium compounds used in the production of brazzein. In addition, please provide a statement that all materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

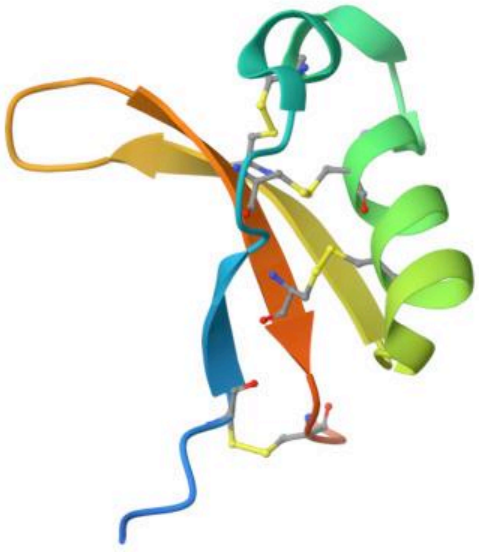
RESPONSE 2: The sodium compounds used as stabilizing agents in the final brazzein products (OFSP) are composed of sodium acetate and sodium chloride. All materials used in the manufacturing process are approved for their respective uses with respect to Part 21 of the U.S. Code of Federal Regulations and are GRAS for their respective use in the U.S.

QUESTION 3: The specifications on p. 16 of the notice include a limit of <0.5 mg/kg for arsenic, cadmium, lead, and mercury. We note that the results of the analyses (p. 17) of three batches of the notified substance for these heavy metals are well below the specified limit. We note that specifications help to ensure that the ingredient is being manufactured in accordance with good manufacturing practices. In addition, FDA's recent "Closer to Zero" initiative focuses on reducing dietary exposure to heavy metals from food. We request that specifications for heavy metals be as low as possible and consistent with the methods used and the results obtained from the batch analyses.

RESPONSE 3: The specifications of heavy metals have been revised to 0.1 ppm for cadmium, lead, and mercury, and 0.2 ppm for arsenic.

QUESTION 4: The structure depicted in Figure 2.1.2-3 (p. 11) appears to be the 54-amino acid isoform of brazzein that includes a N-terminal pyroglutamic acid. Please confirm whether the structure presented in the notice corresponds to the subject of the notice, des-pyroglutamic acid brazzein, or provide an amended figure with the correct structure.

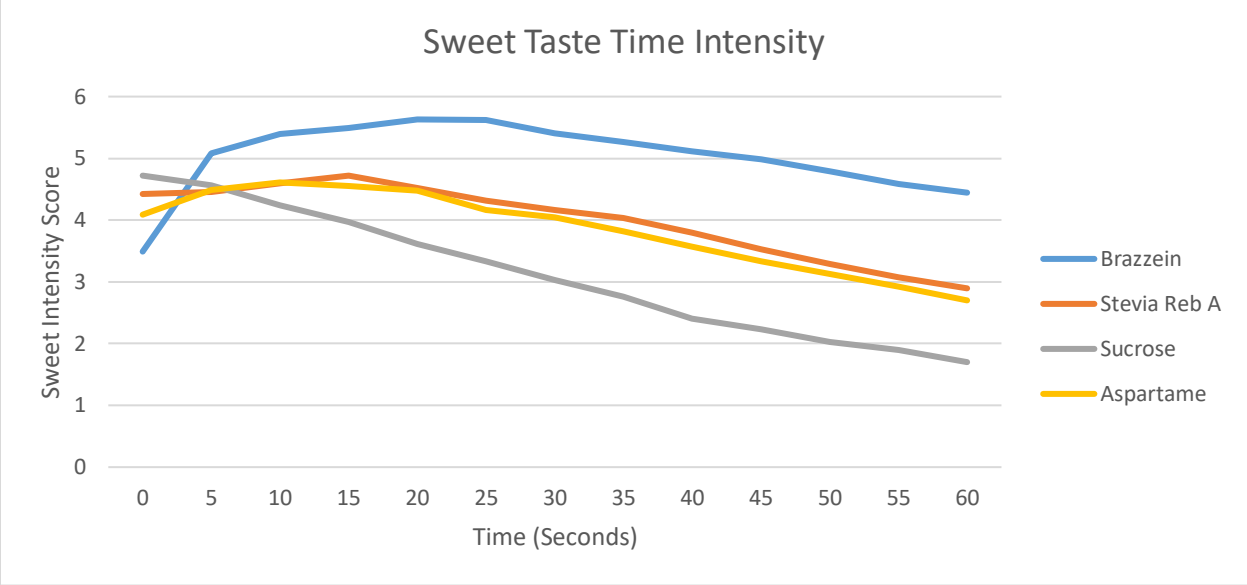
RESPONSE 4: Thank you for noticing the discrepancy. The correct 3D structure of brazzein-53 (des-pyroglutamic acid brazzein) is available on PDB (No. 7W8E) as follows:



Toxicology Questions

QUESTION 1: Is the sweetening effect of brazzein transient, similar to polysaccharide-based sweeteners, or are effects on taste receptors sustained for a significant duration? Please note that such activity has been reported in sensory studies of other protein-based sweeteners, such as miraculin.

RESPONSE 1: The sweetening effect of brazzein was investigated in a time-intensity sensory evaluation. Trained panelists (N=10, 2 replicates) were provided OFSP (brazzein purity 39.7%), Reb A, sucrose, and aspartame solutions at equivalent sweetness. The results are shown in the figure below. Brazzein reached a peak sweetness intensity that was greater than the other sugars at 20 seconds, which then progressively declined within 60 seconds, the length of the experiment. The negative slope trend in the figure below indicates rapid decrease of sweetness after 30 seconds from consumption. These findings suggest that the sweetening effect of brazzein is transient and are not sustained.



Background information in the GRN that provides the basis for the following questions: A BLASTp of the brazzein sequence (PDB ID: 7W8E_A) shows that it has 100% identity with defensin-like protein.

Figure 6.3.1.1.-1 shows that the OFSP band is persistent throughout the entire period of incubation, indicating that brazzein is resistant to pepsin digestion at pH 2.0. Additionally, Figure 6.3.1.1.-2 shows that only about OFSP is not fully digested by pancreatin at pH 7.0 after a significant duration (180 min). Since brazzein has four disulfide bonds and disulfide bonds are difficult to hydrolyze even in acidic pH, it is understandable why brazzein may be resistant to digestion by pepsin in acidic pH. Brazzein from Pentadiplandra brazzeana is apparently also thermostable (PMID 7957951).

Since you state that the OFSP has >35% brazzein by weight, implying that there are significant amounts of other proteins, such as P. pastoris proteins, it is not clear what the digestion percentage represents, that of brazzein or other proteins in OFSP.

FDA notes that pepsin preferentially cleaves certain peptide bonds and not all. Hence, it is not clear that the in silico prediction of digestibility by PeptideCutter can be accurately applied to brazzein, which is resistant to pepsin digestion and nearly resistant to pancreatin digestion. PeptideCutter should more accurately predict pepsin digestibility for proteins that are denatured into a linearized/nearly linearized form following consumption with all/most of the peptide bonds exposed to pepsin action.

QUESTION 2: Since brazzein has 100% amino acid identity with defensin-like protein, does brazzein have potential "defensin-like" function that could be cause for a safety concern to the consumers, especially because brazzein is not digested in the GI tract following oral consumption? Please provide an explanation.

RESPONSE 2: The amino acid sequence of brazzein-53, as provided in Section 2.1.2 of GRN 1142, was searched against the non-redundant protein database of NCBI's Genbank using BLASTp. The results are as follows:

Sequences producing significant alignments Download Select columns Show 100

select all *9 sequences selected* GenPept Graphics Distance tree of results Multiple alignment MSA Viewer

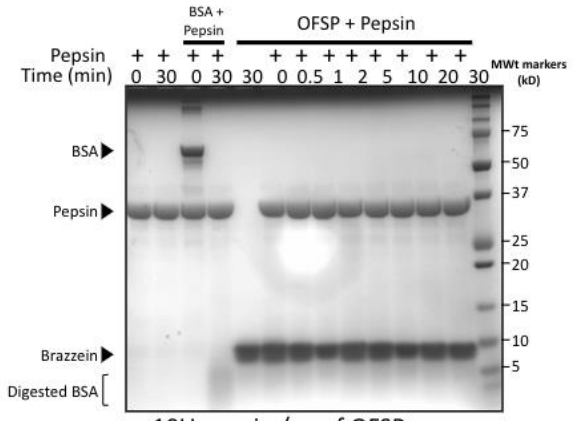
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
brazzein [synthetic construct]	synthetic construct	107	107	100%	5e-29	100.00%	54	AGM38242.1
RecName: Full=Defensin-like protein; AltName: Full=Brazzein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	107	107	100%	5e-29	100.00%	54	P56552.1
Chain A, Brazzein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	107	107	100%	6e-29	100.00%	53	2KGQ_A
Chain A, BRAZZEIN [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	107	107	100%	7e-29	100.00%	54	1BRZ_A
Chain A, Defensin-like protein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	106	106	100%	2e-28	98.11%	69	7W8E_A
Chain A, Defensin-like protein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	105	105	100%	4e-28	98.11%	54	7W8H_A
Chain A, Defensin-like protein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	105	105	100%	5e-28	98.11%	54	2N66_A
Chain A, Defensin-like protein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	102	102	100%	4e-27	96.36%	56	2N69_A
Chain A, Defensin-like protein [Pentadiplandra brazzeana]	Pentadiplandra brazzeana	100	100	94%	2e-26	98.00%	53	2KYQ_A

We note that a number of hits to "defensin-like proteins" sharing 98% to 100% identity with 94% to 100% query coverage was identified, suggesting that Oobli's OFSP containing brazzein-53 may contain similar activity as defensins.

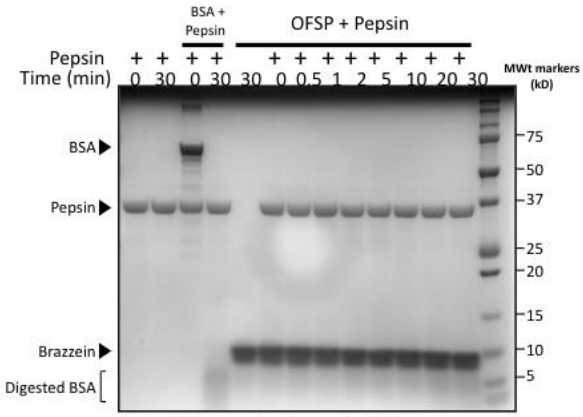
Plant defensins are small cationic, cysteine-rich proteins that are 45 to 54 amino acids in length. These proteins are found in different parts of plants, such as the seeds, leaves, flowers, roots, and stems. [1]Yount and Yeaman (2004) demonstrated that brazzein from P. brazzeana shares a similar motif (γ-core) as other known peptides, including the well-known defensin rs-AFP1 from radish (Raphanus sativus). Indeed, this finding was confirmed through a BLASTp search of the amino acid sequence of brazzein-53 against R. sativus, and 2 matches to defensin proteins from R. sativus (Accession No. KAJ4892244.1 and Accession No. XP_018437331.2) were identified with approximately 42% identity and 58% query coverage. These findings suggest that brazzein-53 may contain "defensin-like" activity, similar to other commonly consumed agricultural products.

[1] Yount NY, Yeaman MR (2004). Multidimensional signatures in antimicrobial peptides. Proc Natl Acad Sci U S A 101(19):7363-7368. DOI:10.1073/pnas.0401567101.

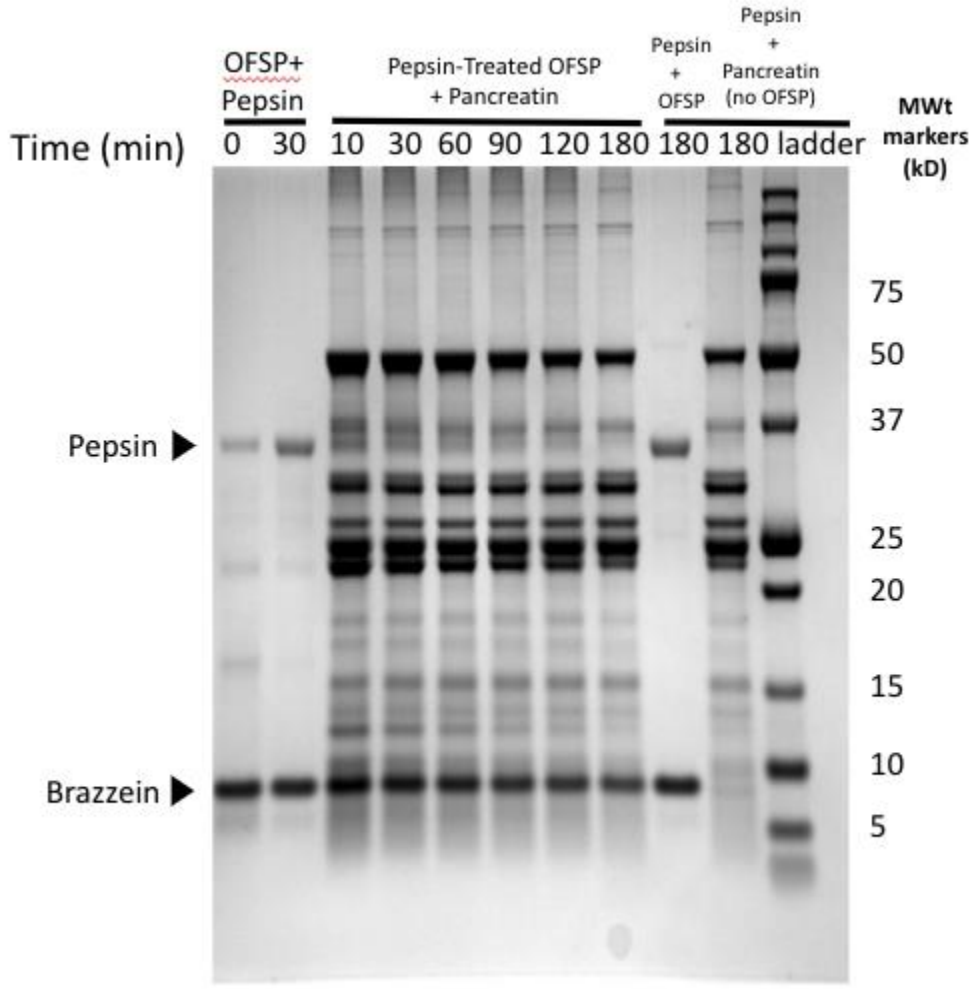
The SDS-PAGE gels within GRN 1142 are revised with the following figures. These figures have been revised to reflect the band of interest (i.e., brazzein) at approximately 6 kDa, the molecular weight of the protein as calculated from its amino acid sequence. The digestion percentage is reflecting that of brazzein rather than other proteins in OFSP. The individual levels of residual *K. phaffii* proteins are low and therefore cannot be detected due to detection limits of this assay.



10U pepsin /ug of OFSP



5U pepsin /ug of OFSP



It is generally recognized that dietary proteins are not absorbed intact and must undergo digestion into amino acids, dipeptides, or tripeptides, prior to absorption within the duodenum or proximal jejunum of the small intestine through transporters (e.g., PepT1 H+/peptide co-transporter)[2] (EFSA, 2021). Within the enterocytes, proteins are digested by peptidases into amino acids and then absorbed into the systemic circulation where they are used in metabolic processes/function. Conversely, highly lipid-soluble peptides may enter the enterocytes via passive diffusion[3] (Miner-Williams et al., 2014). Large polar molecules (>600 Da) cannot pass through the hydrophobic enterocyte cell membrane, and instead are invaginated into vesicles that fuse with lysosomes to form phagolysosomes, whose primary function is the enzymatic digestion of the captured macromolecules (Miner-Williams et al., 2014). Compounds greater than 200 Da are too large for the intercellular space between the enterocytes, and likely would not be absorbed through paracellular transport[4] (Lennernäs, 2007). Proteins that are not fully digested or are partially digested travel to the large intestine where they are ultimately fermented by the gut microbiota[5,6] (Portune et al., 2016; Joye, 2019). In the large intestine, where the microbiota concentration is much higher and the transit time is longer than in the small intestine, the remaining protein is broken down to peptides and amino acids via extracellular bacterial proteases and peptidases[7] (Macfarlane et al, 1986). As a protein that is not fully digested in the upper gastrointestinal tract, brazzein-53 would be expected to be digested/fermented by the gut microbiota within the colon or excreted within the feces.

Taken together, the "defensin-like" activity of brazzein-53 is not expected to pose any safety concerns to final consumers on the basis that the protein is not expected to be absorbed into the systemic circulation, but rather digested or fermented by gut microbiota in the large intestine.

QUESTION 3: The stability of brazzein raises some questions that need to be addressed. In the literature, there are reports that resistance of proteins to gastric digestion and thermal stability may be correlative indicators for potential allergenic risk (e.g., PMID: 9631091, 30134536, FAO/WHO (2001)). However, other recent publications conclude that protein stability is relevant for allergenicity of some proteins, but not for all (e.g., PMIDs: 31063834, 33473251, 21906650). Please provide a scientific narrative that addresses why the stability of brazzein (digestion and thermal) is not a cause for a safety concern when consumed orally.

RESPONSE 3: We recognize that the gastric or thermal resistance of proteins may be indicative of potential allergenicity in consumers, however, this area has been debated within the scientific community and amongst scientific regulators (e.g., EFSA). The pepsin digestion test has been incorporated into the weight-

[2] EFSA (2021). Statement on in vitro protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants (EFSA Panel on Genetically Modified Organisms/GMO) (Question no: EFSA-Q-2020-00314, adopted: 26 November 2020, published: 12 January 2021 by European Food Safety Authority). EFSA J 19(1):6350 [16pp]. DOI:10.2903/j.efsa.2021.6350. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6350.

[3] Miner-Williams WM, Stevens BR, Moughan PJ (2014). Are intact peptides absorbed from the healthy gut in the adult human? Nutr Res Rev 27(2):308-329. DOI:10.1017/s0954422414000225.

[4] Lennernäs H (2007). Intestinal permeability and its relevance for absorption and elimination. Xenobiotica 37(10/11):1015-1051. DOI:10.1080/00498250701704819.

[5] Portune KJ, Beaumont M, Davila A-M, Tomé D, Blachier F, Sanz Y (2016). Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. Trends Food Sci Technol 57(B):213-232. DOI:10.1016/j.tifs.2016.08.011.

[6] Joye I (2019). Protein digestibility of cereal products. Foods 8(6):199 [14pp]. DOI:10.3390/foods8060199.

[7] Macfarlane GT, Cummings JH, Allison C (1986). Protein degradation by human intestinal bacteria. J Gen Microbiol 132(6):1647-1656. DOI:10.1099/00221287-132-6-1647.

of-evidence for allergenicity assessment of novel proteins and has been adopted by the Codex Alimentarius and EFSA. This test has its limitations in that it only evaluates gastric digestion of protein and does not consider proceeding phases along the gastrointestinal tract where protein digestion may occur (e.g., within the small intestine). The pepsin digestion test does not completely mimic the physiological conditions of the human gastric digestion system[8] (EFSA, 2021). As described above and in Section 6.3.1 of GRN 1142, the digestibility of OFSP containing brazzein-53 was investigated using the standard pepsin digestion method as well as a 2-step method which utilized both SGF and SIF to simulate the human gastrointestinal conditions. Brazzein-53 from OFSP was demonstrated to be stable to digestion under both conditions. Both stable and unstable (i.e., digested) proteins may elicit allergenic reactions.

As discussed within GRN 1142, brazzein-53 from OFSP and other residual proteins that may be carried over into the ingredient from the production organism, *K. phaffi*, were evaluated for allergenicity potential using a step-wise in silico approach described by the FAO/WHO, Codex Alimentarius, and EFSA. This in silico approach has been widely employed in the allergenicity assessment of novel protein ingredients, including those that have been the subject of a GRAS Notification that received "no questions" from the FDA (e.g., soy leghemoglobin preparation from GRN 737[9]). This in silico approach has achieved consensus amongst the scientific community as the "best practice" in the allergenicity risk assessment of novel proteins to predict potential cross-reactivity of novel proteins to known allergens. The in silico searches involved searches with the full-length amino acid sequence, 80-amino acid sliding window, and an 8-amino acid exact match. A number of curated allergen databases (e.g., AllergenOnline) were used. No matches to any known allergens were identified that would suggest that brazzein-53 in OFSP is a known allergen or that it would be cross-reactive to other putative allergens. Therefore, based on the totality of evidence, despite the fact that brazzein-53 within OFSP may be resistant to digestion, it is unlikely that this protein would pose any allergenic concern based on the findings of the bioinformatics evaluation.

In addition to the allergenic potential of brazzein-53 from OFSP, the safety of the ingredient has been established from a toxicological perspective. The systemic toxicity of brazzein-53 was evaluated in a 90-day repeated-dose oral toxicity study in rats. The study was conducted in accordance with OECD Test Guideline No. 408 and OECD GLP and yielded a NOAEL of 978 mg/kg body weight/day, the highest dose tested. Based on the proposed conditions of use of OFSP, the observed NOAEL results in a margin of exposure of approximately 230. Considering the resistant nature of brazzein-53 to digestion as discussed above, the intact protein would not be expected to be absorbed into the systemic circulation to elicit any toxic effect.

Based on the available information, given that there is a large safety margin for OFSP containing brazzein-53 under its proposed conditions of use as a sweetening agent, it is not expected that the stability profile of brazzein would be indicative of a safety concern in final consumers.

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[8] EFSA (2021). Statement on in vitro protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants (EFSA Panel on Genetically Modified Organisms/GMO) (Question no: EFSA-Q-2020-00314, adopted: 26 November 2020, published: 12 January 2021 by European Food Safety Authority). EFSA J 19(1):6350 [16pp]. DOI:10.2903/j.efsa.2021.6350. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6350.

[9] U.S. FDA (2018). *Agency Response Letter GRAS Notice No. GRN 737 [Soy leghemoglobin preparation from a strain of Pichia pastoris, Redwood City (CA): Impossible Foods 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=737 [Jul. 3, 2018 - FDA response - no questions].

From: Jason Ryder
To: Kampmeyer, Christopher
Subject: Re: [EXTERNAL] Re: Questions for GRN 001142
Date: Friday, February 2, 2024 3:25:08 PM
Attachments: image001.png
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OFSP_Amendment to GRN 1142.pdf

Hi Chris,

On behalf of Oobli, Inc., I am writing to amend GRAS Notice (GRN) 001142 in order to revise the intended use of Oubli Fruit Sweet Protein Product (OFSP) to reflect the intended use as a general-purpose sweetener, in accordance with current good manufacturing procedures (cGMP). The attached amendment provides updated estimates of daily intakes, along with an updated conclusion in the narrative supporting that use of OFSP as a general-purpose sweetener would not change the original GRAS conclusion. Oobli, Inc. confirms that there have been no changes to the identity, method of manufacture, specifications, or technical effect of OFSP since FDA was notified of the GRAS conclusion on April 20, 2023. The attached amendment therefore addresses only the revised estimate of intake, and an updated conclusion of safety based on comparison of the revised estimated daily intake with the no observed adverse effect level (NOAEL) derived from the pivotal pre-clinical data.

Please kindly confirm receipt of this amendment and let me know if you have any questions.

Best,

Jason

--
Jason Ryder
CTO & Co-Founder

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Amendment to Notice Filed as GRN 1142

Introduction

Oobli, Inc. ("Oobli"), previously concluded that the use of Oubli Fruit Sweet Protein Product, derived from *Komagataella phaffii* and containing ≥35% brazzein by weight (hereinafter "Oubli Fruit Sweet Protein," or "OFSP"), is Generally Recognized As Safe (GRAS) for the intended use as a sweetener in various conventional food and beverage products. Oobli notified offices of the Unites States (U.S.) Food and Drug Administration (FDA) of the GRAS conclusion on April 20, 2023, and the notice was filed as GRN 1142. At this time, FDA is still completing review of the notice.

The intended use of OFSP defined in the notice filed as GRN 1142 is to provide sweetness in select categories of foods (including beverages), with use levels of OFSP ranging from 2 to 99 mg/100 g in the different food categories (see Table 1.3-1 in GRN 1142).

Since the notice was submitted to FDA, Oobli has identified several additional food categories in which the high-intensity OFSP sweetener can be used as a source of sweetness. To include these recently identified uses, as well as other uses that may be feasible, Oobli has revised the intended use of OFSP to reflect the intended use as a general-purpose sweetener, in accordance with current good manufacturing procedures (cGMP), excluding use in infant formula and meat and poultry products. The updated estimates of daily intakes are provided below, along with an updated conclusion in the narrative supporting that use of OFSP as a general-purpose sweetener would not change the original GRAS conclusion. Oobli confirms that there have been no changes to the identity, method of manufacture, specifications, or technical effect of OFSP since FDA was notified of the GRAS conclusion on April 20, 2023. The discussion below therefore addresses only the revised estimate of intake, and an updated conclusion of safety based on comparison of the revised estimate of intake with the no observed adverse effect level (NOAEL) derived from the pivotal pre-clinical data.

Updated Intended Use and Estimated Daily Intake

OFSP is intended for use as a general-purpose sweetener in accordance with current cGMP, excluding infant formula and meat and poultry products.

OFSP contains brazzein, which is a sweet protein. The sweetness intensity of OFSP has been tested using a sweet potency protocol designed to measure the sweetness response of an unknown sample compared to sucrose. Results from this testing demonstrate that brazzein is approximately 750-fold sweeter than sucrose on a weight basis. Given that OFSP is approximately 44% brazzein by weight, OFSP is approximately 330-fold sweeter than sucrose.

The estimated daily intake of OFSP from the intended use as a general-purpose sweetener in foods has been derived using the replacement approach described by Renwick (2008). The approach detailed by Renwick provided estimated intakes of rebaudioside A, a new high-intensity sweetener at the time. Estimates of rebaudioside A intake were based on published data

for intake of other high-intensity sweeteners among populations of children and adults with and without diabetes and the assumption that intake of the new sweetener would fully replace intake of existing high-intensity sweeteners. Replacement of high-intensity sweeteners was calculated based on sweetness intensities relative to sucrose.

The approach reported by Renwick was first used to support the exposure assessment for rebaudioside A in the GRAS conclusion submitted to FDA (GRN 253), to which FDA responded with a "no questions" letter. Since that time, Renwick's replacement methodology has been used to develop estimated daily intakes to support numerous GRAS conclusions for use of rebaudioside A and related sweeteners providing steviol glycosides (e.g., most recently GRN 1106), as well as other high-intensity sweeteners derived from sources such as Luo Han Guo (e.g., GRN 359). FDA issued "no questions" letters to these GRAS notices.

To provide estimates of OFSP intake assuming use as a general-purpose sweetener, the Renwick (2008) approach was applied as follows. The estimated daily intakes of OFSP were calculated by dividing the estimated sucrose equivalent intakes from intense sweeteners reported by Renwick by the relative sweetness determined from the adjusted potency test score for OFSP (i.e., 330-fold). The estimated daily intakes are reported for average and high consumers of intense sweeteners, where "high" consumers are considered to represent intake at or above the 90th percentile consumption level based on data compiled by Renwick (2008).

Table 1 below presents the Renwick estimates of high-intensity sweetener intake by population group along with the estimated intake of OFSP. The estimated daily intakes of OFSP for "average" consumers in populations of non-diabetic adults, diabetic adults, non-diabetic children, and diabetic children are up to 0.77 mg OFSP/kg bw/day, 0.85 mg OFSP/kg bw/day, 1.29 mg OFSP/kg bw/day, and 2.04 mg OFSP/kg bw/day, respectively. The estimated daily intakes of OFSP for "high" consumers in populations of non-diabetic adults, diabetic adults, non-diabetic children, and diabetic children are up to 2.05 mg OFSP/kg bw/day, 2.72 mg OFSP/kg bw/day, 3.00 mg OFSP/kg bw/day, and 2.75 mg OFSP/kg bw/day, respectively.

Table 1. Estimated daily intake of OFSP for populations in the United States using an intense sweetener intake assessment methodology

	Intake of Intense Sweeteners (expressed as sucrose equivalents) (mg/kg bw/day) [a]		**Estimated Intake of OFSP (mg/kg bw/day) [b]**	
Population Group	**Average Consumer**	**"High" Consumer ≥90th percentile**	**Average Consumer**	**"High" Consumer ≥90th percentile**
Non-diabetic adults	255	675	0.77	2.05
Diabetic adults	280	897	0.85	2.72
Non-diabetic children	425	990	1.29	3.00
Diabetic children	672	908	2.04	2.75

[a] Estimates as reported in Renwick, 2008 (Table 7).
[b] Calculated assuming relative sweetness potency of 750-fold for brazzein and assuming 44% brazzein in OFSP.

The estimated daily intake of OFSP by the population of "high" consumer non-diabetic children therefore provides a conservative approach for evaluating the safety of the intended use, as intake by this population represents the highest intake across the four subpopulations. The estimated daily intake of OFSP for "high" consumers among populations of children and adults with or without diabetes is up to 3.00 mg/kg bw/day. The estimates of intake developed with the approach detailed by Renwick are based on actual estimates of high-intensity sweetener intake, yet are conservative as they assume full replacement of the currently approved high-intensity sweeteners with a new sweetener.

Updated Safety Conclusion

In the GRAS dossier submitted to FDA and filed as GRN 1142, the intended use of OFSP in select categories of foods and beverages resulted in a highest estimated dietary intake of 4.25 mg/kg body weight/day (90^{th} percentile in children 2 to 5 years of age; Table 3.1.2-2 in GRN 1142). As noted in the GRAS notice filed as GRN 1142, several assumptions included in the assessment render exposure estimates suitably conservative, including the assumption that all food products within a food category will contain OFSP at the maximum specified level of use.

Under the revised intended use of OFSP as a general-purpose sweetener, in accordance with cGMP, the estimated intake of OFSP by the population of "high" consumers among populations of children and adults with or without diabetes is up to 3.00 mg/kg bw/day. These estimates of intake are based on an established approach for estimating intake of a high-intensity sweetener (Renwick, 2008), and the measured 330-fold sweetness potency of OFSP relative to sucrose. The estimated intake of up to 3.00 mg OFSP/kg bw/day corresponds to intake for children without diabetes. Among adults, the estimated intake of OFSP is up to 2.72 mg OFSP/kg bw/day in the population of adults with diabetes.

The notice filed as GRN 1142 presents a review of the literature available at the time of that notice, which covered the published literature through March 2023. To ensure that the review considers the most recent evidence, a search of PubMed was conducted on January 31, 2024, with the term "brazzein" for literature indexed since March 1, 2023, with no limitations other than the English language. This search identified one study with information pertinent to the GRAS review of OFSP (Novik *et al*., 2023). Findings from the study reported by Novik and colleagues are presented below.

The safety of a recombinant brazzein produced in *Komagataella phaffii* (referenced in the study as *Pichia pastoris,* the previously recognized name for *K. phaffii)* was assessed in acute, sub-chronic, and chronic toxicity tests and genotoxicity tests (Novik *et al*., 2023). The recombinant brazzein test material was stated to be identical to brazzein isolated from the ripe fruits of the *Pentadiplandra brazzeana* plant; purity of the test material was not reported. The reported oral LD_{50} was >5000 mg/kg bw in rats and mice (species not specified). In the subacute (21-day) oral toxicity study, recombinant brazzein was administered via gavage to guinea pigs (9/sex/dose) at 0, 2.17, or 21.7 mg/kg bw/day; no effects were reported at the highest dose tested. In the chronic oral toxicity study in rats, recombinant brazzein was administered via gavage to outbred rats (10/sex/dose; species not specified) at 0 (vehicle control), 2.17, or 21.7 mg/kg bw/day for a

period of 150 days. A positive control group was administered sucrose at 4824 mg/kg bw/day. The authors reported a statistically significant difference in relative body weight gain in male rats at 2.17 mg/kg bw/day (-13%) and 21.7 mg/kg bw/day (-16%) when compared to the control group. However, the dose groups administered sucrose (4824 mg/kg bw/day) also demonstrated a decrease in mean body weight gain (-12%) compared to the control group. In a bacterial reverse mutation assay (*S. typhimurium* strains TA 98, TA 97, and TA 100), recombinant brazzein (up to 50,000 ug/mL) was reported as negative. Recombinant brazzein was reported as negative in *in vivo* micronucleus and *in vivo* chromosome aberrations tests in mice (species not specified) up to 5,000 mg/kg bw/day.

As reviewed in GRN 1142, findings from pivotal pre-clinical studies demonstrate OFSP to be non-mutagenic and non-genotoxic, and the NOAEL from the 90-day study at the highest dose tested was approximately 978 mg/kg body weight/day in males and 985 mg/kg body weight/day in females (Lynch *et al*., 2023). This study remains the pivotal study for the assessment of OFSP safety. Based on the highest estimated dietary intake of 3.00 mg/kg bw/day for the intended use as a general-purpose sweetener ("high" consumers among populations of children without diabetes), the margin of exposure is estimated to be 326-fold, suggesting that OFSP, under its proposed conditions of use, does not pose any safety concerns to end consumers.

Based on the above information, Oobli has concluded that the intended use of OFSP as a general-purpose sweetener, in accordance with cGMP, excluding use in infant formula and meat and poultry products, would not change the GRAS conclusion as previously described in the notice submitted on April 20, 2023, and filed as GRN 1142 and incorporated herein. Oobli concludes that the intended use of OFSP as a general-purpose sweetener, in accordance with cGMP, is GRAS. It is Oobli's view that other experts qualified by scientific training and experience in food safety evaluation would agree with Oobli's conclusion.

References

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