#863

GRAS Notice for Non-Animal Whey Protein from Fermentation by *Trichoderma reesei*

Prepared for:

Office of Food Additive Safety (FHS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Dr.
College Park, Maryland 20740

Submitted by:

Keller and Heckman LLP
1001 G St., NW
Suite 500W
Washington, DC 20001

On behalf of our client:

Perfect Day, Inc.
813 Heinz Ave.
Berkeley, California 94710

Date: March 29, 2019

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Part 1. Signed statements and certification

1. Applicability of 21 C.F.R. part 170, subpart E

We submit this generally recognized as safe (GRAS) notice in accordance with 21 C.F.R. 170, subpart E.

2. Name and address of the notifier

Company:	Perfect Day, Inc.
Name:	Perumal Gandhi
Address:	813 Heinz Avenue Berkeley, California 94710
Phone:	(408) 600-5286
Email:	perumal@perfectdayfoods.com

All communications on this matter are to be sent to Counsel for Perfect Day, Inc.:

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW, Suite 500W
Washington, DC 20005
Tel: 202-434-4222
Fax: 202-434-4646
Email: drozen@khlaw.com

3. Names of the notified substance

Non-Animal Whey Protein (β-Lactoglobulin)
Non-Animal Whey Protein
Microflora-Derived Whey Protein (β-Lactoglobulin)
Microflora-Derived Whey Protein

4. Applicable conditions of use of the notified substance

Perfect Day, Inc. intends to market non-animal whey protein produced via fermentation by *Trichoderma reesei* as a non-animal source replacement for milk and plant proteins for use in foods that currently use protein from milk or plants as a source of dietary protein.

5. Basis for the GRAS determination

Keller and Heckman LLP, on behalf of Perfect Day, Inc., hereby notifies the Agency of its determination that its non-animal whey protein composed of β-lactoglobulin from fermentation by *Trichoderma reesei* is GRAS for its intended use, consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). This GRAS conclusion is based on scientific procedures in accordance with 21 C.F.R. §170.30(a) and (b) and conforms to the guidance issued by the Food and Drug Administration (FDA) under 21 C.F.R. §170.36, 81 Fed. Reg. 54,960 (Aug. 17, 2016). The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with proposed 21 C.F.R. § 170.36. The GRAS

status of β-lactoglobulin from fermentation by *Trichoderma reesei* is based on data generally available in the public domain and on the long history of milk and milk derived protein consumption in human foods.

6. Exclusion from premarket approval

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

7. Availability of data and information

The information for this GRAS conclusion including analytical data, published studies, and information that are the basis for this GRAS determination are available to FDA upon request as required by 21 C.F.R. § 170.225(c)(7)(ii)(A) or (B) by contacting Keller and Heckman LLP at the below address.

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW, Suite 500W
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8. Applicability of FOIA exemptions

Perfect Day, Inc. is not claiming any information in Parts 2 through 7 of this document as trade secret, confidential or financial information that is privileged or confidential. Thus, all information and data in this submission are not exempt from the Freedom of Information Act (FOIA), 5 U.S.C. Section 552.

9. Certification

We certify on behalf of our client, Perfect Day, Inc., that this GRAS conclusion is based on representative data from Perfect Day, Inc. required for the safety and GRAS status for non-animal whey protein from fermentation by *Trichoderma reesei*. To the best of our knowledge it is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

10. Signature and name and title of the person signing this GRAS notice:

Date: March 28, 2019

Melvin S. Drozen
Partner
Keller and Heckman LLP

Part 2. Identity, method of manufacture, specifications, and physical or technical effect

1. Scientific data and information that identifies the notified substance

(a) Common or usual name:

Non-Animal Whey Protein (β-Lactoglobulin)
Non-Animal Whey Protein
Microflora-Derived Whey Protein (β-Lactoglobulin)
Microflora-Derived Whey Protein

2. Identity

β-lactoglobulin is the major whey protein found in ruminant milk. In bovine (*Bos taurus*) milk, β-lactoglobulin is typically found at concentrations ranging from 2-3 g/L, which represents approximately 7-9% of the total protein content.[1] Perfect Day, Inc. produces a highly purified protein extract comprised of ≥ 90% β-lactoglobulin via fermentation, using a fungal strain commonly used for production of food enzymes, *Trichoderma reesei*. The resulting product is a homogenous white to cream colored powder that can be incorporated into foods at usage levels matching other purified dairy protein products.

3. Material specifications

(a) Host strain

The host microorganism used to construct the β-lactoglobulin producing strain is the filamentous fungus *Trichoderma reesei* strain QM6a. The host strain is auxotrophic for uracil production through deletion of the endogenous *PYR4* gene, which is analogous to *URA3* in *Saccharomyces cerevisiae. T. reesei* is the anamorph (asexual reproductive stage) of the fungus *Hypocrea jecorina* and is classified as a Biosafety Level 1 (BSL-1) organism and is widely used to produce enzymes utilized in foods worldwide.

(b) Production strain

To optimize expression of β-lactoglobulin and obtain the purest product possible, Perfect Day employs several common and well-characterized genetic modification techniques: 1) the host strain was genetically modified with one or more expression cassettes to produce β-lactoglobulin from the domestic cow (*Bos taurus)*; 2) the genetic sequence for β-lactoglobulin has been codon-optimized for expression in the host fungal strain, but the amino acid of the sequence remains unchanged from the donor organism; 3) a series of well understood and characterized endogenous or synthetic promoters that are used to promote the expression of the β-lactoglobulin gene via induction by the sugar-based media; and 4) selective up-regulation or

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[1] Kontopidis, G., *et al*. Invited Review: β-Lactoglobulin: Binding Properties, Structure, and Function. *J Dairy Sci* 2004; 87; 785 – 796.

attenuation of endogenous transcription factors and/or inclusion of exogenous transcription factors from non-pathogenic and non-toxigenic source organisms. Each β-lactoglobulin expression cassette contains: (1) DNA homologous to the desired integration site, (2) the codon-optimized β-lactoglobulin gene under the control of an endogenous or synthetic *T. reesei* promoter, as well as signal and terminator sequences, and (3) the endogenous *PYR4* (orotidine 5'-phosphate carboxylase) gene for use as a selectable marker which is recycled (*i.e*. removed) in the final production strain. The expression cassette is stably inserted into the host strain genome, as evidenced by multi-generational studies showing consistent levels of β-lactoglobulin production. *T. reesei* strains are commonly used in biotechnological processes because of their known stability. The expression cassette does not contain any antibiotic resistance genes or mobile genetic elements. Finally, the *T. reesei* genome has been shown to have a low risk for unwanted transfer of genetic material due to a relative lack of repetitive DNA sequences and extant functional transposable elements.[2]

4. Raw Materials and Processing Aids

The raw materials, processing aids (*e.g.*, antifoam), filtration aids, and pH adjustors used in the fermentation and recovery processes are safe and suitable standard ingredients that meet predefined quality standards. The raw materials conform to either specifications set out in the Food Chemical Codex, 11th edition, 2018 or to other applicable regulatory standards.

(a) Description of the method of manufacture

β-Lactoglobulin is manufactured under current good manufacturing practices (cGMP) for food (21 C.F.R. Part 117, Subpart B) and meets appropriate food grade specifications. β-lactoglobulin is manufactured by submerged fermentation of a pure culture of the filamentous fungus *T. reesei* that has been genetically modified as described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by undesired microorganisms. During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are conducted periodically to ensure the absence of foreign microorganisms and confirm the production strain's identity.

A new lyophilized stock culture vial of the modified *T. reesei* production strain is used to initiate a seed fermentor at the beginning of production for each batch. Production then moves from a seed to production fermentor for the main fermentation phase. β-lactoglobulin is secreted from the fungus and remains solubilized in the fermentation media until the recovery process begins. Recovery begins immediately after the fermentation phase and is a multi-step process that begins with a primary solid/liquid centrifugation step to separate biomass from the fermentation media that contains soluble β-lactoglobulin. This step is followed by a pH adjustment step which allows for concentration of β-lactoglobulin. An intermediate filtration and polishing step may be further used to decrease impurities. The β-lactoglobulin is then

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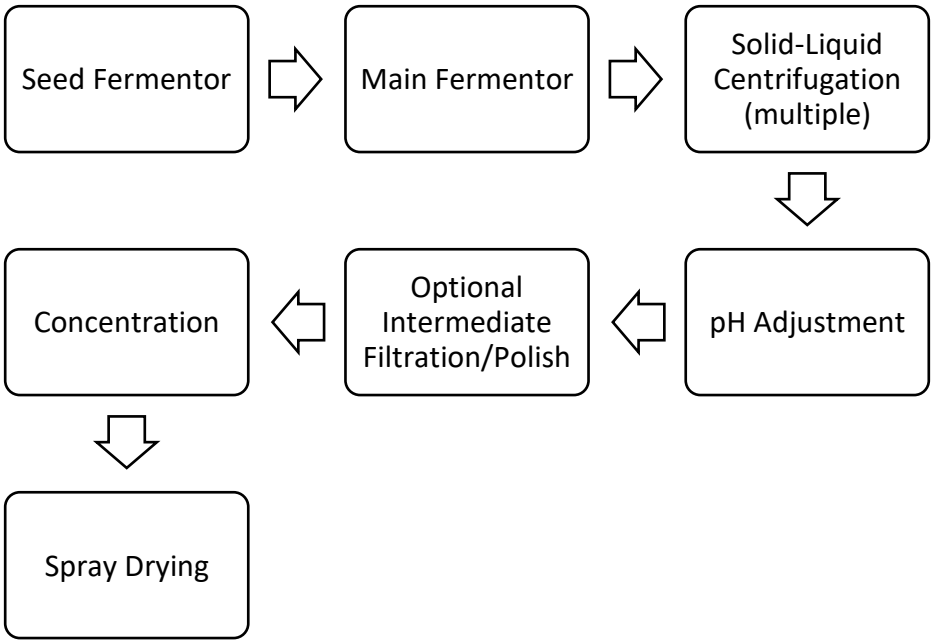
[2] Kubicek, C.P., *et al*. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. *Genome Biology* 2011; 12 (4) p R40; Martinez, D., *et al.* Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Biotechnology* 2008; 26; p. 553–560.

concentrated via an ultrafiltration/diafiltration step and spray dried. The finished product is a white to off-white powder consisting of ≥90% β-lactoglobulin. The manufacturing process is summarized in **Figure 1**.

Figure 1. Manufacturing process of β-lactoglobulin.



5. Product Specifications and Batch Analyses

(a) Physical, Chemical, and Microbiological Specifications

The product specifications for β-lactoglobulin produced by fermentation of *T. reesei* are presented in **Table 1**.

Table 1. Physical and Microbiological Characteristics of β-Lactoglobulin Produced by Fermentation

Analysis	Specification	Reference Method
Protein Dumas/Kjeldahl	>85 wt%	AOAC 968.06 or 992.15
Protein DMB	>90%	AOAC 968.06
β-Lactoglobulin as % of Protein	>90 %	HPLC & Densitometry
Moisture	<6 wt%	AOAC 925.45
Ash	<3 wt%	AOAC 942.05
Fat	<1.5 wt%	AOAC 989.05
Total Carbohydrates	<2 wt%	By difference

(b) Batch Analyses

Data from the analysis of three non-consecutive lots of by fermentation, which demonstrate the consistency of manufacturing process and compliance with the physical and chemical specifications, are presented in **Table 2**.

Table 2. Physical, Chemical, and Microbiological Product Analysis for Three Non-Consecutive Lots Produced by Fermentation

Analysis	Lot 519	Lot 503	Lot 535
Protein Dumas	94.1	97.2	96.3
Protein DMB	98.2	98	99.6
β-Lactoglobulin as % of Protein	99.9	100	99.5
Moisture (%)	4.1	1	3.3
Ash (%)	1.2	1	1.3
Fat (%)	<0.1	0.8	<0.1
Total Carbohydrates (%)	<0.6	<0.1	<0.1

Part 3. Dietary exposure

1. Estimate of Dietary Exposure

As noted above in Part 1, Perfect Day, Inc. intends to market non-animal whey protein produced via fermentation by *T. reesei* as a non-animal source replacement for milk and plant proteins for use in foods that currently use protein from milk or plants as a source of dietary protein. Examples of the typical food uses of β-lactoglobulin from fermentation of *T. reesei* are summarized in Table 3.

Table 3. Typical Food Uses and Use Levels of β-Lactoglobulin from Fermentation of *T. reesei.*

Nutritional Products	Meal Replacements and Supplements	Emulsifier, source of high-quality protein	5-15%
	Powdered Nutritional Beverages	Source of high-quality protein	10-25%
	Nutritional Bars	Source of high-quality protein, texturizer	5-35%
	Sports Beverages	Source of high-quality protein	5-20%
Dairy and Dairy-Based Products	Milk Products (including beverages and coffee creamer)	Source of high-quality protein, texturizer	1-15%
	Yogurt and Fermented Milk Products	Texturizer, thickener	1-5%
	Spreads, Dips, and Cream Substitutes	Texturizer	1-15%
	Frozen Dairy Desserts and Mixes	Emulsifier	1-10%
Sugar Based Products	Desserts and Mousses	Texturizer	<5%
	Confections (including chocolate confections)	Texturizer, flavor	1-10%
	Snack Foods	Texturizer, flavor	1-10%
	Coatings and Fillings	Texturizer, flavor	1-10%
Dressings	Salad Dressings	Emulsification, flavor	<5%

Due to the relative novelty of high purity β-lactoglobulin products, specific consumption data are not available at this time. However, because β-lactoglobulin products are equivalent to traditional whey protein products from the standpoint of nutritional properties and safety, and because β-lactoglobulin products effectively will substitute for traditional whey protein and other

protein products in the marketplace, we anticipate no issues related to dietary exposure to this protein that is already an existing part of the diet.

Most of the population's protein intake is derived from, and will continue to be derived from unprocessed foods, including meat, poultry, fish, and legumes. Moreover, for those processed foods to which the β-lactoglobulin will be added, there are competitive products on the market. Thus, the addition of β-lactoglobulin simply will serve as a replacement for these other competitive protein sources and will not increase consumer exposure to protein. Therefore, we do not realistically expect that the actual consumption of foods containing β-lactoglobulin will contribute to a significant portion of total protein intake. That said, based on the intended uses summarized above and taking into account potential use levels and serving sizes for the foods of interest, we believe that a reasonable worst-case estimate for intake of β-lactoglobulin is up to 20% in sports beverages. Assuming that the sports beverages have a serving size of 240 ml, the 20% maximum use level of β-lactoglobulin in that application would be 48 g, which is within the range of the Institute of Medicine's Recommended Dietary Allowance (RDA) for protein of 56 g/day for adult males and 46 g/day for adult females.[3] Given the history of β-lactoglobulin's presence in the diet from dairy sources and as discussed in Part 6 below, we believe that the non-animal β-lactoglobulin described in this notice is safe at the levels proposed above.

[3] Institute of Medicine of The National Academies Nutrient reference values, accessed January 2019. Available at: http://www.nationalacademies.org/hmd/~/media/Files/Activity%20Files/Nutrition/DRI-Tables/3_RDA%20AI%20AMDR%20Values_Total%20Water%20and%20Macronutr.pdf?la=en

Part 4. Self-limiting levels of use

The use of β-lactoglobulin is not self-limiting. The maximum use levels in food are described above.

Part 5. Experience based on common use in food before 1958

While the basis for this GRAS Notice is scientific procedures, rather than common use in food, we note that β-lactoglobulin is a component of milk. Milk and products derived from milk, such as whey, have a long history of safe consumption by humans at all ages in the form of fluid milk, in dried form (*i.e.*, milk powder), or as milk-derived ingredients. Therefore, the history of milk consumption provides support for the safety GRAS status of β-lactoglobulin's intended use.

Part 6. Narrative

1. Overview of Safety of β-lactoglobulin produced via fermentation of *T. reesei*

Considerations regarding safety of β-lactoglobulin produced via fermentation of *T. reesei* involve the safety of both the production organism (modified *T. reesei*) and the safety of the end use product (bovine-identical β-lactoglobulin). *T. reesei* has a long history of safe use in industrial scale food enzyme production. The safety of this species as an industrial enzyme production organism has been reviewed multiple times; it is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under submerged fermentation conditions.[4]

The protein in cow's milk is 80% casein protein and 20% whey protein, where the whey protein is a collection of soluble, globular proteins comprised primarily of β-lactoglobulin (~50-65%), and α-lactalbumin (~25%).[5] While high purity β-lactoglobulin products are relatively novel, they are equivalent to traditional whey protein and other purified milk protein products from the standpoint of nutritional properties and safety.

2. Safety of *Trichoderma reesei*

(a) Safety of the Parental Strain

T. reesei was first isolated from nature in the 1940's and subsequently has been the subject of intense research due to its established safety and usefulness in the production of a variety of food enzymes, including cellulases, proteases, and amylases.[6] Some literature has proposed that *T. reesei* was synonymous with *T. longibrachiatum,* however, evidence emerged indicating that the two species are not identical.[7] *T. reesei* has been determined to be an

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[4] Nevalainen, H., *et al*. On the safety of *Trichoderma reesei*. *J Biotechnol* 1994; 37; 193-200; Blumenthal C. Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regul Toxicol Pharmacol* 2004; 39; ,214-228.

[5] Haug, A., *et al*. Bovine milk in human nutrition – a review. *Lipids Health Dis* 2007; 6; 25.

[6] Olempska-Beer, *et al.* Food-processing enzymes from recombinant microorganisms—a review. *Regul Toxicol Pharmacol* 2006; 25; 144-158. Nevalainen, H., *et al*. On the safety of *Trichoderma reesei*. *J Biotechnol* 1994; 37; 193-200; Blumenthal C. Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae,* and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regul Toxicol Pharmacol* 2004; 39; 214-228.

[7] Bissett, J. A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. nov. *Can J Bot* 1984; 45; 144-158; Meyer, W., *et al*. The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. *Curr Genet* 1992; 21, 27-30.

anamorph, or asexual reproductive phase, of *Hypocrea jecorina*, and the U.S. National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina* and no longer includes it in the genus *Trichoderma.*[8] Therefore, the names *Trichoderma reesei, Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different regulatory approval documents and positive lists, but these names refer to essentially the same microorganism species.

A review of the public scientific literature uncovered no reports that implicate *T. reesei* in any way with human, animal, or plant disease or allergenicity among healthy adults.[9] The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended). It is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

While it is accepted that secondary metabolite production does not typically occur under relevant industrial production conditions (*i.e*. absent nutrient starvation or competition from other microorganisms), recent research regarding the ability of *T. reesei* to produce secondary metabolites, including mycotoxins, indicates that the organism's ability to produce such compounds is minimal or non-existent in any circumstance.[10] Recent bioinformatic studies on whole genome sequences of *T. reesei* indicate the presence of relatively few (27) putative secondary metabolite gene clusters.[11] By comparison, other industrially relevant fungal strains of *A. niger* and *A. oryzae* contain 78 and 75 such gene clusters, respectively.[12] *T. reesei* has been previously reported to have the ability to produce trichodermin.[13] However, this was later proven not to be the case with the error attributed to culture contamination with other

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[8] Kuhls, K., *et al.* Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecornina*. *Proc Natl Acad Sci USA* 1996; 93; 7755-60.

[9] Conducted December 2018.

[10] Frisvad, J.C., *et al.* (2018) Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger, Aspergillus oryzae,* and *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* 102 (22):9481-9515.

[11] Zeilinger, S., *et al*. (2016) Secondary metabolism in *Trichoderma*—chemistry meets genomics. *Fungal Biol Rev.* 30:74–90.

[12] Lind, A.L., *et al.* (2015) Examining the evolution of the regulatory circuit controlling secondary metabolism and development in the fungal genus *Aspergillus*. *PLoS Genet*. 2015;11:e1005096; Wasil, Z., *et al.* (2018) Oryzines A & B, maleidride congeners from *Aspergillus oryzae* and their putative biosynthesis. *J Fungi*. 4:96.

[13] Watts, R., *et al*. (1988) Isolation of a new antifungal metabolite of *Trichoderma reesei. Plant Soil.* 107:81–84.

Trichoderma species.[14] A putative gene cluster for gliotoxin has been reported to be present in the genome of *T. reesei*, though no reports of detection of this toxin in *T. reesei* cultures exist in the literature.[15]

Certain strains of *T. reesei* have been reported to produce paracelsin compounds that exhibited antibiotic activity.[16] However, these compounds' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those employed for commercial protein production for food uses. The U.S. Environmental Protection Agency (EPA) published a risk assessment to support tiered exemption status for *T. reesei* and its derivates, in which EPA acknowledged that under normal submerged fermentation conditions paracelsin is not produced.[17]

T. reesei has a long history of safe use in industrial scale enzyme production for food use. FDA's GRAS Notice Inventory lists 17 notices involving production of both endogenous and exogenous enzymes using fermentation of genetically modified *T. reesei* that have received "no questions" letters from the FDA (**Table 4**). We incorporate by reference the relevant safety data on *T. reesei* in the notices listed below.

Table 4. Summary of GRAS Notices for Food Enzymes Produced Using *T. reesei* Fermentation

GRN No.	Enzyme	Use
32	Pectin lyase from *A. niger*	Juice clarification
230	Chymosin from *B. Taurus*	Cheese production
315	Transglucosidase from *A. niger*	Brewing
333	Acid fungal protease from *T. reesei*	Grain processing and brewing
372	Glucoamylase from *T. reesei*	Grain processing and brewing
524	Phospholipase A2 from *A. nishimurae*	Degumming oil and egg yolk hydrolysis

[14] Nielsen, K.F., *et al.* (2005) Trichothecene production by *Trichoderma brevicompactum*. *J Agric Food Chem*. 53:8190–8196.

[15] Zeilinger, S., *et al*. (2016) Secondary metabolism in Trichoderma—chemistry meets genomics. *Fungal Biol Rev*. 30:74–90; Martinez, D., *et al*. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*) *Nat Biotechnol.* 26:553–560; Kubicek, C.P., and Druzhinina, I.S. (2016) *Trichoderma* mycoses and mycotoxins. In: Paterson, R.R.M., Lima N., editors. Molecular biology of food and water borne mycotoxigenic and mycotic fungi. Boca Raton: CRC Press.

[16] Bruckner, H., *et al*. Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma rees*ei Simmons Part A., *Experientia* 1983; 39; 528-530.

[17] 77 Fed. Reg. 54, 499-511 (September 5, 2012).

557	Polygalacturonase from *A. tubingensis*	Beverages
558	Pectin esterase from *A. tubingensis*	Beverages
566	Mannase from *T. reesei*	Fruit and vegetable processing
567	Xylanase from *A. niger*	Brewing and baking
628	Xylanase from *T. flexuosa*	Grain processing, brewing and baking
631	Triacylglycerol lipase from *F. oxysporum*	Baking
653	Lysophospholipase from *A. nishimurae*	Sweetener production
675	Xylanase from *T. leycettanus*	Grain processing, brewing and baking
680	〈-L-arabinofuranosidase from *T. pinophilus*	Corn milling
707	Glucose oxidase from *P. amagasakiense*	Edible oil refining
727	Trehelase from *T. reesei*	Brewing and organic acid production

(b) Safety of Production Strain

As described in Section 2b, the host strain was modified with an expression cassette containing a codon optimized sequence for bovine-identical β-lactoglobulin which has been stably inserted into the genome of *T. reesei*. The cassette also contains appropriate, well-described regulatory sequences and a *PYR4* auxotrophic selection marker which can be recycled as needed. No antibiotics or antibiotic selection markers were used during the production strain construction process. Based on the long history of safety for the host strain and the nature of the genetic modifications made to the host and the long history of use of modified *T. reesei* in industrial food enzyme production, it can be concluded that the production strain poses no risk to human health.

3. Safety of β-lactoglobulin

Due to the substantial similarities between β-lactoglobulin and traditional whey protein, as well as the substantial similarities between these products and the concentrated milk proteins that are the subject of GRN000504, the safety discussion related to concentrated milk proteins is directly applicable to establishing the safety and GRAS status of β-lactoglobulin. In particular, we view the safety overview provided in GRN000504 concerning the safety of concentrated milk protein as being relevant to β-lactoglobulin, as follows:[18]

> Given the long history of human consumption of milk, milk and milk proteins are of little toxicological concern to humans or animals. With the exception of particularly sensitive populations — namely milk-allergic and lactose-intolerant individuals, whom we address below — we are not aware of adverse effects associated with consumption of concentrated milk proteins. In addition, a literature search does not yield any reported adverse effects.

[18] GRN 504, American Dairy Products Institute. Concentrated Milk Proteins. Available at https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=504&sort=GRN_No&order=DESC&startrow=1&type=basic&search=504.

As a protein identical to the protein in milk, these safety conclusions are relevant to Perfect Day, Inc.'s β-lactoglobulin, and we agree with these conclusions as well.

With the exception of sensitive populations who are allergic to milk proteins or who are lactose-intolerant, we are not aware of adverse effects associated with consumption of milk or milk derived products in general or β-lactoglobulin specifically. We note that lactose-intolerant populations are not at risk from consumption of β-lactoglobulin from fermentation of *T. reesei* due to the notified product not being sourced from milk directly and, therefore, lacking any lactose content. A literature search (December 2018) did not yield any reported adverse effects other than allergy issues, discussed below.

4. Allergenicity

Milk is one of the eight major food allergens.[19] Because the notified substance is chemically identical to the protein found in bovine milk and isolated milk proteins, it will produce a milk protein allergy when consumed. All products containing non-animal whey protein will indicate that the product contains an allergen (*e.g.*, a protein also found in milk) to inform those consumers who are allergic to milk and address food allergen labeling requirements.

In an effort to further examine allergenic potential posed by residual *T. reesei* proteins that remain in the β-lactoglobulin product after processing, Perfect Day sponsored an analysis of its β-lactoglobulin product which was conducted by the Food Allergy and Resource Program (FARRP) at the University of Nebraska-Lincoln (Appendix 1).[20] FARRP is recognized worldwide as a leader in food allergy research and provides analytical services for the food industry. FARRP analyzed samples (via LC-MS/MS) from the production strain using two different processing streams and compared the results to commercial β-lactoglobulin (cBLG).[21] The analysis also attempted to identify levels and identity of residual *T. reesei* proteins in the Perfect Day samples. FARRP concluded that the analysis indicated no differences between the Perfect Day samples and the cBLG with regards to peptide abundance. FARRP identified residual *T. reesei* protein levels of up to 6.7%. The sample with higher residual protein was dominated by two (6.6% combined) proteins identified as extracellular membrane CFEM-domain proteins (Accession #A0A024RVF3 and G0RX84), while the most abundant residual proteins from the lower residual sample were a variety of *T. reesei* proteins involved in cell wall metabolism and substrate mobilization (0.01-0.18%). FARRP determined that neither sample contained sufficient residual fungal proteins to present allergenicity concerns.

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[19] Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA), Pub. L. 108-282, title II (Aug. 2, 2004).

[20] Mass Spectrometry for Perfect Day Product, December 8, 2018.

[21] Sigma Aldrich, β-Lactoglobulin from bovine milk, available at https://www.sigmaaldrich.com/catalog/product/sigma/l0130?lang=en&region=US.

5. Summary of Basis for GRAS Determination

Perfect Day, Inc. has determined that β-lactoglobulin produced by fermentation of *T. reesei* is GRAS for the intended use in food based on the following:

- The fact that β-lactoglobulin is manufactured under cGMP for food (21 C.F.R. Part 117) and meets appropriate food grade specifications;
- That potential contaminants, such as heavy metals, mycotoxins, and pathogenic microbes, are either absent (not detected) or below toxicological and regulatory limits;
- The intended uses and the estimated consumption of β-lactoglobulin;
- The proper labeling of the products;
- The long history of safe use of the production organism, *Trichoderma reesei*, in the industrial scale production of food enzymes and data supporting the organism's non-pathogenic and non-toxigenic nature; and
- The long history of safe use of milk and milk protein as food.

Part 7. List of supporting data and information

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From:	Drozen, Melvin S.
To:	McMahon, Carrie
Cc:	Rainer, Natalie
Subject:	RE: GRAS Notice 863: beta-lactoglobulin
Date:	Wednesday, July 03, 2019 1:28:10 PM
Attachments:	image003.png 2019-06-28_GRN 863_filing letter_signed.pdf

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Hi Dr. McMahon,

Following up on your email below and our discussion last week, this will confirm that the ingredient is not intended for use in products regulated under USDA/FSIS jurisdiction or in infant formula.

Please let us know if you have any further questions.

Have a good holiday.

Regards,

Mel Drozen.

Melvin S. Drozen
Partner
tel: +1 202.434.4222 | fax: +1 202.434.4646 | drozen@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

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Keller and Heckman LLP is pleased to announce its Practical Food Law Seminar, taking place on 2-3 October 2019 in Brussels, Belgium. This conference will provide members of the food industry with an understanding of the applicable statutory and regulatory framework in the United States for foods (including dietary supplements). The course will focus on food safety, labeling and advertising, and enforcement. The seminar presenters will also contrast the U.S. regulatory requirements with the

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From:	Drozen_Melvin S.
To:	McMahon_Carrie
Cc:	Rainer_Natalie; Fulmer_Preston A.
Subject:	GRAS Notice 863
Date:	Wednesday, August 14, 2019 2:03:55 PM
Attachments:	LGB_PAGE.tif RE GRAS Notice 863 - status update.msg

Dr. McMahon,

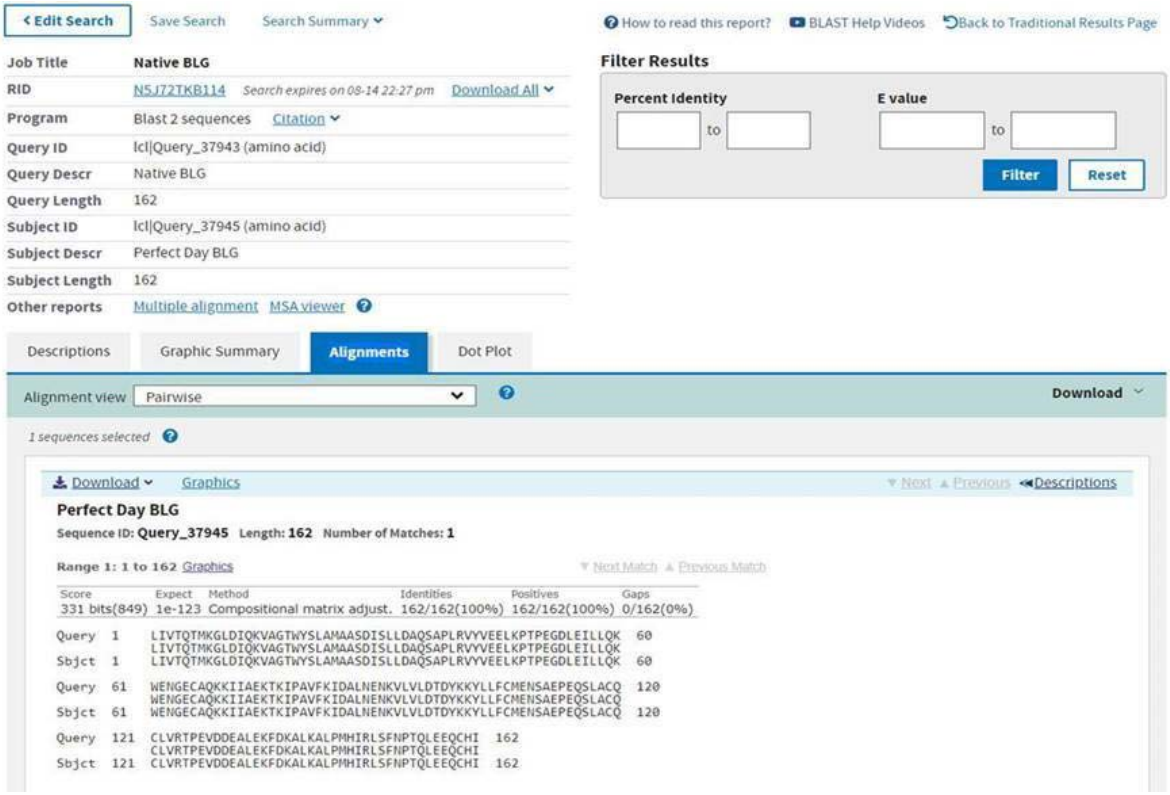
Thank you for your August 6th email update(attached) message regarding GRN 863 (concerning Perfect Day's b-lactoglobulin). You indicated that, while you had not received feedback from the full review team, it might be prudent for Perfect Day to gather data to substantiate the assertion that Perfect Day's b-lactoglobulin is identical to that found in cow's milk. While you suggested that we await feedback from the review team, we thought we would share the information gathered and reviewed to date. As detailed below, we believe that the Food Allergy Research and Resource Program (FARRP) report (provided as Appendix 1 to the GRAS Notice), sequence alignment (discussed below), and SDS-PAGE gel (discussed below) support the conclusion that Perfect Day's b-lactoglobulin produced via fermentation by *T. reesei* is identical to native b-lactoglobulin found in cow's milk.

FARRP Analysis

GRN 863 discusses a FARRP report undertaken on behalf of Perfect Day by FARRP, and the full report was provided as Appendix 1. In part, this report compared Perfect Day's b-lactoglobulin (produced via two different production streams using the same production strain) with that of a commercially available b-lactoglobulin (Sigma-Aldrich) from cow's milk via protein via mass spectrometry. FARRP found no differences between the native form and that produced via fermentation.

Sequence Alignment

Bovine b-lactoglobulin is the major protein (~65%) found in whey protein with a molecular weight of approximately 18 kDa. The native bovine protein is 178 amino acids, consisting of a 16 amino acid N-terminal signal sequence that is cleaved upon excretion from the cell to form the 162 amino acid final protein found in cow's milk (UniProtKB - P02754 (LACB_BOVIN). b-lactoglobulin produced by Perfect Day's process has been codon-optimized for production in *T. reesei*, and the 16 amino acid bovine signal sequence has been replaced with a 17 amino acid signal sequence specific to *T. reesei*. As with the native protein, the signal sequence is cleaved during excretion from the cell and is not present in the final product. The amino acid sequences of b-lactoglobulin found in cow's milk and that produced by Perfect Day are 100% identical as evidenced by the included BLAST alignment of the two sequences.



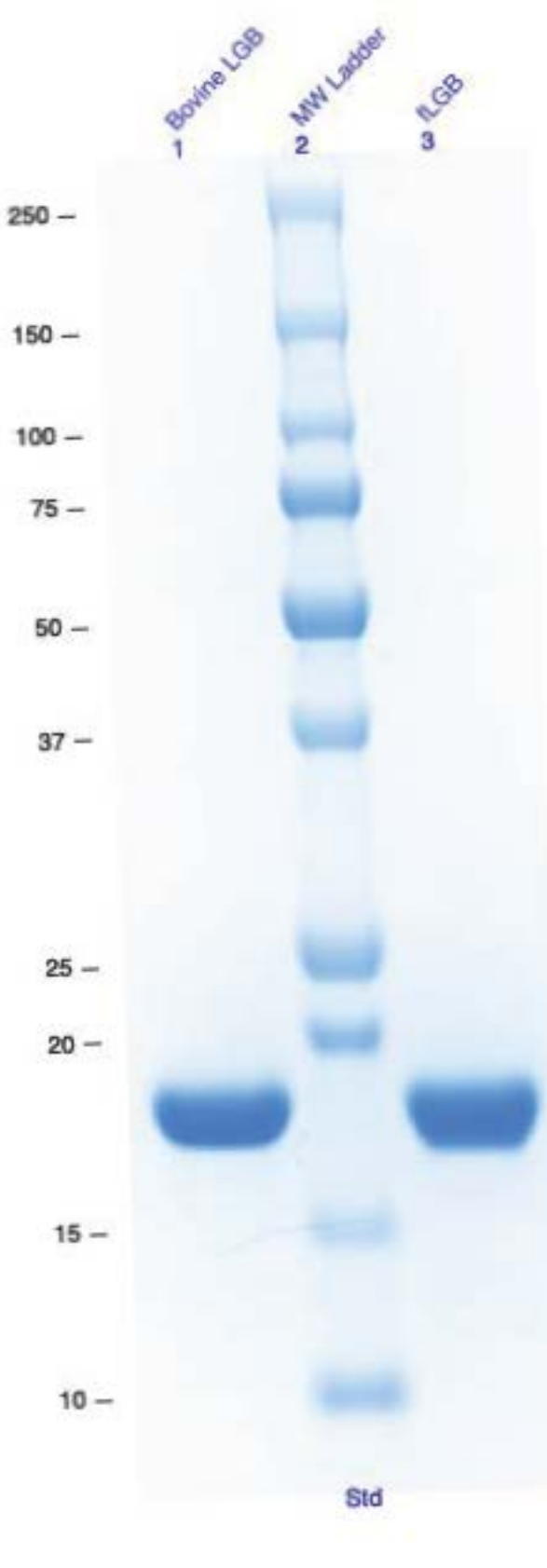
SDS-PAGE

Perfect Day has also conducted an SDS-PAGE analysis comparing commercially available bovine b-lactoglobulin with that produced by Perfect Day via fermentation. The attached image file indicates that bovine b-lactoglobulin (Lane 1) and Perfect Day's b-lactoglobulin (Lane 3) show identical bands at approximately 18 kDa.

We trust that the information above provides sufficient support for, and conclusion that, Perfect Day's b-lactoglobulin and bovine b-lactoglobulin are identical. Please let us know if you or the review team have any further questions about the equivalence issue or any other aspect of the GRAS Notice.

Sincerely,

Mel Drozen.


Bovine LGB
1
MW Ladder
2
rLGB
3
250
150
100
75
50
37
25
20
15
10
Std

From:	Drozen, Melvin S.
To:	McMahon, Carrie
Cc:	Rainer, Natalie; Fulmer, Preston A.
Subject:	RE: GRAS Notice 863 - status update
Attachments:	image001.png image002.jpg image003.jpg image004.jpg image005.jpg image006.jpg

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Hi Dr. McMahon,

Thanks for getting back to me so quickly. We will gather the information and will await further suggestions from you and or the review team. Best regards. Mel.

From:	Drozen, Melvin S.
To:	McMahon, Carrie
Cc:	Rainer, Natalie; Fulmer, Preston A.
Subject:	GRAS Notice 863
Date:	Tuesday, September 10, 2019 1:27:59 PM
Attachments:	image001.png APPENDIX 1 for GRN 863.pdf

Dr. McMahon

Thank you for the update on your progress in reviewing GRN 863. We apologize for the missing Appendix 1 and have included it here. It likely was omitted by accident after amending the filing and resubmitting based on Dr. Stice's initial feedback regarding cross-referencing previously filed GRAS notices. We look forward to hearing the team's questions.

Best Regards,

Mel Drozen.

From: McMahon, Carrie <Carrie.McMahon@fda.hhs.gov>
Sent: Tuesday, September 10, 2019 10:09 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Rainer, Natalie <rainer@khlaw.com>; Fulmer, Preston A. <fulmer@khlaw.com>
Subject: RE: GRAS Notice 863

Dear Mr. Drozen,

REGARDING: GRN 863

You are correct recalling that I had informed you that the GRN 863 review team would meet in mid-August to discuss their evaluations. However, you provided an amendment for their consideration on August 14th, which I received after the team met and prior to my relaying the team's questions. Consequently, the review team now must evaluate the data and information in the amendment and consider revisions to their questions for Perfect Day based on the additional information. We appreciate your patience.

To facilitate our review, please send a copy of Appendix 1. While the notice (PDF page 18) and your August 14 email refer us to "Appendix 1" for the FARRP report, I could not locate this in the GRAS notice submission. (Note: the submission's Table of Contents does not indicate inclusion of an appendix.)

Regards,
Carrie McMahon, Ph.D.
Consumer Safety Officer

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1202
Carrie.McMahon@fda.hhs.gov

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APPENDIX 1

Protein Mass Spectrometry for Perfect Day Foods Product

Materials and Methods (further details available on request). 7 March 2019

Samples were provided by the Sponsor. For ease of presentation, this report will refer to these samples as following :

cBLG : control BLG sample (likely commercial source)

NIKE : BLG from culture - Nike

VENUS : BLG from culture - Venus

All samples were provided to the UNL Proteomics and Metabolomics Facility. In brief, samples were reduce, alkylated and digested prior to LC-MS/MS (Thermo QE-HF mass spectrometer attached to a nanoAcquity nanoLC). MS settings were optimized for a short-column, 1h separation 'discovery' workflow.

Data analysis was performed using PEAKS-Q 8.5 (Bioinformatic Solutions Inc.) using the three databases provided ('uniprot-HYPJE', uniprot-HYPJQ' and 'uniprot-HYPJR'). Each database was appended with sequences for the A and B isoforms of bovine BLG. Searches required carbamidomethylation of cysteine (result of alkylation) and allowed for oxidized methionine (commonly occurring during sample preparation. Only tryptic peptides, and those with one missed tryptic cleavage site were accepted. Relative quantitation of proteins was performed using a label-free approach (relying on parent ion abundance) using the mean of the abundance of the three most abundant peptides of the given protein (if available). Microsoft Excel was used for data recording, manipulations and storage.

Equivalency of BLG

The major BLG isoform present in all three samples appears to be BLG-B.

Identification of BLG-B in NIKE, VENUS and cBLG is equivalent. Coverage of BLG in representative MS analyses is shown in Figure 1. It is likely that the BLG-B present in each of this samples is similar with respect to primary (sequence and modifications) structure.

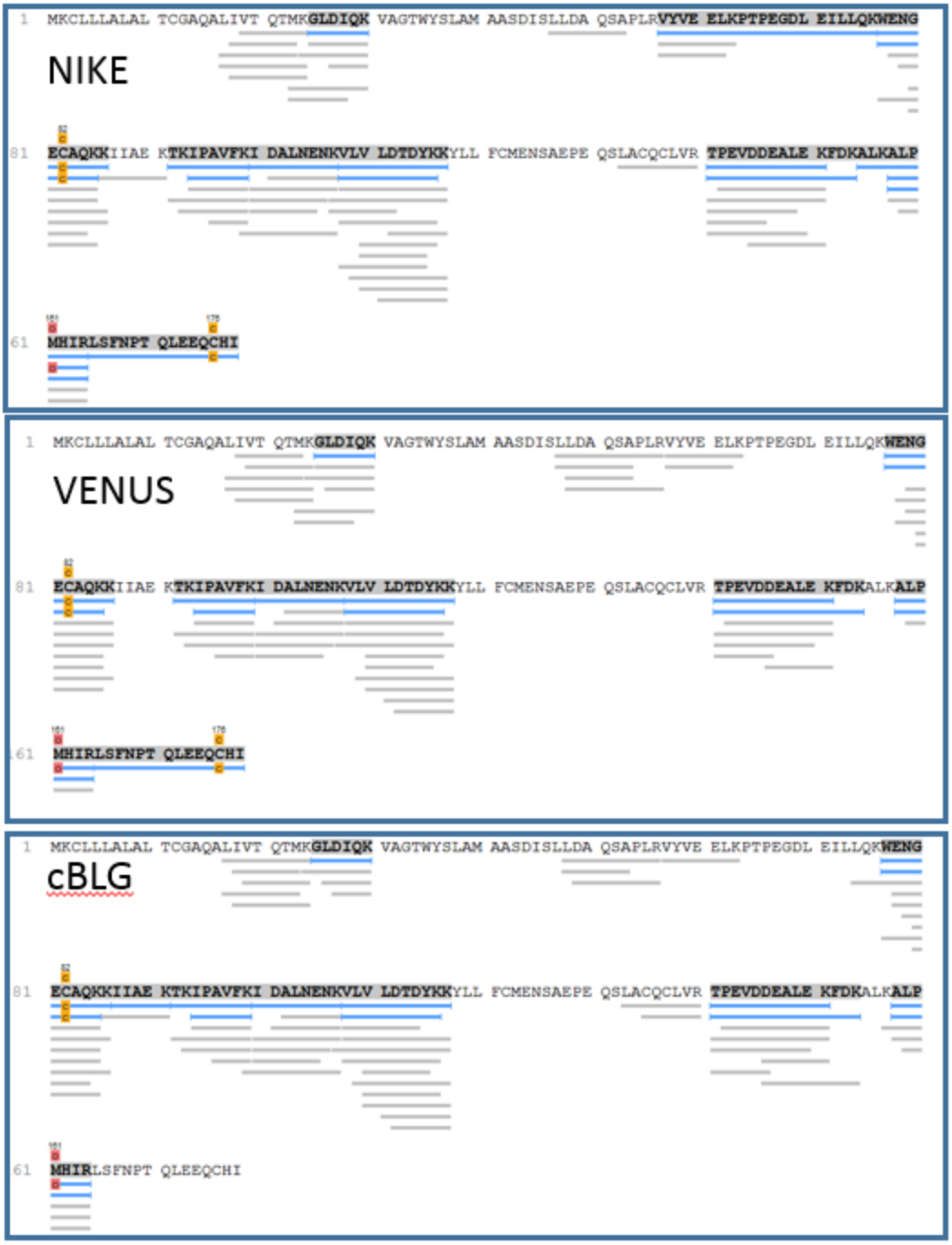

Figure 1. **Coverage of BLG-B in representative MS analyses of NIKE, VENUS and cBLG samples.** The sequence of BLG-b is shown. Blue lines represent identification of peptides consistent using the PEAKS database search algorithm. Grey lines indicate peptides identified by *de novo* data analysis.

Relative quantitation of individual peptides derived from BLB-b allows quantitation of BLB-B itself. The amount of BLG-B derived peptides in the provided samples was compared to the abundance in the cBLG samples. This comparison is shown in Figure 2. The NIKE sample was more similar to the cBLG sample with respect to peptide recovery. The peptides VLVLDTDYK and WENGECAQKK appeared to recover

less well from Venus samples. This may be due to modification of one or more amino acids within these peptides, or to differences in tryptic digestion due to the presence of interfering molecules in the sample. However, the relative recovery of peptides was, overall, similar to that of the cBLG samples.

The slope of the regression lines allows calculation of relative amounts of BLG in each of the three samples. If **cLBG contains 1 unit** of BLG-b, **NIKE would contain 0.70 units**, and **VENUS 4.27 units**. There is therefore approx.. 6 -fold difference between the amount of BLG-b in NIKE and VENUS. This may be important for your yield/product.

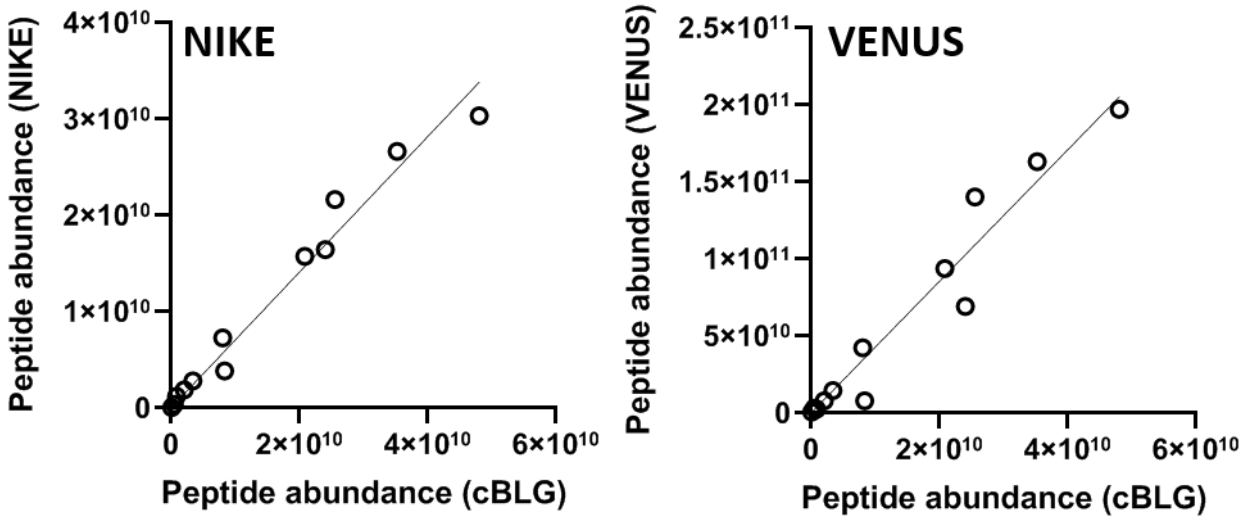

Figure 2. Abundance of BLG-B peptides in control (cBLG) samples vs that in NIKE and VENUS samples. An analysis showing a perfect straight line would indicate that all observed peptides were present in the same ratio as that in cBLG. Line indicates a simple (x-y) regression forced through the x-y intercept. Data indicate the mean of three analytical replicates.

Presence of contaminating proteins from *Trichoderma reesii*

MS data for cBLG, NIKE and VENUS were searched against databases provided by the Sponsor. These databases contained fungal proteins, and were named 'uniprot-HYPJE', uniprot-HYPJQ' and 'uniprot-HYPJR'. Searches against these databases allowed the detection of fungal proteins. Table 1 contains details on fungal proteins identified. Quantitation of these fungal proteins was performed using BLG-B as a standard, with all fungal protein contaminants presented as a % of the abundance of BLG-B in that sample. Proteins with only one identified peptide were removed as likely false identifications (and in any case extremely low abundance).

The **NIKE** sample contained significant (>5%) amounts of an extracellular CFEM-domain protein (A0A024RVF3) and of an uncharacterized protein (G0RX84) with homology to other CFEM-domain proteins. These proteins appear to have a role in in general as pathogenesis response proteins and are relatively abundant.

The **VENUS** sample contained lower amounts (0.01-0.18) of extracellular Trichoderma proteins involved in sugar/wall metabolism and presumably therefore with a role in substrate mobilization. An enzyme of diphthamide (modified histidine) synthesis was also identified.

Accession & Database	Cov. (%)	#Peptides	NIKE	VENUS	cBLG	Description
BLGB	59	16	100.000	100.000	100.000	BLGB
A0A024SIP8	5	6	0.000	0.184	0.000	Diphthamide biosynthes
G0RDA3	13	8	0.000	0.184	0.000	Aldose-1-epimerase OS=
G0RNW6	7	4	0.000	0.115	0.000	Glycoside hydrolase fa
G0RWC5	2	3	0.002	0.069	0.000	Glycoside hydrolase fa
A0A024RWH3	2	3	0.002	0.068	0.000	Family 31 glycosyl hyd
G0RVZ7	8	3	0.000	0.062	0.000	Predicted protein OS=H
G0RDP2	9	6	0.000	0.044	0.000	Beta-hexosaminidase OS
A0A024S8R0	9	6	0.000	0.044	0.000	Beta-hexosaminidase OS
A0A024RX70	8	3	0.000	0.029	0.000	Uncharacterized protei
G0RSR6	1	2	0.000	0.014	0.000	Glycoside hydrolase fa
A0A024RYU5	1	2	0.000	0.014	0.000	Uncharacterized protei
G0R911	4	3	0.000	0.013	0.000	Glycoside hydrolase fa
A0A024SL31	4	3	0.000	0.013	0.000	Alpha-1 2-mannosidase
G0RVH0	5	2	0.009	0.012	0.000	Predicted protein OS=H
Q92457	2	2	0.000	0.007	0.000	Alpha-galactosidase 2
G0RVT1	2	2	0.000	0.007	0.000	Alpha-galactosidase OS
A0A024RZH1	2	2	0.000	0.007	0.000	Alpha-galactosidase OS
A0A024RVF3	27	4	5.312	0.001	0.000	CFEM-domain-containing
G0RX84	45	3	1.312	0.000	0.000	Predicted protein OS=H
G0R7T9	12	2	0.005	0.000	0.000	Predicted protein OS=H
G0R8E8	7	2	0.025	0.000	0.000	Predicted protein OS=H
A0A024S4D1	12	2	0.005	0.000	0.000	Uncharacterized protei
A0A024SN77	7	2	0.011	0.000	0.000	Uncharacterized protei

Table 1. Identification and relative quantitation of fungal proteins in samples. The % of protein covered by detected peptides (Cov. %), number of peptides from given proteins (#peptides), the relative amount of protein relative as a % of the amount of BLG for each sample (NIKE, VENUS, cBLG) and the first 22 characters of the Uniprot protein description is given.

The *Trichoderma reesii* proteins identified suggest that both fungally expressed recombinant BLG preparations are derived from fungal proteins that are secreted from the cell. Further, although BLB-B dominates the protein content of both NIKE and VENUS, proteins from *T. reesii* are readily identifiable and in modest to appreciable quantities. The differences in concentration of the fungal proteins between NIKE and VENUS, suggest that their production differs in fundamental ways (strain used, growth, harvesting or purification) and is likely not a simple batch-batch variation. Further experiments could precisely quantify contaminating proteins if necessary.

Overall conclusions

VENUS is more enriched for BLG-B (more pure). I would suggest the BLG-B present in VENUS is equivalent to that of the cBLG in spite of observed peptide recovery differences. NIKE also produces intact BLG that is sequentially identical to cBLG, but it contains less BLG and is less pure than is VENUS. The other proteins in NIKE are from *T. reesii*.

Further evaluation of proteins based on part on your questions last week about labeling for fungal proteins. The proteins from *T. reesii* that were detected above 0.001% of BLG, using BLG as the 100% relative standard. There were many other lower abundance proteins with peptides determined by LC-MSMS. RG compared the amino acid sequences of the whole detected proteins to AlleregnOnline.org version 18B. The results are described here.

TABLE 2. Proteins used in comparisons to AllergenOnline.org veron 18B

MS #	Acc #	Cover	Pept #	Nike %	Venus %	cBLG %	Avg Mass	Des	Data base
	BLGB	59	16	100.000	100.000	100.000	19883	------	HYPJE
1	A0A024SIP8	5	6	0.000	0.184	0.000	92653	Diphth	HYPJR
2	G0RDA3	13	8	0.000	0.184	0.000	44577	Aldose	HYPJQ
3	G0RNW6	7	4	0.000	0.115	0.000	50349	Glycos	HYPJQ
4	G0RWC5	2	3	0.002	0.069	0.000	98544	Glycos	HYPJQ
5	A0A024RWH3	2	3	0.002	0.068	0.000	101780	Fam 31	HYPJR
6	G0RVZ7	8	3	0.000	0.062	0.000	25376	Pred	HYPJQ
7	G0RDP2	9	6	0.000	0.044	0.000	64261	Betahex	HYPJQ
8	A0A024S8R0	9	6	0.000	0.044	0.000	64261	Betahex	HYPJR
9	A0A024RX70	8	3	0.000	0.029	0.000	25376	Unch	HYPJR
10	G0RSR6	1	2	0.000	0.014	0.000	116642	Glycos	HYPJQ
11	A0A024RYU5	1	2	0.000	0.014	0.000	116642	Unch	HYPJR
12	G0R911	4	3	0.000	0.013	0.000	89022	Glycos	HYPJQ
13	A0A024SL31	4	3	0.000	0.013	0.000	89022	Alpha	HYPJR
14	G0RVH0	5	2	0.009	0.012	0.000	43285	Pred	HYPJQ
15	Q92457	2	2	0.000	0.007	0.000	82080	Alpha	HYPJE
16	G0RVT1	2	2	0.000	0.007	0.000	82093	Alpha	HYPJQ
17	A0A024RZH1	2	2	0.000	0.007	0.000	82093	Alpha	HYPJR
18	A0A024RVF3	27	4	5.312	0.001	0.000	18684	CFEM	HYPJR
19	G0RX84	45	3	1.312	0.000	0.000	9907	Pred	HYPJQ
20	G0R7T9	12	2	0.005	0.000	0.000	18071	Pred	HYPJQ
21	G0R8E8	7	2	0.025	0.000	0.000	28117	Pred	HYPJQ
22	A0A024S4D1	12	2	0.005	0.000	0.000	18071	Unch	HYPJR
23	A0A024SN77	7	2	0.011	0.000	0.000	28117	Unch	HYPJR

Alignments of the *T. reesii* sequences that were identified which were above 0.001% of the total predicted mass based on BLG, were tested in the bioinformatics searches described in Table 3. Three of the proteins #3, (with 15 matches to *Cryptomeria* sp. and *Juniperus* sp. pollen allergenic homologes beween 35% and 38.5%), identical sequences #7 and #8 (with one match of 42.5% to *Penicillium chrysogenum* protein over 112 AA) and one protein #14 (with matches of ~ 40% over 80 amino acids to a protein in house dust mite (HDM) allergen in both *Dermatophagoides farina* and *Dermatophagoides pteronyssinus*). Those alignments are low in identity match and are not likely to represent a real risk, especially since the proteins represent low concentrations of between 0.012% and 0.115% in the VENUS BLG material. More detail can be provided if needed.

Table 3 UNIQUE sequences were compared to AllergenOnline.org version 18B as shown in Table 3. The amino acids are included (but duplicate sequences left out). The Database they matches are shown. The output in terms of >35% identity over 80 AA are shown (if any). There were a few alignments identitied by Overall FASTA, but those were generally below 35% identity.

MS #	Acc #	**Amino acid sequences are shown along with a description of which ones were identical, and which ones had significant matches.**	Database
1	A0A024SIP8	MKLTGSPLAVFALAASLPLTAAAAKSGGGPDKDGKYWIRAKGIEASFIPYGASISNLFIDDRYG IRRDLVGGFDNASYYGIDAQHPHFGGVPGRYANRIKNSTFSIDGTTYRVLPNENPTKAEPNG TDTLHGGPDGWDWRNFTVTAHTPTSITFSIVDPDGKEGFPGEVVSHITYTVKEHQWDLKM VALATTKKTPIMLSSHTYWNLDGFANNETQTALNHTLHLPYSGQRVDVDGYLIPTGDILANK KGTANDFWSRPKQLGKGFEQSGIKNNCGNNCTGFDTCYIVNRDALQPFDWRTEGPVASLS SAWSGIHLDVYSDQTAFQVYSCNGQNGTLALKKTQGLHGNKRFPRTIPQYGCLVMEVEDW IDGINNPEWGRKQVYGPGDEPALVDLGAAADIEEARLEVHTTESETTKNETPATTTTTRQPK RRFVGRRAADEAAAAKATTEQGGSGAVQAAKPRRAPRLLNRVPPEISEDPNLKEAIALLPAN YNFEIPKTIHRVRESGARKVALQMPEGLLLFATTISDIITQFCPGVETLIMGDVTYGACCIDDYT ARALGCDLLVHYAHSCLIPVDVTKIKTLYVFVDISIDTAHLIASLERNFASGKTIAIVGTIQFNATI HGVRSSLEAAGFSVVVPQIGPLSKGEILGCTSPRLRDEDGVDLILYLGDGRFHLESIMIHNPAIP AYRYDPYSRKLTRETYGHDEMQSVRRSAIQTARKARRWGLILGSLGRQGNPHTLAMIERELA ERGIPKVDLLLSEIFPGKLAMMSDVECWVQVACPRLSIDWGYAFPRPLLTPYEALVALEKRG GWSKEEGGDGIYPMDYYGRDGLGRTKPLEGVAA	0 match by sliding 80mer
2	GORDA3	MKLTGSPLAVFALAASLPLTAAAAKSGGGPDKDGKYWIRAKGIEASFIPYGASISNLFIDDRYG IRRDLVGGFDNASYYGIDAQHPHFGGVPGRYANRIKNSTFSIDGTTYRVLPNENPTKAEPNG TDTLHGGPDGWDWRNFTVTAHTPTSITFSIVDPDGKEGFPGEVVSHITYTVKEHQWDLKM VALATTKKTPIMLSSHTYWNLDGFANNETQTALNHTLHLPYSGQRVDVDGYLIPTGDILANK KGTANDFWSRPKQLGKGFEQSGIKNNCGNNCTGFDTCYIVNRDALQPFDWRTEGPVASLS SAWSGIHLDVYSDQTAFQVYSCNGQNGTLALKKTQGLHGNKRFPRTIPQYGCLVMEVEDW IDGINNPEWGRKQVYGPGDEPYVLQASYRFSIDGKKSA	0 match by sliding 80mer

3	G0RNW6	MVALSSIILAALPIALAVSSSAPDLMGREANAAQTESHWANHAAAQGRHFCYVRPDADGG DDAPAIMDALNNKCNSRSLVIFPGPVYNIQTNMTTLNLEDVVIYQFGRMLWSTDIDYWLSV SMPVGFQNQSTVWYFGGNNVIWDGWGVGTLDGNGQVWYDWARSQGNLPHRPMNIN LRTLTNSVIRRMRFVQSQMWTMAITYSQHVELDDIYVNSTSTSQWSTLNTDGCDTIFSDSIT FRRWTVSNGDDAIALKMNSSNIAVYDSYFENGQGIAIGSMGQYNGRYEYLENFYAKNITLK NTAHVSYLKTWAGISRGYPPNGGGGGYGVARNITIEDVKLIGGRQQPFFAWQCENYSGYA GQDCDSSLFKMEDVAWRRVSGTVQSGVTEAAYFQCSAAAGGCDDFEVTGFDVTKEGTDEL LAIWDCFNVNNPVGFTCTESQAQKMSSDVTGGHGSNNK (Cryptomeria, Juniperus: 35% to 38%)	15 match by sliding 80mer 35 to 38%
4	G0RWC5	MVYWKALLYAGLASAAALFKKDNATAAGLDQCPGYKASNVRVTATGVTADLTLAGAACNV YGTDLPHLTLQVTYQTEDRIHVLIQDQGNQVYQVPESVFPRPGGSVWSQTSKLKFSYTANPF SFKITRAKTGEVIFDTSAASLVFESQYLRLRTSLPANPNLYGLGEHSDSLRLETTNYIRTMWNQ DSYGIPSHANLYGTHPFYLEQRATGAHGVFFLNSNGMDIIINKDASGNQYLEYNTIGGVFDF YFVAGPTPVAAVQQYGEFAGFPTMQPYWGLGFHQCRYGYRDAFDVAEVVQNYSLAGIPLE TMWTDIDYMDRRRVFTLDPDRFPLSKMRELVDHLHAHDQHYVVMVDPAVAYQNYPPAN QGLEDNVFMLRSNGSVWIGVVWPGVTVFPDWFSANITRYWNGQFQTFFDADTGLDIDAL WIDMNEPSNFPCNFPCDDPYKAAIGYPPAPPPVRAPPRPLPGWPIRNIVPNNKPSSGRGDQ KGLPGRDLLYPKYAIHNKAAYQDSWNADKGGISNHTVNTDLIHQNGLAMYDTHNLYGTM MSSASRDAMEARRPGLRPLVITRSTFAGAGSKVGHWLGDNMSQWSYYTVSIRTMLAFTSL FQFGFVGSDVCGFGGNTNEELCARWASLGAFNTFYRNHNDYGNIGQEFYRWPSVASAAKK AIDIRYRLLDYIYTALWRQSTDGTPAVSPMFFQYPEDPATWGLELQFFFGPGLVVAPVTQQG STSVNVYLPSGVFYDWYTHARIDGGATNHAITGVDITSIPLFIRGGAILPLRVKSANTTTELRK QNFELLIALDASGSASGELYLDDGVSIHQRATTHVTFTYKKGIFILGGSFSLRVPFLISKVTILGG RPSAAAKSSSAGGSNSESFDVHLPFTGPASVRIG (= #5 below)	0 match by sliding 80mer
5	A0A024RWH3	Findings the same as protein 4	0 match by sliding 80mer
6	G0RVZ7	MQRILALTAAAAGLFVTALGTKVPSVNVPSCPRIGSVSYTTSVPDRTPFPRTQVDLCYTDDSL ELTFIAYDEVNYFFNASQGTNDDIWEYEVMEAFIYKGTEDPQTYVELEVNPNNVTYQAFVYN PSKNRTAGAPFDHFFISDPATDGFKAKTILNKPAKTWRSTLTVPLGIFNVDVGKAKGTSWRM NFFRTVVSPEIYPNQILGGWGVPDQASFHITKYFGKVKFI (= #9 and 10)	0 match by sliding 80mer
7	G0RDP2	MMLLPKAVLAIAALAFSPANALWPIPQKISTGDGVLFIDQAVRVTYNGVPIITIGYTPPASSHF DSRQIVQGGVSRALQSIFSTNYVPWKLHPRNSNFEPKLALQNRVQTIAIQQTGKDSASTFKP RAGDVDESYSLTVSKTGQVSITAKSSTGVLHALETFSQLFYKHSAGPFYYTTQAPVSITDSPKY PHRGIMLDLARNYQTVDDIKRTIDAMSWNKLNRLHLHITDSQSWPLVIPSLPKLSQAGAYHP SLVYTPADLAGIFQYGVARGVEVITEIDMPGHIGVVDLAYNDLIVAYEQMPYQYYCAEPPCG AFSMNSSKVYDFVDALFDDLLPRVAPYSAYFHTGGDELNANDSMLDPHIRSNATDVLQPLL QKFLNFAHAKIRAAGLSPFVWEEMVTTWNLTLGNDTVVQSWLGGTAVKDLAESGHKVIDT DYNFYYLDCGRGQWVNFPNGASFDTYYPFGDWCAPTKNWRLIYSHDPAAGISASHAKNVL	1 match by sliding 80mer

		GGELAVWSEMIDASNLDNIIWPRASAAGEVWWSGNVDAATGQNRSQLEVVPRLNEFRER MLARGVSAMPIQMTYCTQLNATACALFP (Same as #8 below, one match of 42.5% to Penicillium chrysogenum over 112 AA)	
8	A0A024S8R0	Finding the same as protein 7 above	0 match by sliding 80mer
9	A0A024RX70	Finding the same as protein 6 and # 10 below	0 match by sliding 80mer
10	G0RSR6	Finding the same as protein 6 and # 10 below	HYPJR
11	A0A024RYU5	MRSTVTSAAALLSLLQLVSPVHGTTLVDRVTKCLSRHDGSDAESHFSKNVYKTDFAGVTWDE DNWLLSTTQLKQGAFEARGSVANGYLGINVASVGPFFEVDTEEDGDVISGWPLFSRRQSFA TVAGFWDAQPQMNGTNFPWLSQYGSDTAISGIPHWSGLVLDLGGGTYLDATVSNKTISHF RSTYDYKAGVLSWSYKWTPKGNKGSFDISYRLFANKLHVNQAVVDMQVTASKNVQASIVN VLDGFAAVRTDFVESGEDGSAIFAAVRPNGVANVTAYVYADITGSGGVNLSSRKIVHNKPYV HANASSIAQAVPVKFAAGRTVRVTKFVGAASSDAFKNPKQVAKKAAAAGLSNGYTKSLKAH VEEWATVMPESSVDSFADPKTGKLPADSHIVDSAIIAVTNTYYLLQNTVGKNGIKAVDGAPV NVDSISVGGLTSDSYAGQIFWDADLWMQPGLVAAHPEAAERITNYRLARYGQAKENVKTA YAGSQNETFFSASAAVFPWTSGRYGNCTATGPCWDYEYHLNGDIGISLVNQWVVNGDTKD FEKNLFPVYDSVAQLYGNLLRPNKTSWTLTNMTDPDEYANHVDAGGYTMPLIAETLQKANS FRQQFGIEQNKTWNDMASNVLVLRENGVTLEFTAMNGTAVVKQADVIMLTYPLSYGTNYS AQDALNDLDYYANKQSPDGPAMTYAFFSIVANEISPSGCSAYTYAQNAFKPYVRAPFYQISE QLIDDASVNGGTHPAYPFLTGHGGAHQVVLFGYLGLRLVPDDVIHIEPNLPPQIPYLRYRTFY WRGWPISAWSNYTHTTLSRAAGVAALEGADQRFARKPITIHAGPEQDPTAYRLPVKGSVVI PNKQIGSQQTYAGNLVQCHAASSPNDYVPGQFPIAAVDGATSTKWQPASADKVSSITVSLD KEDVGSLVSGFHFDWAQAPPVNATVIFHDEALADPATALASAHKHNSKYTTVTSLTNIELSD PYVSTKDLNAIAIPIGNTTNVTLSHPVAASRYASLLIVGNQGLDPVDVKAKNGTGATVAEWAI FGHGKEHSGKPSSHSKRRLNVRTAATLSNPRSFMRRRL	0 match by sliding 80mer

12	G0R911	MHFQQVVAYWPLLLGLSQAAEEKHSQFDVLDYVDPLIGTANGGHVFPGASLPYGMAKSVADGNKEIQGGYASNDGLITGFSHMHDSGTGGGASLGNFPLFAQTGCLNDDINNCYFPSSLRASEKINSTVVAKPGYFALQLNTSVKAEMTVTNHTALYRFSFPTDGTPTKLPADGKTPYSPLILADLADLSGSRSAAVISVDAHTGRISGNGTFRPSFGIGTYNAYFCTDFKGAKLRNTGIFVNNRAGSDHKSFRAYDDGQSPVLPAGAWAQFHPPTNNQILARVGLSFISTAQACHNAEKEIPNFDFEAVRSDAEDAWRKKLSVIKVDNTGVSDSLQRTFWSGIYRTLLSPQDYTGENPLWKSNEPYFDSYYCIWDSFRSTHPLLTLVDPHAQALMVRSLIDIYRHEGKLPDCRMSLCKGFTQGGSNADNLLADSYLKGLKDGIDWDTGYKAVVSDAEEEGQIWTVEGRGGLQSWKKLGYIPTDDWDPNGYGLFTRSISRTIEYSYNDFCIAEMARDLGKHADAEKYLKRSGNWKNMFDPHSKSMLNLTGTQDPSQFTDSGFTGFLQPRYLNGTFGFQDPAICTDLYNFEGCYLNPGGHETYEAGSWLYTFYVPHDQATLITTLGGPDEFVRRLRYMHDTPGLFYIGDEQAYLLLFIFHYAGRPGISAEYAHKYIPSAFNDTASGLPGNDDSGAMGSFTALTMLGLYPMSGQDVYIIIPPFFPEVKLTDPRTGKTAIIRNINFDAGYKNIYIQSAKLNGKPYTKGWVTHSFFADGGVLELTLGPKENTKFGTSEKDFPPSASKDFWK (Same findings as #13 below)	0 match by sliding 80mer
13	A0A024SL31	(Same findnigs as #12 above)	0 match by sliding 80mer
14	G0RVH0	MKLLGLSVVLSALQAVDARDPACRPAAPSCRFVSPWTARECSAIIHSHHIRLSTCEAPEKTITKTVVVTHHPTFTHTKTVKPSPSTKTTTKVITQTDPVTQKDTTTAWATATAVSTTTTVTSDFTTATTTTTSVETETDSTTVTTTQDVTTTVSWAPEVCTPTVTLAPTLAPSKRSDKRNDYRIPRDCSCFLTSTKSCGPRATKVVTRIEEDRPRYITKTVTDRRKFETVTITKTSITHINGAPPPKQTVTSTTTSTVITTATSTSTVEQTTTATEATTTTVVESATVTTTHPVTATEDPCNPANVNKYLLSGPPSNPNVVLGFRGDGNNNPGICCQNCVMNADCVYWKLSAGGSICEGYFTSRTAPVEGCTTNACRRGHPFMAVSPQSDGNTYGMGQCGVFGVAF (two matches to House dust mites, Dermatophagoides farina and Dermatophagoides pteronyssinus, 40% ID)	2 matches by sliding 80mer (HDM 40% ID)
15	Q92457	MLGAPSPRRLADVLAVTAGLVASVRAASPISVSGKSFALNGDNVSYRFHVDDDSKDLIGDHFGGPATEDGVFPPIIGPIQGWVDLIGRQRREFPDLGRGDFRTPAVHIRQAAGYTVSDFQYKSHRVVEGKPALRGLPSTFGDAGDVSTLVVHMYDNYSSVAADLTYSIFPKYDAIVRSVNITNMGKGNITIEKLASLSVDLPYEDFDMLELKGDWAREGKRLRRKVDYGSQGFGSTTGYSSHLHNPFFSLITPTTTESQGEAWGFSLVYTGSFSVEVEKGSQGLTRAAIGVNPYQLSWPLGPGETFSSPEAVAVFSTTGVGGMSRKFHNLYRKHLIKSKFATQMHPVLLNSWEGLGFDYNDTTILHLAQESADLGIKLFVLDDGWFGVKHPRVSDNAGLGDWEANPKRFPQGLPDFISDVTKLKVANSSDHLQFGLWFEPEMVNPNSTLYMEHPDWAIHAGSYPRTLTRNQLVLNVALPEVQDFIIESLSNILSNASISYVKWDNNRGIHEAPYPGLDYAYMLGLYRVFDTLSSKFPNVRWEGCASGGGRFDPGVLQYFPHIWTSDDTDAVERIAIQFGTSLVYPPSAMGAHVSAVPNGQTQRTTSIAFRAHVAMMGGSFGFELTPAEMPEDDKAQIPGIIALAEKVNPIVVKGDMWRLSLPEESNWPAALFISQDGSQAVLFYFQIRANINNAWPVLRLQGLDASAKYKIDGNQTFSGATLMNIGLQYQFNGDYDSKVVFLEKQT (Findings = 16 and 17 below)	0 match by sliding 80mer
16	G0R	Findings equal # 15 and # 17	0 match by

	VT1		sliding 80mer
17	A0A024RZH1	Findings equal # 16 and # 17	0 match by sliding 80mer
18	A0A024RVF3	MKTAFVALALAALAQAQTRADIPSCALPCLDDAVKANTKCSTTDYACICKNFDAVQGAATG CVISKCGTDVAINKVLPATQALCAANSGGSGSSGSSSAAAGTTAAQQTTSAAQETTTVAQTT VAQTTVVSSVVSSPPVQTITTTTPAGPVGSGTGVVPPPAGNRTTTGAPTAPTNAGSALLPGL AMLALGALAL	0 match by sliding 80mer
19	G0RX84	MKTAFVALALAALAQAQTRADIPSCALPCLDDAVKANTKCSTTDYACICKNFDAVQGAATG CVISKCGTDVAISMSCPLPRPVPFDGREPHDNRC	0 match by sliding 80mer
20	G0R7T9	MKFFATLLFAAAGVSAAGSATAPATPGSTCLADYILEDCLSSTTKTANSCKPTDYECLCAAYQ AVLTCYNNCPNDIRAPSVQQQVDSYCRTATLLNPKTTATKAKPSQSQESSGSEETSASSPSN NDEASATDAGPSQTSGSRTSATTAATASSSTNAAATMMGSVAGIVMAVAGAAAAMV	0 match by sliding 80mer
21	G0R8E8	MKSVVIALCTLVAATAAQGSANLAACGQTCAANMLSADKADELGCKQNDLRCLCANKNFL YGLRDCSAAICSAEDARKVVEYGISVCAGAGVAIQTSSGGGSGGASRTASVSGSATDSVSTLV TATASGAITETLLTTVTSDGTTITTGIATATGNASNGVVSTFTTAVTDSDGNVHTSTGQTTLSG TISVTLTGSVPTATGTDSAALTTVTSGSSAIVKTLTTKSETATVTESATQTESTNSGAEATETET ATETASSSASSTSSTGAGVPQKTAGPVGIIAAAGVALLML (Findings are identical to those of #23)	0 match by sliding 80mer
22	A0A024	Findings are identical to those of #20	0 match by sliding 80mer

	S4D1		
23	A0A024SN77	Findings are identical to those of #21	0 match by sliding 80mer

More information can be provided about the methods for LC-MSMS if needed and about the bioinformatics searches. Our understanding (PJ and RG) is that any food that these ingredients are put in would need to be labeled as Cow's milk, or derived from cows milk for the safety assessment, and if the food developers use a "CONTAINS" statement, cows milk would need to be on it.

Since the proteins from *T. reesii* are so low in abundance and they are not the intended ingredient, there does not need to be a label of Trichoderma or fungal proteins.

Clearly you may want to consider which manufacturing procedure you use to optimize yield and minimize T. reesii proteins, but that may take additional samples to know. It is possible that the strains differ, that the purification method differs or the culture conditions differ.

If you have questions, please contact us at your convenience.

Dept. of Food Science & Technology, FARRP, UNL.

From:	Drozen, Melvin S.
To:	McMahon, Carrie
Cc:	Fulmer, Preston A.; Rainer, Natalie
Subject:	RE: GRN 863 (Perfect Day) - technical review team questions
Date:	Monday, December 30, 2019 3:32:36 PM
Attachments:	image007.png 2019-11-25_GRN 863_technical review questions.pdf GRN 863 Technical review questions response_final.pdf COA_P0164_Covance.pdf COA_P0193_Covance.pdf COA_P0210_Covance.pdf

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Dear Dr. McMahon,

Please find attached our letter responding to the technical review team questions sent via your email of November 26(below). We trust that we have completely responded to the questions and that you will let us know if you need anything further. In the meantime, we look forward to successful completion of FDA review for GRN 863 as soon as possible and receipt of a close out letter. Happy New Year. Best regards. Mel Drozen.

From: Drozen, Melvin S.
Sent: Tuesday, November 26, 2019 2:38 PM
To: McMahon, Carrie <Carrie.McMahon@fda.hhs.gov>
Cc: Alsobrook, Lisa P. <alsobrook@khlaw.com>; Fulmer, Preston A. <fulmer@khlaw.com>
Subject: FW: GRN 863 (Perfect Day) - technical review team questions

Dear Dr. McMahon,

Thank you for your questions. We are reviewing them, will review with our client and get back to you quickly since certainly the client wants to obtain a “no questions” letter ASAP. Of course, do let us know if you have a specific time frame in mind. In the meantime, have a nice Thanksgiving.

Regards,

Mel Drozen.

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December 30, 2019

Dr. Carrie McMahon
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Office of Food Additive Safety
Washington, DC

Re: GRN 863: Technical Review Questions

Dear Dr. McMahon:

We are writing to respond to the technical review questions attached to your email on November 25th. Please find below Perfect Day's (PD's) responses to your team's questions and the attached files containing CoAs for three non-consecutive production lots. We first set forth the FDA question, followed by our response.

Identity: When the source of a notified substance is a biological material, your GRAS notice must include both taxonomic information (*e.g.*, genus, species), including as applicable data and information at the sub-species level (*e.g.*, variety, strain). See 21 CFR § 170.230(a)(2).

1. On page 6, you state that the host strain was genetically modified with "one or more expression cassettes." Please explain what you mean by <u>one *or* more</u> with respect to the production strain.

   Response:

   "One or more expression cassettes" refers to the differences between the current strain and future iterations which may provide improved yield and purity of the β-lactoglobulin product. Perfect Day's current "best producing" strain contains a single insert. In the future, Perfect Day (PD) would like the flexibility to iterate the strain for increased production through targeted host gene knockouts. These strain improvements would have the effect of deleting host genes and replacing them with β-lactoglobulin expression cassettes. Potential target genes include proteases and/or enzymes involved in metabolism of carbohydrates which are not utilized in the PD process such as mannase or xylanase. Targeted deletion of these host genes and replacement with additional expression cassettes would serve the dual purpose of increasing β-lactoglobulin while decreasing host protein

production (and thereby increasing purity of the target protein). The Company believes that this approach is appropriate for β-lactoglobulin produced via fermentation of *T. reesei* due to the extensive library of safety data on the production organism, all of which indicates *T. reesei* is an extremely safe production organism and therefore deletion of additional non-essential host genes would have no impact on the safety of the organism as the production organism for β-lactoglobulin.

2. Please provide the name of production strain. You should provide sufficient information to distinguish the production strain from the parent strain (*T. reesei* strain QM6a).

   Response:

   The current production strain is *Trichoderma reesei* QM6a-PD1. QM6a-PD1 was produced via the introduction of a single expression cassette for β-lactoglobulin to the host strain QM6a.

3. Please describe the method you used confirmed the identity of the production strain (for example, to confirm the nucleotide sequence and copy number of the integrated expression cassette(s)).

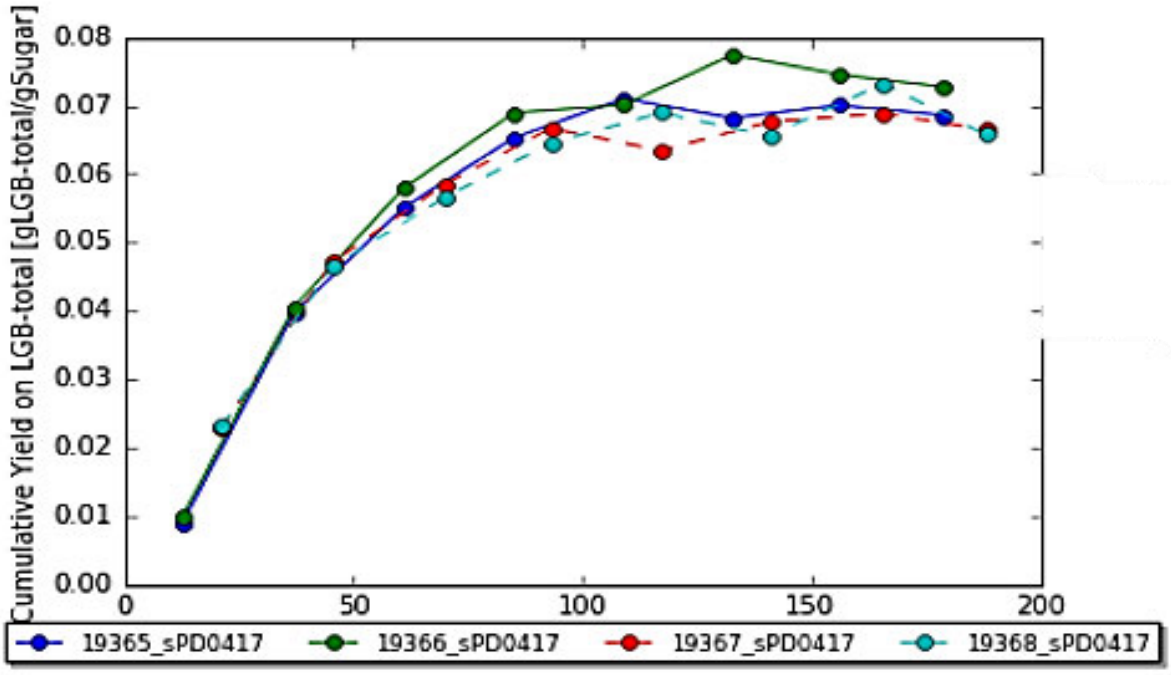
   Response:

   Correct insertion of the β-lactoglobulin expression cassette is confirmed via PCR, and insertion copy number is confirmed via qPCR.

4. On page 7, you state that "the expression cassette is stably inserted into the host strain genome as evidenced by multi-generational studies showing consistent levels of b-lactoglobulin." Please provide a brief summary of the multi-generational studies. Your summary should include the method and results on which you based your conclusion.

   Response:

   Perfect Day has conducted studies which show that the expression cassette for β-lactoglobulin has been stably inserted into the host genome due to consistent expression of β-lactoglobulin for many generations. Perfect Day has monitored the yield (gram of β-lactoglobulin/gram dextrose) of β-lactoglobulin over the course of approximately 200 hours. The figure below indicates that there is no difference in β-lactoglobulin yield between early (~25) and extended (~70) generation production runs. Runs 19365 (blue) and 19366 (green) are early generation runs and runs 19367 (red) and 19368 (teal) are extended generation runs.



Method of Manufacture and Specifications: In Part 2 of your GRAS notice, you must include a description of the method of manufacture of the notified substance with sufficient detail to evaluate the safety of the notified substance as manufactured and specifications for food-grade material. See 21 CFR § 170.230(b) and (c).

5. Please identify any materials used in the production and formulation of the finished, food-grade b-lactoglobulin that are derived from major allergens (other than milk allergens) and state whether these will be present in the final product. If none, please provide a statement confirming this.

   Response:

   There are no materials used in the production of Perfect Day's β-lactoglobulin that are derived from major allergens, and no major allergen (other than milk allergens) will be present in the final product.
6. Please provide specifications for pH, heavy metals (lead, arsenic, and mercury), and microbial limits.

   Response:

   The below requested specifications will be added to those already present in GRN 863:

Test	Specification	Method

pH	6.0-8.0	AOAC 945.27
Arsenic	<0.01 ppm	AOAC 2011.19 and 993.14
Cadmium	<0.01 ppm	AOAC 2011.19 and 993.14
Lead	<0.01 ppm	AOAC 2011.19 and 993.14
Mercury	<0.01 ppm	AOAC 2011.19 and 993.14
Total Plate Count	<10,000 CFU/g	FDA BAM Chapter 3/AOAC 966.23
Yeast & Mold	<50 CFU/g	CMMEF Chapter 20
Coliforms	<10 CFU/g	CMMEF Chapter 8

7. On page 9, the notice contains select data from the analysis of three non-consecutive lots of b-lactoglobulin produced by fermentation. Please provide certificates of analysis (COAs) for the three lots demonstrating the consistency of the manufacturing process and compliance with physical and chemical specifications, as well as specifications for limits on microorganisms.

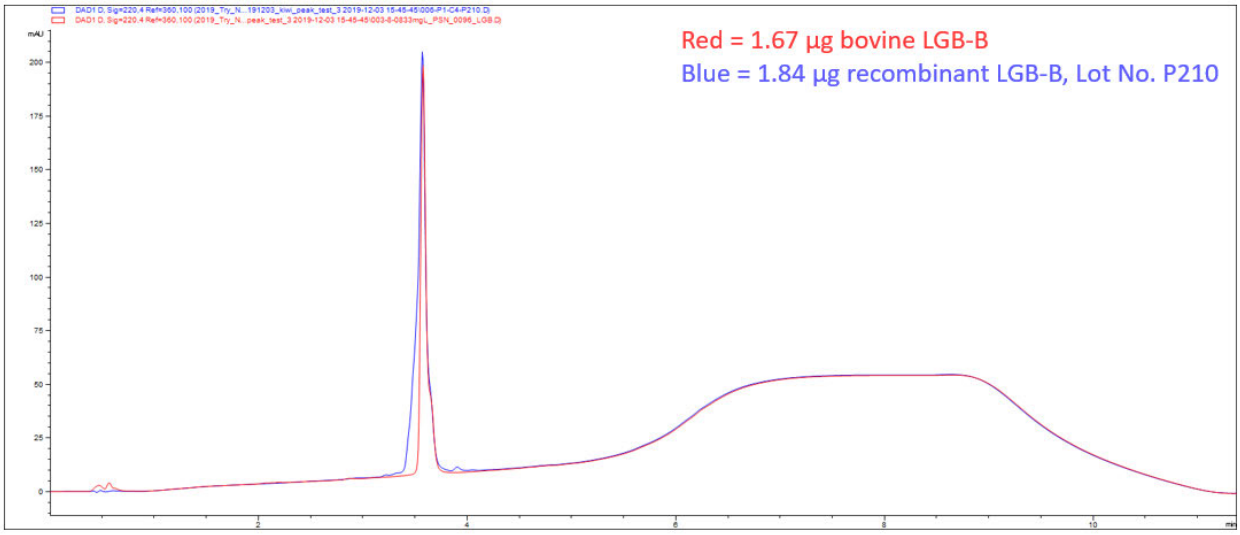
   Response:

   Please find attached CoAs (Attachments 1-3) for non-consecutive lot numbers 163, 193, and 210 which reflect analyses for all previous specifications as outlined in GRN 863, as well as the new specifications as requested by FDA in Question 6, with the exception of pH. pH has not been routinely determined as PD did not believe it to be a major determinant of safety for this product, however we will add this test to the specifications (see #6 above) moving forward.

8. Table 1 specifies the purity (% protein) of b-lactoglobulin as determined using high-performance liquid chromatography (HPLC) and densitometry. Please provide a representative HPLC chromatogram and densitometry trace to support the stated purity of β-lactoglobulin reported in Table 2.

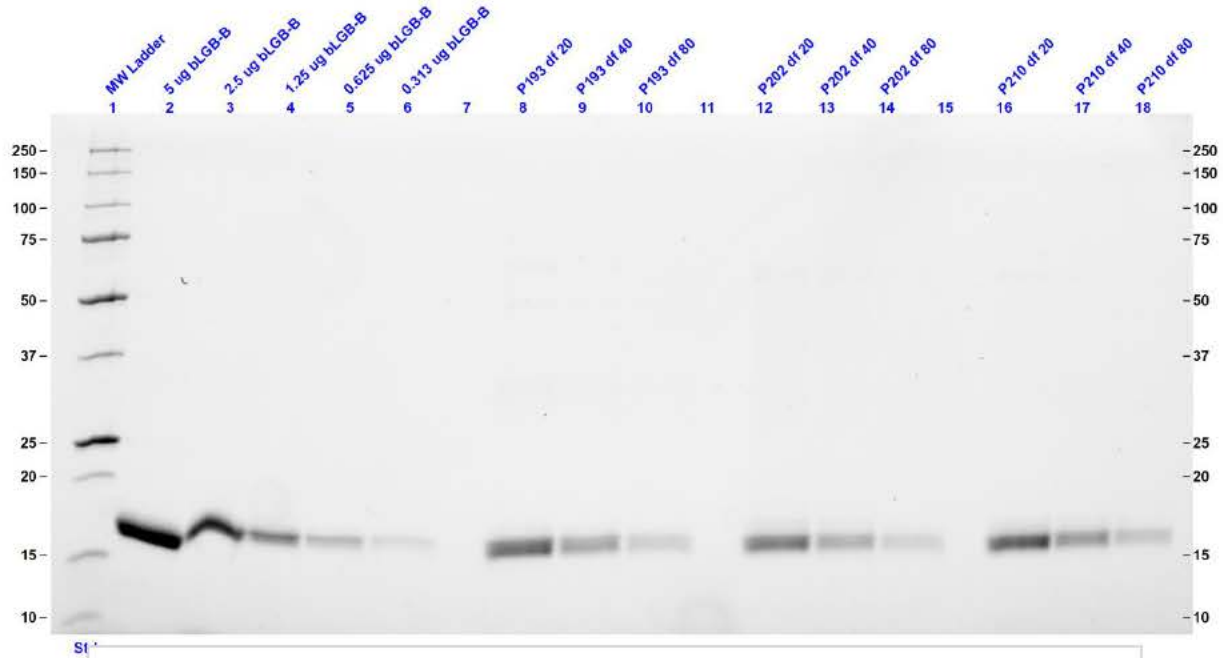
   Response:

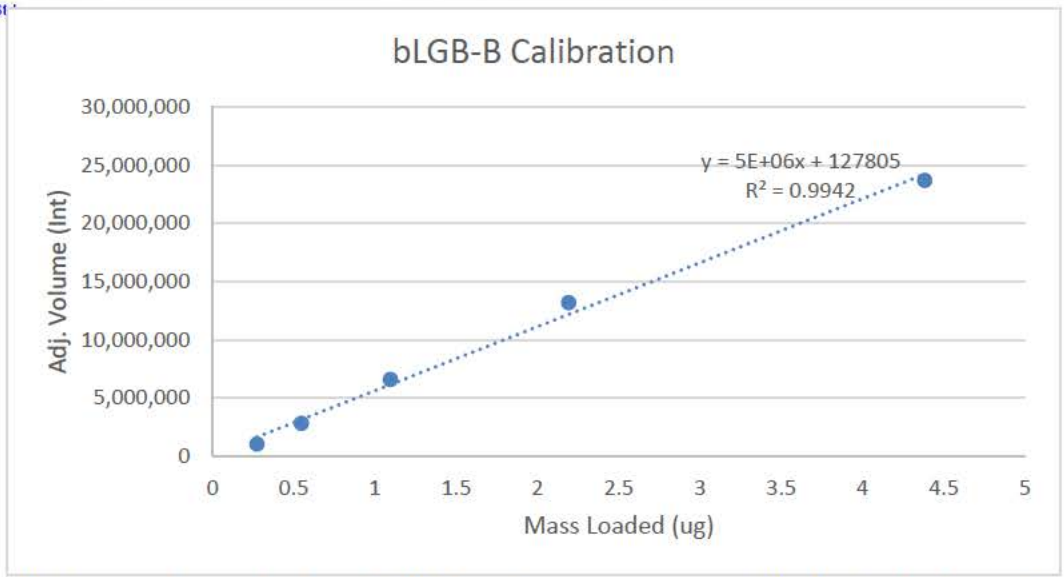
   As HPLC is a more accurate and less time-consuming method, Perfect Day will only utilize HPLC to determine the purity (% protein) of β-lactoglobulin in the future. However, as requested we have provided representative HPLC chromatogram and densitometry trace for Perfect Day production Lot 210 showing comparative purity of a commercially available bovine β-lactoglobulin (Sigma-Aldrich) and Perfect Day's β-lactoglobulin produced via fermentation. This lot is also included in the SDS-PAGE image which is included in our response to question #10b below. β-lactoglobulin purity for lot P210 was 98.2% of total protein and was processed using the intermediate polish step as described in GRN 863 and question 10a below.



*Approximate masses based on corrected Direct A280 Nanodrop measurement

For the densitometry trace, β-lactoglobulin powders were dissolved to 10 g/L in MilliQ water and hydrated overnight at 4˚C. The total protein concentration was determined by measuring the absorbance at 280 nm (A280) and calculating protein concentration using the extinction coefficient of β-lactoglobulin (E280nm = 9.63 cm-1·M-1). The powder solutions were diluted serially to a dilution factor of 20, 40, and 80. The dilutions were prepared for PAGE by diluting in loading dye (Bio-Rad, Part No. 1610747) with TCEP reducing agent (Thermo Fisher Scientific, Part No. 77720), and by heating the samples at 95˚C for 5 minutes. These samples were loaded in equivolume amounts into an 8-16% polyacrylamide gel (Bio-Rad, Part No. 5678104). The PAGE was run at constant 200 volts for 30 minutes. Bands were visualized using the gel's stain free capabilities and BioRad's Chemidoc imaging system on the "Stain Free Gel 590/110, UV Trans" setting with 45 sec activation and 2 sec exposure. Densitometry was determined using BioRad's ImageLab software. The standard curve was determined using commercially available bovine β-lactoglobulin (Lanes 2-6) was then used to calculate the mass loaded into the measurement lanes of the powder solution samples, and we convert the calculated mass to concentration based on the volume loaded per lane, and the standard curve is shown below the gel image.





Dietary Exposure: In part 3 of your GRAS notice, you must provide data and information about dietary exposure (i.e., the amount of relevant substances that consumers are likely to eat or drink as part of a total diet). You must provide an estimate of dietary exposure to the notified substance that includes exposure from its intended use. See 21 CFR § 170.235(a).

9. [We provide the following comment for your information; no response is expected:] In the notice, you state that you believe a reasonable worst-case estimate for the intake of b-lactoglobulin is up to 20% in sports beverages. You then estimated exposure to b-lactoglobulin from this use and stated that it was below the Institute of Medicine's Recommended Daily Allowance (RDA) for protein for adult males and females. We note

that the intended uses for b-lactoglobulin are in multiple food categories. Therefore, it is not appropriate to only estimate exposure based on a single food category. However, you also stated that there are competitive products on the market for the processed foods to which b-lactoglobulin will be added. Accordingly, you explain that b-lactoglobulin will serve as a replacement for the protein sources in those products and consumer exposure to protein is not expected to increase. This latter rationale is an acceptable argument to estimate exposure to b-lactoglobulin from the proposed uses. Alternatively, you could have provided an estimate the dietary exposure for all the food categories in which b-lactoglobulin is intended to be used.

Response:

Thank you for the comments.

Narrative: In Part 6 of your GRAS notice, you must include a narrative that provides the basis for your conclusion of GRAS status, in which you must explain why the data and information in your notice provide a basis for your view that the notified substance is safe under the conditions of its intended use. See 21 CFR § 170.250(a)(1).

10. On August 14 and September 10, 2019, you provided data supporting your statements about the identity of the *T. reesei*-produced β-lactoglobulin, including its amino acid sequence by mass spectrometry and Molecular Weight by SDS-PAGE. The August 14 email from Perfect Day's representative states that the FARRP Analysis compared β-lactoglobulin produced via two different 'production streams.' We have the following questions:

    a. Regarding the mass spectrometry analysis: please state the source of the protein samples and provide a general description of the sample preparation methods. Please include in your response a description of the two different production streams. How do these production streams compare to the manufacturing process described on page 7-8 of Perfect Day's GRAS notice?

    Response:

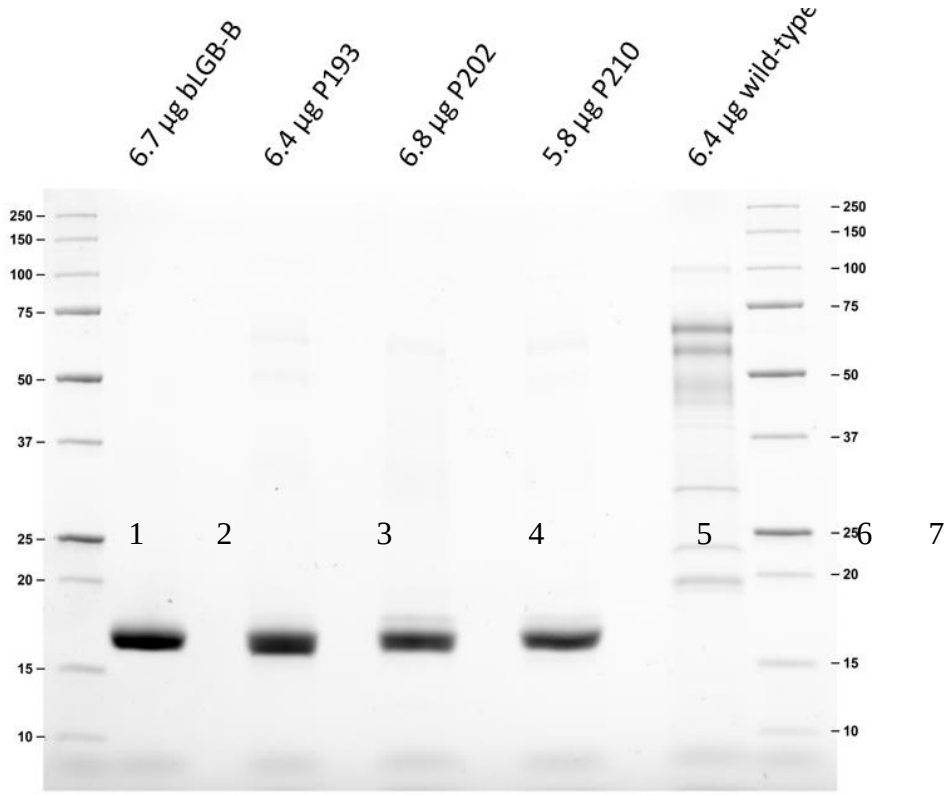
    The difference between the products in the FARRP report (Nike-~93% BLG and Venus-~98% BLG) is the presence (Venus) or lack of (Nike) the "optional intermediate filtration/polish" step.

    b. Regarding the SDS- PAGE analysis: please state the source of the protein samples and provide a general description of the sample preparation and analysis. Please provide an SDS-PAGE analysis that includes a negative control (i.e., the unmodified host strain) and indicate the amount of protein loaded to the gel for each of the

samples. Which of the two production streams was used to generate the *T. reesei*-produced sample used in the SDS-PAGE?

Response:

Please refer to the SDS-PAGE image below which includes molecular weight markers (lanes 1 and 7), commercially available (Sigma-Aldrich) bovine β-lactoglobulin (2) and 3 production lots (3-5) of Perfect Day's b-lactoglobulin produced using the "Venus" (i.e. with the intermediate polish step) process. Lane 6 contains protein from the wild type *T. reesei*. The approximate total protein mass is given above the respective lanes. β-lactoglobulin powders were dissolved to 10 g/L in MilliQ water and hydrated overnight at 4˚C. The total protein concentration was determined by measuring the absorbance at 280 nm (A280) and calculating protein concentration using the extinction coefficient of β-lactoglobulin (E_{280nm} = 9.63 $cm^{-1} \cdot M^{-1}$). The samples were prepared for PAGE by diluting in loading dye (Bio-Rad, Part No. 1610747) with TCEP reducing agent (Thermo Fisher Scientific, Part No. 77720), and by heating the samples at 95˚C for 5 minutes. These samples were loaded in equivolume amounts into an 8-16% polyacrylamide gel (Bio-Rad, Part No. 5678105. The PAGE was run at constant 200 volts for 30 minutes. It was stained for protein using Coomassie stain (Bio-Rad, Bio-Safe Coomassie Stain, Part No. 1610786) for 1 hour and de-stained overnight.



*Approximate masses based on corrected Direct A280 Nanodrop measurement

c. Please state whether there are differences between post-translational modification (*e.g.*, glycosylation) of the *T. reesei*- and bovine-derived β-lactoglobulin. Please provide either your rationale or additional data to support your conclusion. If there are differences, please discuss these in the context of your GRAS conclusion.

Response:

As can be seen in the SDS-PAGE above, there are no obvious molecular weight differences between a commercially available bovine β-lactoglobulin and Perfect Day's β-lactoglobulin produced via fermentation. While we acknowledge that this analysis would only elucidate post-translational modifications which would result in "significant" molecular weight differences, we also note that the included FARRP analysis relied on comparisons between commercially available β-lactoglobulin and Perfect Day's β-lactoglobulin as analyzed via LC-MS/MS, which is a far more sensitive analytical tool than SDS-PAGE. FARRP concluded that their analysis indicated no significant differences between bovine β-lactoglobulin and Perfect Day's β-lactoglobulin. While they did not comment directly on post-translational modifications, we believe that any such differences would have been readily apparent when analyzed via LC-MS/MS and therefore conclude that there are no significant differences in post-translational modifications between β-lactoglobulin produced in the bovine vs. β-lactoglobulin produced via fermentation with *T. reesei*. Further, a literature search has not produced any published reports which would lead Perfect Day to believe that there would be any expected differences in post-translations modification between native β-lactoglobulin and β-lactoglobulin produced via fermentation of *T. reesei.* Therefore, there is no impact from post-translational modifications on the safety of Perfect Day's product.

11. [We provide the following comment for your information; no response is expected:] On page 18 of your notice, you state that "FARRP determined that neither sample contained sufficient residual fungal proteins to present allergenicity concerns." In Appendix 1, which you provided on September 10, FARRP concludes that while three *T. reesei* proteins showed some similarity (ranging from 35-42%) to known allergens, "those alignments are low in identity match and are not likely to represent a real risk, especially since the proteins represent low concentrations" in the samples analyzed by FAARP. *As the available evidence does not support population-based 'thresholds' for known oral allergens at this time (See https://www.fda.gov/food/food-labeling-nutrition/approaches-establish-thresholds-major-food-allergens-and-gluten-food), we disagree with reliance on protein abundance (unless absent) as a basis for concluding that the fungal proteins do not present allergenicity concerns.* However, on page 15 of your notice, you state that a review of the published literature uncovered no reports implicating *T. Reesei* in allergenicity among healthy adults and you refer to the history of its use by the food industry for production of food ingredients. This is an acceptable rationale to support the

conclusion that *T. reesei* proteins present in the finished product would not present allergenicity concerns.

Alternatively, you could have searched publicly available protein databases such as UniProt using the three aligning sequences to determine whether they are also present in commonly consumed foods that do not have a history of being allergenic. If so, this information would add support to a weight-of-evidence conclusion that proteins from *T. reesei* detected in the fermentation-derived b-lactoglobulin preparation do not present a known oral allergen concern.

Response:

Thank you for the comments.

We trust that this responds to your technical review questions. Please let us know if you have further questions or if there is anything else we can do to help reach a satisfactory and speedy conclusion to your review of GRN 863.

Sincerely,

Melvin S. Drozen

Attachments (3)

- COA_P0164_Covance
- COA_P0193_Covance
- COA_P0210_Covance

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Report Number:	**2580005-0**
Report Date:	**02-Aug-2019**
Report Status:	**Final**

Certificate of Analysis

Perfect Day, Inc.

Sample Name:	**PDF_00748**	**Eurofins Sample:**	**8680867**
Project ID	PERFECT_DA-20190729-0053	**Receipt Date**	30-Jul-2019
PO Number	PA7	**Receipt Condition**	Ambient temperature
Lot Number	P0164	**Login Date**	29-Jul-2019
Sample Serving Size		**Date Started**	30-Jul-2019
		Online Order	11494-1201B86F

Analysis	Result
Fat by Base Hydrolysis	
Fat	0.9 %
Protein (N x 6.38) Dumas Method	
Protein	94.6 %
Ash	
Ash	1.35 %
Moisture by M100_T100	
Moisture	4.12 %
Yeast and Mold Plate Count	
Yeast Plate Count	<10 CFU/g
Mold Plate Count	<10 CFU/g
Aerobic Plate Count	
Standard Plate Count	370 CFU/g
Elements by ICP Mass Spectrometry	
Arsenic	16.8 ppb
Cadmium	<5.00 ppb
Lead	59.3 ppb
Mercury	<5.00 ppb
Coliform Count *	
Coliform Plate Count	<10 CFU/g

Method References	Testing Location
Aerobic Plate Count (APC)	**Food Integ. Innovation-Madison NE**
FDA BAM Ch. 3 AOAC 966.23 CMMEF Ch. 8	
Ash (ASHM_S)	**Food Integrity Innovation-Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	

*** This analysis or component is not ISO accredited.**

Certificate of Analysis

Perfect Day, Inc.

Method References	Testing Location

Coliform Count (COLIPC) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods: Enterobacteriaceae, Coliforms, and Escherichia coli as Quality and Safety Indicators, Chapter 8, 4th Edition, 2001.

Elements by ICP Mass Spectrometry (ICP_MS_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Method 2011.19 and 993.14, AOAC INTERNATIONAL, (Modified).
Pequette, L.H., Szabo, A., Thompson, J.J., "Simultaneous Determination of Chromium, Selenium, and Molybdenum in Nutritional Products by Inductively Coupled Plasma/Mass Spectrometry: Single-Laboratory Validation," Journal of AOAC International, 94(4): 1240 - 1252 (2011).

Fat by Base Hydrolysis (FAT_BH_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Methods 989.05, 932.05, 986.25, 945.48B, AOAC INTERNATIONAL (modified)

Moisture by M100_T100 (M100T100_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).

Protein (N x 6.38) Dumas Method (DGEN_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Yeast and Mold Plate Count (YM_SPRD) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods, Yeasts and Molds, 4th Edition, Chapter 20, 2001, (Modified)

*** This analysis or component is not ISO accredited.**

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Report Number:	**2577407-0**
Report Date:	**31-Jul-2019**
Report Status:	**Final**

Certificate of Analysis

Perfect Day, Inc.

Testing Location(s)	**Released on Behalf of Eurofins by**

Food Integrity Innovation-Madison [redacted] **- Director**

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

ilac-MRA A2LA ACCREDITED
2918.01

Food Integ. Innovation-Madison NE [redacted] **- Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

ilac-MRA A2LA ACCREDITED
2918.05

These results apply to the sample as received and only to the items tested. This certificate of analysis shall not be reproduced, except in its entirety, without the written approval of Eurofins.

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Report Number: 2704783-0
Report Date: 27-Nov-2019
Report Status: Final
Supercedes :50-0 2704738-0

Certificate of Analysis

Perfect Day, Inc.

Sample Name:	**PDF_00889**	**Eurofins Sample:**	**8903231**
Project ID	PERFECT_DA-20191009-0078	**Receipt Date**	09-Oct-2019
PO Number	PA7	**Receipt Condition**	Ambient temperature
Lot Number	P0193	**Login Date**	09-Oct-2019
Sample Serving Size		**Date Started**	09-Oct-2019
		Online Order	11494-125F70A1

Analysis	Result
Fat by Base Hydrolysis	
Fat	0.1 %
Protein (N x 6.38) Dumas Method	
Protein	96.1 %
Ash	
Ash	1.42 %
Moisture by M100_T100	
Moisture	2.78 %
Yeast and Mold Plate Count	
Yeast Plate Count	<10 CFU/g
Mold Plate Count	10 CFU/g
Aerobic Plate Count	
Standard Plate Count	20 CFU/g
Elements by ICP Mass Spectrometry	
Arsenic	<10.0 ppb
Cadmium	9.09 ppb
Lead	<5.00 ppb
Mercury	<5.00 ppb
Coliform Count *	
Coliform Plate Count	<10 CFU/g

Method References	Testing Location
Aerobic Plate Count (APC)	**Food Integ. Innovation-Madison NE**
FDA BAM Ch. 3 AOAC 966.23 CMMEF Ch. 8	
Ash (ASHM_S)	**Food Integrity Innovation-Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	

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Certificate of Analysis

Perfect Day, Inc.

Method References	Testing Location

Coliform Count (COLIPC) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods: Enterobacteriaceae, Coliforms, and Escherichia coli as Quality and Safety Indicators, Chapter 8, 4th Edition, 2001.

Elements by ICP Mass Spectrometry (ICP_MS_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Method 2011.19 and 993.14, AOAC INTERNATIONAL, (Modified).
Pequette, L.H., Szabo, A., Thompson, J.J., "Simultaneous Determination of Chromium, Selenium, and Molybdenum in Nutritional Products by Inductively Coupled Plasma/Mass Spectrometry: Single-Laboratory Validation," Journal of AOAC International, 94(4): 1240 - 1252 (2011).

Fat by Base Hydrolysis (FAT_BH_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Methods 989.05, 932.05, 986.25, 945.48B, AOAC INTERNATIONAL (modified)

Moisture by M100_T100 (M100T100_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).

Protein (N x 6.38) Dumas Method (DGEN_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Yeast and Mold Plate Count (YM_SPRD) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods, Yeasts and Molds, 4th Edition, Chapter 20, 2001, (Modified)

*** This analysis or component is not ISO accredited.**

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Report Number: 2704783-0
Report Date: 27-Nov-2019
Report Status: Final
Supercedes :50-0 2704738-0

Certificate of Analysis

Perfect Day, Inc.

Testing Location(s)	Released on Behalf of Eurofins by

Food Integrity Innovation-Madison — [redacted] - Director

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

ilac-MRA A2LA ACCREDITED
2918.01

Food Integ. Innovation-Madison NE — [redacted] - Business Unit Manager

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

ilac-MRA A2LA ACCREDITED
2918.05

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Report Number: 2704739-0
Report Date: 27-Nov-2019
Report Status: Final
Supercedes : 40-0 2687839-0

Certificate of Analysis

Perfect Day, Inc.

Sample Name:	**PDF_00928**	**Eurofins Sample:**	**8964686**
Project ID	PERFECT_DA-20191029-0086	**Receipt Date**	30-Oct-2019
PO Number	PA7	**Receipt Condition**	Ambient temperature
Lot Number	P0210	**Login Date**	29-Oct-2019
Sample Serving Size		**Date Started**	30-Oct-2019
		Online Order	11494-127B0D10

Analysis	Result
Fat by Base Hydrolysis	
Fat	0.3 %
Protein (N x 6.38) Dumas Method	
Protein	95.7 %
Ash	
Ash	0.800 %
Moisture by M100_T100	
Moisture	3.53 %
Yeast and Mold Plate Count	
Yeast Plate Count	<10 CFU/g
Mold Plate Count	<10 CFU/g
Aerobic Plate Count	
Standard Plate Count	<10 CFU/g
Elements by ICP Mass Spectrometry	
Arsenic	20.4 ppb
Cadmium	<5.00 ppb
Lead	5.48 ppb
Mercury	<5.00 ppb
Coliform Count *	
Coliform Plate Count	<10 CFU/g

Method References	Testing Location
Aerobic Plate Count (APC)	**Food Integ. Innovation-Madison NE**
FDA BAM Ch. 3 AOAC 966.23 CMMEF Ch. 8	
Ash (ASHM_S)	**Food Integrity Innovation-Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	

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Certificate of Analysis

Perfect Day, Inc.

Method References	Testing Location

Coliform Count (COLIPC) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods: Enterobacteriaceae, Coliforms, and Escherichia coli as Quality and Safety Indicators, Chapter 8, 4th Edition, 2001.

Elements by ICP Mass Spectrometry (ICP_MS_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Method 2011.19 and 993.14, AOAC INTERNATIONAL, (Modified).
Pequette, L.H., Szabo, A., Thompson, J.J., "Simultaneous Determination of Chromium, Selenium, and Molybdenum in Nutritional Products by Inductively Coupled Plasma/Mass Spectrometry: Single-Laboratory Validation," Journal of AOAC International, 94(4): 1240 - 1252 (2011).

Fat by Base Hydrolysis (FAT_BH_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis, Methods 989.05, 932.05, 986.25, 945.48B, AOAC INTERNATIONAL (modified)

Moisture by M100_T100 (M100T100_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).

Protein (N x 6.38) Dumas Method (DGEN_S) — **Food Integrity Innovation-Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Yeast and Mold Plate Count (YM_SPRD) — **Food Integ. Innovation-Madison NE**

Compendium of Methods for the Microbiological Examination of Foods, Yeasts and Molds, 4th Edition, Chapter 20, 2001, (Modified)

*** This analysis or component is not ISO accredited.**

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Report Number: 2704739-0
Report Date: 27-Nov-2019
Report Status: Final
Supercedes : 40-0 2687839-0

Certificate of Analysis

Perfect Day, Inc.

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	[redacted] - Director
Eurofins Food Chemistry Testing US, Inc. 3301 Kinsman Blvd Madison WI 53704 800-675-8375	2918.01
Food Integ. Innovation-Madison NE	[redacted] - Business Unit Manager
Eurofins Food Chemistry Testing US, Inc. 2102 Wright Street Madison WI 53704 800-675-8375	2918.05

These results apply to the sample as received and only to the items tested. This certificate of analysis shall not be reproduced, except in its entirety, without the written approval of Eurofins.

*** This analysis or component is not ISO accredited.**

From: Drozen, Melvin S.
To: McMahon, Carrie
Cc: Rainer, Natalie; Fulmer, Preston A.
Subject: RE: GRN 863 - follow up question to Dec 30 amendment
Date: Tuesday, January 28, 2020 4:02:47 PM
Attachments: image001.png
RE GRN 863 (Perfect Day) - technical review team questions.msg

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Dear Dr. McMahon,

Thank you for alerting us to the discrepancy between the heavy metal limits stated in our response to your technical questions and the levels as tested in the CoAs. This issue is due to an inadvertent error; and the heavy metal specifications should have been stated as "< 0.1 ppm," rather than "<0.01 ppm." As requested, we have included a table with all of Perfect Day's specifications for their ß-lactoglobulin produced by fermentation of *T. reesei* below:

Analysis	Specification	Reference Method
Protein Dumas/Kjeldahl	= 85 wt %	AOAC 968.06 or 992.15
ß-Lactoglobulin as % of Protein	= 90 %	HPLC
Moisture	< 6 wt%	AOAC 925.45
Ash	< 3 wt%	AOAC 942.05
Fat	< 1.5 wt%	AOAC 989.05
Total Carbohydrates	< 2 wt%	By difference
pH	6.0-8.0	AOAC 945.27
Arsenic	< 0.1 ppm	AOAC 2011.19 and 993.14
Cadmium	< 0.1 ppm	AOAC 2011.19 and 993.14
Lead	< 0.1 ppm	AOAC 2011.19 and 993.14
Mercury	< 0.1 ppm	AOAC 2011.19 and 993.14
Total Plate Count	< 10,000 CFU/g	FDA BAM Chapter 3/AOAC 966.23
Yeast & Mold	< 50 CFU/g	CMMEF Chapter 20
Coliforms	< 10 CFU/g	CMMEF Chapter 8

The specifications summarized above are the current specifications for the Company's beta-lactoglobulin. For ease of reference we have attached our December 30 email and letter.

We trust that this email responds to your inquiry in full, and please let us know if you have any further questions.

Best regards,

Mel Drozen.