From: Kevin Gillies
To: Hice, Stephanie
Cc: Kritika Mahadevan
Subject: Re: [EXTERNAL] GRN 000967 FDA questions / Clara Foods response
Date: Sunday, April 4, 2021 1:20:57 PM
Attachments: image001.png
GRN000967_NSEWP_Clara Foods Reveiw Questions Response 04022021.pdf

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Dear Dr. Hice:

I hope this mail finds you well. Please see Clara Foods responses to the GRN 000967 technical review questions sent to us in your mail of February 10 attached to this email. Should the review team have further questions, please don't hesitate to contact me.

Best,
Kevin Gillies
Kevin O. Gillies Consulting Services, LLC
1759 Grape St.
Denver, CO 80220

Tel:+1 816 590 9836

On Wed, Mar 24, 2021 at 9:08 AM Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:

> Dear Mr. Gillies,
>
> Thank you for the update – we sincerely appreciate it.
>
> Sincerely,
>
> Stephanie Hice
>
> **Stephanie Hice, PhD**
>
> *Staff Fellow (Biologist)*
>
> *Division of Food Ingredients*
>
> **Center for Food Safety and Applied Nutrition**

Office of Food Additive Safety

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

From: Kevin Gillies <kevin.o.gillies@gmail.com>
Sent: Wednesday, March 24, 2021 9:21 AM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Cc: Kritika Mahadevan <kritika@clarafoods.com>; Joel Kreps <joel@clarafoods.com>
Subject: Re: [EXTERNAL] GRN 000967 FDA questions / Clara Foods response

Good morning Dr. Hice,

Yes, enjoying late winter snows here in Denver as you may have heard. Thank you for your follow up on our response to the FDA technical questions relating to GRN 000967. To update you, we just received the final intake report from Exponent, Inc. yesterday and are incorporating the assessment into the Clara Foods responses. This will be quite simple as the Exponent intake assessment is in substantial agreement with the intake assessment in the GRAS notice submission. I apologize for being a bit late on our mid-March timeline projection, but it took longer than expected to get the contractual aspects of the intake assessment project finalized. We should be able to send the response letter early next week. If there are any further delays, I will alert you.

Best,

Kevin

Kevin O. Gillies Consulting Services, LLC

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On Wed, Mar 24, 2021 at 6:30 AM Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:

> Dear Mr. Gillies,
>
> Good morning, and I hope this email finds you well.
>
> As we are nearing the end of March, would you be able to provide us with an update regarding the status of the amendment to GRN 000967?
>
> Thank you for your consideration; please let me know if you have any questions.
>
> Sincerely,
>
> Stephanie Hice
>
> **Stephanie Hice, PhD**
>
> *Staff Fellow (Biologist)*
>
> *Division of Food Ingredients*
>
> **Center for Food Safety and Applied Nutrition**
>
> **Office of Food Additive Safety**
>
> **U.S. Food and Drug Administration**

stephanie.hice@fda.hhs.gov

U.S. FOOD & DRUG ADMINISTRATION

From: Hice, Stephanie
Sent: Tuesday, February 23, 2021 3:55 PM
To: 'Kevin Gillies' <kevin.o.gillies@gmail.com>
Cc: Kritika Mahadevan <kritika@clarafoods.com>; Joel Kreps <joel@clarafoods.com>
Subject: RE: [EXTERNAL] GRN 000967 FDA questions / Clara Foods response

Dear Mr. Gillies,

Good afternoon, and thank you for your email.

Thank you for providing us with an update – we sincerely appreciate it.

After discussing with my managers, aiming for mid-March for receipt of the amendment is acceptable. As mentioned during our teleconference, I will relay this information to the other members of the review team.

Thank you again; please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD

Staff Fellow (Biologist)

Division of Food Ingredients

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

From: Kevin Gillies <kevin.o.gillies@gmail.com>
Sent: Tuesday, February 23, 2021 9:24 AM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Cc: Kritika Mahadevan <kritika@clarafoods.com>; Joel Kreps <joel@clarafoods.com>
Subject: [EXTERNAL] GRN 000967 FDA questions / Clara Foods response

Dear Dr. Hice,

I hope this note finds you well. Thanks again for the productive discussions last week. Following up on our discussion of the timing of Clara Foods responses, I wanted to inform you that we are in discussion with a third party that will assist Clara in making the recommended intake assessment changes. As we discussed, we will not be able to respond in the 10 working day window that FDA originally requested in your letter of February 10, 2021 because of the additional data collection timeline, but we will work expeditiously to obtain the needed intake assessment data and come back with our responses. Preliminary discussions indicate that we will have the updated data by mid-March. We will then incorporate the additional assessment with the accompanying narrative into our responses. Your team indicated during the call a preference to receive

all the responses in one document and that is our intention.

Should there be a change in your team's preference, please don't hesitate to contact me.

Best,

Kevin Gillies

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April 2, 2021

Dr. Stephanie Hice
Staff Fellow
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Re: GRN 000967: Technical Review Questions

Dear Dr. Hice:

We are writing to respond to the technical review questions attached to your email on February 10, 2021. Please find below Clara Foods, Inc.'s responses to the review team's questions. We first set forth the FDA question, followed by our response.

1. On pages 2-4, 6, 27, 30-32 and 40, the notifier lists the citation for the seven parts of a GRAS notice as 21 CFR 570.225, 21 CFR 570.230, 21 CFR 570.235, 21 CFR 570.240, 21 CFR 570.245, 21 CFR 570.250 and 21 CFR 570.255, respectively. We note that 21 CFR 570 corresponds to food additives for use in animal drugs, feeds, and related products. As such, the appropriate CFR citations for the seven parts of a GRAS notice are 21 CFR 170.225, 21 CFR 170.230, 21 CFR 170.235, 21 CFR 170.240, 21 CFR 170.245, 21 CFR 170.250 and 21 CFR 170.255, respectively. 21 CFR 170 corresponds to food additives for use in human conventional foods. For the administrative record, please make a statement that corrects this reference.

   The correct references on pages 2-4, 6, 30-32 and 40 for the seven parts of a GRAS notice are 21 CFR 170.225, 21 CFR 170.230, 21 CFR 170.235, 21 CFR 170.240, 21 CFR 170.245, 21 CFR 170.250 and 21 CFR 170.255, respectively.

2. On page 5, the notifier lists the citation for the certification statement (Section 1.7) as 21 CFR 570.225(c)(9). The appropriate citation is 21 CFR 170.225(c)(9). The notifier should provide a corrected certification statement with the appropriate citation in 21 CFR.

   The appropriate citation for Section 1.7 is 21 CFR 170.225(c)(9).

3. Large sections of the description of the production microorganism in Section 2.2.1 (page 13) appear to be copied from the website pichiagenome.org from the section on the website titled "Taxonomy and Natural Isolates of *Pichia pastoris*" (http://pichiagenome-ext.boku.ac.at:8080/apex/f?p=100:1:12386721196681::NO::::YES; last accessed February 10, 2021). These sections should be re-written and appropriately cited.

> The type strain for *Pichia pastoris*, now part of the genus *Komagataella* (Yamada 1995), was isolated in 1922 from a chestnut tree in France and described by A. Guillermond[1]. The type strain was given the accession number NRRL Y-1603 for the US-based stock center and CBS704 for a European stock center. Later versions of *Pichia pastoris* were isolated by H. Phaff from trees in California (Phaff et al., 1956). NRRL Y-1603 was used, along with other strains, by Phillips Petroleum to develop improved versions that were deposited back into the US stock center. One of these new strains, NRRLY-11430 (CBS7435), was the base strain for the development of *Komagataella phaffii* into a protein production platform (Cregg et al., 1985).
>
> Recent phylogenetic work, using molecular information such as 26S RNA sequence information (C. Kurtzman, 2005), established new species designations within the genus *Komagataella*. Additional analyses of the original type strain and the main strains being used for protein production determined that the modern strains actually represent two different species *K. pastoris* and *K. phaffii* (C. Kurtzman, 2009). *K. phaffii* was shown to be descended from the strain isolated by Phaff in the US (C. Kurtzman, 2009). The NRRL Y-11430 strain was used by the company BioGrammatics (Carlsbad, CA, USA) to develop strain BG08 that was further modified to create BG10 through the loss of endogenous plasmids. This work by BioGrammatics is described, along with the genome sequence for BG10, in a recent publication (Sturmberger, et al. 2016). Clara Foods further modified BG10 to develop a methanol-utilization slow (mutS) phenotype that reduces the strain's ability to consume methanol. This base strain is called DFB-001.

[1] http://gcm.wfcc.info/Strain_numberToInfoServlet?strain_number=CBS%20704

Yamada, Y., Matsuda, M., Maeda, K., Mikata, K. 1995. "The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of Komagataella gen. nov. (Saccharomycetaceae)." *Biosci Biotechnol Biochem 59* 439-44.

Phaff, H., M Miller , and M Shifrine. 1956. "The taxonomy of yeasts isolated from Drosophila in the Yosemite region of California." *Antonie Van Leeuwenhoek 22* 145-61.

Cregg, J. M., K. J. Barringer, A. Y. Hessler, and K. R. Madden. 1985. "Pichia pastoris as a host system for transformations." *Mol Cell Biol 5* 3376-85.

Kurtzman, C. 2005. "Description of Komagataella phaffii sp. nov. and the transfer of Pichia pseudopastoris to the methylotrophic yeast genus Komagataella." *Int J Syst Evol Microbiol 55* 973-6.

Kurtzman, C. P. 2009. "Biotechnological strains of Komagataella (Pichia) pastoris are Komagataella phaffii as determined from multigene sequence analysis." *J Ind Microbiol Biotechnol. 36* 1435-8.

Sturmberger, L., T. Chappell, M. Geier, F. Krainer, K. J. Day, U. Vide, S. Trstenjak, et al. 2016. "Refined Pichia pastoris reference genome sequence." *J Biotechnol. 235* 121–131.

4. Please state whether *Komagataella phaffii* (previously classified as *Pichia pastoris*) strain "DFB-003" has been deposited in a recognized culture collection and provide the non-trade name designation.

   Strain *Pichia pastoris* (*Komagataella phaffii*) DFB-003 has been deposited in American Type Culture Collection (ATCC)[2] under the non-trade name *Komagataella phaffii* accession number GSD-1209.

5. For the administrative record, please provide a brief description of the production strain including phenotypic characteristics (e.g., production of antibiotics, production of secondary metabolites), and whether this poses a safety concern.

   Section 2.2.1 discusses the safety of the production organism DFB-003 in detail. *P. pastoris* (now *Komagataella phaffii)* is a yeast that is not known for making antibiotics, or toxic secondary metabolites. Yeasts, in general, are not known to make antibiotics (I. C. MacWilliams, 1959[3]) and the Phaff Yeast Culture Collection (UC Davis) holds over 7500 strains of yeast, none of which are known to produce antibiotics[4].

   In particular, *P. pastoris* SMD1168, derived from the same ancestor as DFB-003, has been tested and shown to not make any toxic metabolites in preparation for a 90-day toxicology study with rats (Ciofolo et al., 2006[5]). As the authors noted: "Mycotoxin activity was analyzed using HPLC (high performance liquid chromatography) for aflatoxins and ochratoxin A and TLC (thin layer chromatography) for T-2 toxin and sterigmatocystin. The limits of detection (LOD) for the mycotoxins tested were as follows: aflatoxin B1 (1.0 ppb), aflatoxin B2 (1.0 ppb), aflatoxin G1 (1.0 ppb), aflatoxin G2 (1.0 ppb), ochratoxin A (2 ppb), T-2 toxin (0.1 ppm), and sterigmatocystin (200 ppb).

   As noted in GRN 000204, "In addition, *P. pastoris* itself has been approved by FDA as a source of animal feed protein for use in broiler feed up to 10% of the total feed (FDA, 1993). Toxicity studies done in support of the above-referenced *P. pastoris*-approved animal feed (including a pathogenicity study in mice, an acute oral toxicity study in rats, a subacute oral toxicity study in rats, and a two-generation teratology study in rats) also demonstrated-per **FDA's** review in 1993-that *P. pastoris* is neither pathogenic nor toxigenic (FDA, 1993)"[6].

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[2] https://www.atcc.org/

[3] MacWilliams, I. C. 1959. A survey of A survey of the antibiotic powers of yeasts. *J. Gen. Microbiol.* 21: 410-414.

[4] https://phaffcollection.ucdavis.edu/searchable-fields-strain-database#8

[5] Ciofalo, V., Barton, N., Kreps, J., Coats, I., and Shanahan, D. 2006. "Safety evaluation of a lipase enzyme preparation, expressed in Pichia pastoris, intended for use in the degumming of edible vegetable oil." *Regulatory Toxicology and Pharmacology* 45, 1-8

[6] FDA 1993. *21 CFR Part 573. [Docket No. 87F-02211* Food additives permitted in feed and drinking water of animals: Pichia Pastoris dried yeast. Federal Register 58, 59169-59170.

In addition, a PubMed search (March 12, 2021) using the key word terms "*Pichia pastoris* AND antibiotic" yielded 176 references. The returned references were papers that described the heterologous expression of antimicrobials in a *P. pastoris* expression hosts or the use of antibiotic resistance markers introduced in *P. pastoris* for selection of desired recombinant *P. pastoris*. None of the references describe antibiotic production by native *Pichia pastoris* strains.

6. On page 14, the notifier states, "The genome of DFB-003 is fully sequenced and well-characterized". Please discuss whether the full genomic sequences are publicly available and provide the corresponding NCBI accession number.

   We have not published the genome of our strain and consider it confidential business information. Clara Foods has characterized the genome of the production strain to ensure the rOVD production genes are inserted as intended.

   For reference the full genome sequences of the 4 chromosomes of the base strain BG10 (*Komagataella phaffii* CBS 7435) are publicly available as GenBank accession numbers LT962479, LT962478, LT962477, LT962476.

7. Please describe the origin and source of the donor genes (e.g., are they *de novo* synthesized or of bacterial origin).

   Original genetic material used to transform BG10 was synthesized *in vitro* by two DNA supply companies IDT[7] and Atum[8], inserted into suitable transformation cassettes and then propagated in *E. coli* K12[9] to amplify the material[10]. The *E. coli*-amplified DNA was used to transform the production base strain BG10 to produce strain DBF-003. Genome sequencing of DFB-003 showed that no bacterial genes, such as antibiotic resistance genes or transformation plasmid codon components such as origins of replication are present in the genome of strain DBF-003.

8. Please provide the accession number (NCBI or UniProt) of the non-animal soluble egg white protein (NSEWP) sequence that has been expressed in *K. phaffii* (previously classified as *P. pastoris*) strain "DFB-003".

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[7] https://www.idtdna.com/pages/
[8] https://www.atum.bio/
[9] Stellar Cells from Takeda https://www.takarabio.com/documents/User%20Manual/PT5055/PT5055-2.pdf, 10betas and STbL from New England Biolabs, https://www.neb.com/products/c3040-neb-stable-competent-e-coli-high-efficiency#Quality,%20Safety%20&%20Legal_Specifications
[10] https://blog.addgene.org/plasmids-101-common-lab-e-coli-strains)

NSEWP amino acid sequence has not be submitted to NCBI or UniProt databases but is fully described in Section 2.1.3 and in response to Question 10 below (Table 3).

UniProt number for native hen egg ovomucoid (nOVD) is P01005.1[11] . We note that this UniProt entry is the pro+mature sequence of the protein that has a molecular mass of 28 kDa.

The molecular mass of the mature nOVD protein, found in hen eggs, is ~ 20 kDa. Table 3 (Question 10 below) shows the mature sequence for the nOVD protein along with Molecular Weight for the mature form of the proteins (native ovomucoid as well as NSEWP).

9. Please provide a discussion of the extent of *K. phaffii* (previously classified as *P. pastoris*) proteins in the final preparation. We understand that the secretory signal sequences aid in the secretion of these proteins in the environment, but usually there are residual proteins from the host in the medium. Please provide a description why the residual *K. phaffii* (previously classified as *P. pastoris*) proteins are not a safety concern.

   Residual *K. phaffii* proteins are not a safety concern for several reasons as described in Section 6.4.2 in the notice 000967.

   Previous GRAS notice submissions (GRN 000204 and GRN 000737) have tested protein preparations from *K. phaffii* in well-controlled animal toxicology studies and demonstrated the safety of those preparations. GRN 000204 test article material was produced in a process similar to the process for NSEWP fermentation and recovery of a secreted protein after a methanol-based fermentation.

   In GRN 000737 the test article materials contained 14 to 29% w/w host protein. They identified the 17 most abundant host proteins in their test article material and characterized them by comparison to databases of known allergens[12], and genomes of commonly consumed microbes from the genus *Saccharomyces*. They concluded that "The long history of consumption of these close homologs of all 17 *Pichia pastoris* proteins with no reports of allergenicity or toxicity offers strong general evidence for their safety in food (Annex 9)". The material from GRN000737 is now being consumed around the USA as part of the Impossible Burger. And, as noted in GRN000204: "In addition, *P.* pastoris itself has been approved by FDA as a source of animal feed protein for use in broiler feed up to 10% of the total feed (FDA, 1993). Toxicity studies done in support of the above-referenced *P.* pastoris-approved animal feed (including a pathogenicity study in mice, an acute oral toxicity study in rats, a subacute oral toxicity study in rats,

[11] https://www.ncbi.nlm.nih.gov/protein/P01005.1
[12] (https://farrp.unl.edu/resources/allergenonline

and a two generation teratology study in rats) also demonstrated-per **FDA's** review in 1993-that *P.* pastoris is neither pathogenic nor toxigenic (FDA, 1993)".

Analysis of NSEWP lots showed that they contained ~7 to 12% host protein, w/w (Table 1, below). The identities of the host proteins were determined using LC MS/MS analysis (Colgrave et al., 2014[13]). The identities of the proteins found are listed in Table 2 (below). Protein sequences were analyzed by BlastP for similarity to known allergens at the FARRP Allergens Online[14], to known virulence factors[15], and for similarity to other proteins in the NCBI database using BlastP[16]. All of the DFB-003 host proteins detected in the NWSEP fell into safe categories, strongly matching *Saccharomyces* proteins, or failing to match any known allergen or known toxin sequences.

As noted in GRN000737, there is a strong history of safe consumption of *Saccharomyces* proteins, and the NSEWP proteins that are also found in *Saccharomyces* are expected to be safe. We note that a typical nutritional Saccharomyces yeast product recommends a 15 g/day dose, and the nutritional label estimates it to have 8 g protein (manufacturer's site[17]), Further, people who maintain a vegan diet consume nutritional yeast as a source of certain B vitamins that they would normally get from consuming meat. Several websites suggest keeping daily intake of nutritional yeast below 32 g/day to avoid over-dosing on Niacin (D. Cudmore, 2020[18]). The intake of the proteins from the consumption of nutritional yeasts far exceeds the consumption of such proteins from ingestion of the NWESP preparation.

Two protein sequences did not match protein sequences from *Saccharomyces cerevisiae*, one (F2QYL8) did have a strong match to an endoglucanase from *Rhizopus oryzae*, a well-characterized microbe found in several foods (Cantabrana et al, 2015[19]). The F2QYL8 endonuclease was not homologous to proteins in the FARRP database. The putative protein, C4R3C4, was not homologous to proteins in the UniProt database but the C4R3C4 amino acid sequence was not homologous to proteins in the FARRP database or homologous to known protein toxins. The putative protein, however, has a well-known subtilisin protease domain. Subtilisins are not known allergens or toxins and *Bacillus subtilis* subtilisin is GRAS for use as a food processing aid and was tested in a 90-day feeding study (GRN 000714). Based upon this analysis, the two putative proteins are not likely to present risks to consumers.

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[13] Colgrave, M. L., et al. 2014. "Using mass spectrometry to detect hydrolysed gluten in beer that is responsible for false negatives by ELISA." *J. Chromatogr.* 105–114

[14] https://farrp.unl.edu/resources/allergenonline

[15] at http://www.mgc.ac.cn/VFs/search_VFs.htm

[16] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

[17] https://www.bobsredmill.com/nutritional-yeast.html

[18] Cudmore, D. 2020. "How much nutritional yeast a day? Can you eat too much?" https://vegfaqs.com/how-much-nutritional-yeast-per-day-is-too-much/

[19] Cantabrana, I., et al. 2015. "Uses of *Rhizopus oryzae* in the kitchen". *Int. J. of Gastronomy and Food Science* 2, 103-111

Table 1: % ovomucoid present in total protein in NSEWP composition

Analysis Parameter	**SOL19303**	**SOL19317**	**SOL19351**
% Protein by combustion	75.31	75.06	79.94
% OVD in powder determined by HPLC	66	68	74
% OVD as % of protein (calculated)	87.6	90.6	92.6

Table 2: Proteins detected in NSEWP composition by LCMS/MS (Colgrave et al., 2014[13])

	Uniprot Protein ID	**Protein Description**	**Match to Either Allergen or VFDB Database?**	**Closest Protein *S. cerevisiae* (s288c)**
	P01005[1]	Ovomucoid	Yes - Allergen DB Match	N/A
1	F2QY66	Superoxide dismutase [Cu-Zn]	Yes - Allergen DB Match	P00445 - Sod1
2	F2R0E1	Cell wall biogenesis involved protein	No	Nca3
3	F2QUR1	Endochitinase	No	Cts1
4	F2QXH5	Extracellular protein X1	No	Pry2
5	F2QQT7	Putative glucanase	No	Sun4
6	F2QUG8	Vacuolar aspartyl protease (Proteinase A)	No	Yps3
7	F2QSQ9	Uncharacterized protein	No	gag-pol
8	F2QPF8	Acyl-CoA-binding protein	No	Acb1
9	F2QYV4	Glycosidase	No	Crh1
10	F2QS11	Protein with internal repeats 1, cell wall protein	No	Pir3
11	F2QYW1	ATPase involved in protein folding	Yes - Allergen DB Match	Ssa3
12	F2QUE4	GDP-bound Gsp1p interacting protein	Yes - Allergen DB Match	P33331 - Ntf2
13	F2QX14	ATPase involved in protein folding	Yes - Allergen DB Match	Ssa3
14	F2QNG1	Putative glucanase	No	Scw4
15	F2QPL8	Endo-beta-1,3-glucanase	No	SCW4
16	F2QVU1	Carboxypeptidase Y inhibitor	No	Tfs1
17	F2QY94	Polyubiquitin [Cleaved into: Ubiquitin]	No	Uba1
18	F2QYL8	Endo-glucanase	No	No Hits
19	F2QZM1	Protein with internal repeats 2, cell wall protein	No	Pir1

	Uniprot Protein ID	Protein Description	Match to Either Allergen or VFDB Database?	Closest Protein *S. cerevisiae* (s288c)
20	F2QQH9	Protein with similarity to the human NPC2/He1	No	Npc2
21	F2QU52	Putative glucanase	No	Scw11
22	F2QUV5	Cell wall beta-glucan assembly glycoprotein	No	Kre9
23	C4R3C4	Uncharacterized protein	No	No Hits
24	Q56D08	Kar2p, protein chaperone	Yes - Allergen DB Match	Kar2
25	Q9C1Z8	Protein disulfide-isomerase	No	Pdi1
26	Q0QCW1	1,3-beta-glucanosyltransferase	No	Gas1

[1]P01005(OVD) is an internal control and demonstrates detection of NSEWP as homologous to the hen egg ovomucoid.

The *P. pastoris* proteins found in NSWEP preparations are not described in GRN 000737. There are two points to consider when comparing these current results with those in GRN 000737: (1) GRN 00737 describes host protein identities that are based on analysis of a cell-lysate, as their product is produced intracellularly, while Clara Foods's NSEWP preparation is secreted outside the cell and the carryover production host proteins (also secreted proteins) are a minor subset of the total protein component of the yeast. We note that these extracellular proteins (in the NSEWP preparation) would not be likely be detected in a harvested whole cell lysate preparation especially by SDS-PAGE, which is a relatively insensitive measure of total protein composition. In addition, the GRN 000737 results were based on LC MS/MS analysis of the proteins detectable by PAGE, meaning only the most abundant proteins that are visible in the Coomassie Blue stained PAGE gel were further analyzed[20]. The list of proteins from Clara Foods NSEWP preparation, Table 2, come from LC MS/MS analysis of all detectable NSEWP preparation proteins, not just the most abundant.

A more relevant comparison of the protein composition would be to the phospholipase C preparation that is the subject of GRN 000204. While total protein composition was not analyzed in GRN 000204, it is likely that the secreted phospholipase C preparation described therein contained host-derived secreted proteins similar to NSEWP preparation as the two preparations share the same production host background, fermentation process and similar purification steps. We note that toxicological analysis of the phospholipase C preparation did not reveal test article-related adverse effects.

Finally, as further evidence that the *P. pastoris*-derived proteins in the NSEWP preparation are unlikely to present safety concerns, a PubMed literature search

[20] Appendix 9 of GRN000737: "The 17 proteins were identified using proteomic analysis from 10 stainable protein bands in a one-dimensional SDS PAGE as the most abundant residual proteins from the yeast in the Soy Leghemoglobin Preparation."

using the keywords "*Pichia pastoris*" and "toxin" did not find any references to toxin production in *P. pastoris* except those references to the expression of heterologous toxins in a *P. pastoris* production host.

10. In Section 2.1, the notifier provides the chemical identity of hen egg ovomucoid (OVD) and describes that NSEWP is substantially equivalent to the native hen egg OVD. However, compared to native OVD, the observed molecular weights in the SDS-PAGE gel, glycosylation, and n-terminal amino acid sequence of NSEWP are different from that of the native OVD. Therefore, please provide information on the structural characteristics of NSEWP produced using the *K. phaffii* (previously classified as *P. pastoris*) strain "DFB-003" expression system. In particular, we suggest that the notifier describe in detail the physical and chemical properties of NSEWP, including the molecular mass, amino acid residues in the polypeptide chain, isoelectric point, carbohydrate chain and identity of the carbohydrates, and any other major proteins in the NSEWP by percentage.

See response to Question 9 above for a detailed description of production host-derived proteins in the NSEWP preparation in addition to rOVD.

The physical characteristics of the mature protein forms of nOVD and rOVD described in Table 3 (below) are substantially equivalent in molecular weight, isoelectric point, glycosylation sites. The two proteins differ in glycoform as described in detail in Section 2.1.3.

Table 3: Physical characteristics of native and recombinant OVD

Protein	**Amino acid sequence of mature protein without glycosylation**	**Molecular Mass**	**Isoelectric point without glycosylation[1]**	**Number of residues glycosylated**	**Carbohydrate identities[2]**
Native OVD (mature form)	AEVDCSRFPNAT DKEGKDVLVCNK DLRPICGTDGVT YTNDCLLCAYSIE FGTNISKEHDGE CKETVPMNCSSY ANTTSEDGKVMV LCNRAFNPVCGT DGVTYDNECLLC AHKVEQGASVD KRHDGGCRKEL AAVSVDCSEYPK	20 kDa	4.47	5[21]	N-acetyl glucosamine, mannose, galactose

[21] Besler, M. and Mine, Y. 1999. "*Mini-Review:* The Major Allergen from Hen's Egg White: Ovomucoid *(Gal d 1)." Internet Symposium on Food Allergens 1(4): 137-46*. http://www.food-allergens.de

	PDCTAEDRPLCG SDNKTYGNKCNF CNAVVESNGTLT LSHFGKC				
Recombinant OVD	**EAEA**AEVDCSRF PNATDKEGKDVL VCNKDLRPICGT DGVTYTNDCLLC AYSIEFGTNISKE HDGECKETVPM NCSSYANTTSED GKVMVLCNRAF NPVCGTDGVTY DNECLLCAHKVE QGASVDKRHDG GCRKELAAVSVD CSEYPKPDCTAE DRPLCGSDNKTY GNKCNFCNAVV ESNGTLTLSHFG KC	20.5 kDa	4.36	4	N-acetyl glucosamine

[1] Calculated isoelectric point

[2] Refer to Figure 1 in the GRN 000967 (page 7) for description of Carbohydrate residues and the relevant glycoforms.

The predominant protein in NSEWP preparation is recombinant ovomucoid (rOVD). Native hen egg ovomucoid as listed in the Uniprot database has a molecular weight of 28 kDa (ovomucoid is listed at 28 kDa due to the presence of a secretion signal that is present in a pro+ mature form but removed during secretion to generate the mature only form with a molecular weight of~ 20 kDa) and an isoelectric point of 4.1, and comprises 11% of egg white proteins (Besler et al., 1999). It is this mature 20 kDa form of nOVD that is equivalent to the rOVD in the NSEWP preparation.

The SDS-PAGE gel (GRN 000967: Figure 2, page 10; reproduced below as Figure 1 in this document) indicates the molecular weight for the fully glycosylated nOVD between 25 kDa and 37 kDa when compared to the molecular weight size standards (Lane 1). Deglycosylation of nOVD (Lanes 2 and 3 prior to deglycosylation and Lanes 4 and 5 after deglycosylation) to the same glycoform as the rOVD (Lanes 6-11), results in nOVD and NSEWP having similar mobility in the SDS-PAGE gel and thus equivalent molecular weights at approximately 20 kDa in agreement with the molecular weights determined by amino acid analysis (a slight difference in molecular weight is accounted for by the addition of 4 amino acids to the N-terminal end of the rOVD sequence as described in Section 2.1.3). The presence of the deglycosylating enzyme PNGaseF is also detected in the deglycosylated nOVD samples at a MW of approximately 28kDa (Lane 4 and 5).

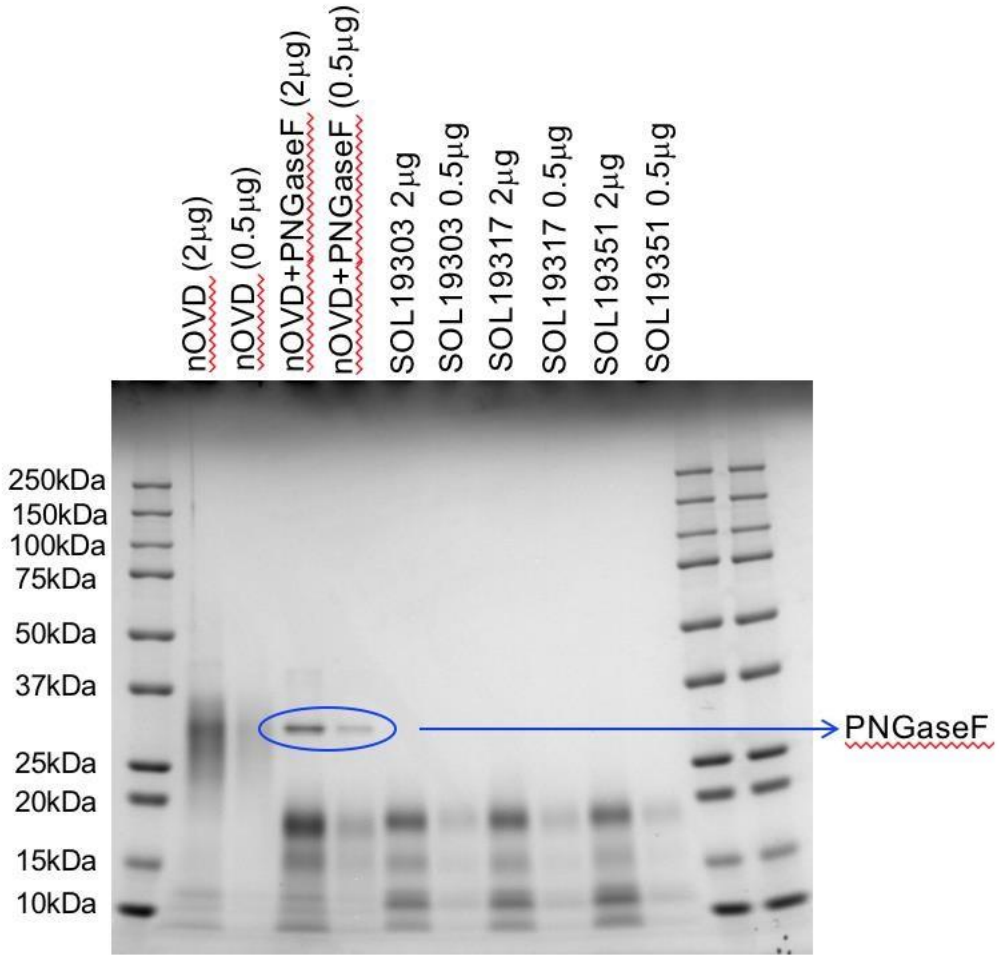

Figure 1. SDS-PAGE analysis of nOVD, nOVD deglycoslysated and NSEWP preparation. Lane 1: MW standards; Lane 2 and 3 nOVD; Lanes 4 and 5: deglyosylated nOVD with PNGaseF; Lanes 6-11: 3 lots of NSEWP preparation.

The data demonstrate that the deglycosylated nOVD and the rOVD in the NSEWP preparation are equivalent by amino acid sequence, molecular weight (demonstrated by SDS-PAGE mobility and amino acid sequence); nOVD and the rOVD in the NSEWP preparation are equivalent by isoelectric point, carbohydrate glycoform core moiety, immunoreactivity, *in vitro* digestibility, and bovine trypsin inhibition.

11. In Section 2.4.3, the notifier states the NSEWP is purified by centrifugation followed by micro-filtration, and then further purified by pH adjustment and ultrafiltration. Please describe how the final NSEWP preparation is standardized to a final concentration of protein (> 75% or > 80% by dry weight powder). In addition, please describe what analytical methods are used to monitor the identity and purity of the in-process and final products.

The fermentation process is carried out for a set number of days to ensure a standardized protein titer. The NSEWP preparation is then centrifuged, washed and filtered. The protein concentration, protein composition and microbial purity are monitored at each unit operation. The purified preparation is then spray dried.

In process samples are taken at the completion of each unity operation in the production process. The in-process samples are analyzed for purity of culture and checked to ensure there is no microbial contamination. Protein identity produced is monitored by SDS PAGE. Identity and purity of the final dried NSEWP preparation product is evaluated by a combination of analyses. The concentration of recombinant ovomucoid in the preparation is quantified using HPLC. Proximate analyses, heavy metal and microbial analyses are carried by an ISO 17025 accredited laboratory to ensure the sample meets the product specifications.

12. In Section 2.1.3, the notifier describes the differences in the glycosylation form between hen egg OVD and NSEWP. Considering that NSEWP has a simplified glycoform compared to native hen egg OVD, please further describe the thermostability of NSEWP by providing and comparing the melting temperature (T_m) with that of hen egg OVD.

Clara Foods has not included analytical data related to thermal stability (melting temperature) of the NSEWP in the notice since this parameter does not reflect stability of the product during production as determined by in-process testing, nor does this parameter impact the functionality or safety of the protein. It is understood that melting temperature may be an important parameter for a protein where the native conformation may be important for the functionality in a therapeutic application but the use of NSEWP preparation as a macronutrient in food presupposes that the protein will be denatured and digested. We believe pepsin digestion percentage is the relevant parameter in this regard. Section 2.1.3, Table 2 of GRN 000967 describes the results of an *in vitro* protein digestibility assay where three (3) lots of NSEWP preparation and native chicken OVD were digested at >90%.

We understand that determining the melting temperature of the native OVD and NSEWP would add another point of comparison between the two proteins, but we believe that the similarity in the two proteins is adequately described by the comparison of amino acid sequence, molecular weight, glycosylation structure, trypsin inhibition characteristic, *in vitro* digestibility and immunoreactivity.

Furthermore, the in-process Quality Control testing of the NSEWP preparation from the fermentation stage until after spray drying as described in our response to Question 11 indicates that the product is sufficiently stable throughout the process.

13. In Section 2.4.4, the notifier states that NSEWP meets the purity specifications set forth in the Food Chemicals Codex, 11th Edition (FCC 11). However, there is no monograph for NSEWP in FCC 11. The notifier should indicate which monograph they intend to reference.

> It is correct that there is no monograph for NSEWP in FCC 11, which is understandable as NSEWP is a newly developed product. We have based the microbial and purity specification on Enzyme Preparations Monograph (Food Chemical Codex, 11th Edition) as a guide to appropriate food grade specifications for proteins.

14. In section 2.4.4, the batch analyses demonstrate levels of arsenic, mercury, and cadmium to be below 0.01 mg/kg, and levels of lead to be below 0.2 mg/kg (Table 4, page 26). However, the specifications for these heavy metals were stated to be < 1 mg/kg. In order to keep exposure to heavy metals as low as possible, please consider lowering the specification limits for heavy metals to be consistent with the results of the batch analyses.

> Clara Foods understands FDA's interest in ensuring that heavy metal concentrations in foods are as low as possible and will consider lowering specification limits as appropriate. We note that there is a limited data set of quality testing results during the product development stage of NSEWP and we believe it is premature to lower the specifications below the currently accepted QC limits for proteins in the industry.

15. In Table 3 (page 24), the notifier lists the specification for coliforms as ≤ 30 CFU/g, however, in Table 4 (page 26), the notifier lists the specification for coliforms as < 10 CFU/g. Please clarify this discrepancy.

> Thank you for pointing out the discrepancy in Table 3 and Table 4 listing of specifications for coliforms. This was an inadvertent error. The specification for coliforms in Section 2.4.4 Table 4 (page 26) should read ≤ 30 CFU/g. The Lot test results are < 10 CFU/g as stated.

16. The notifier states that the method used to detect *Salmonella* serovars is AOAC 2003.09 (page 24), which corresponds to enumeration of *Salmonella* serovars in frankfurters, raw ground beef, raw ground chicken, raw frozen tilapia fish, orange juice, and mozzarella cheese. Please clarify if this method is appropriate and fit for purpose.

> The method used to detect Salmonella (AOAC 2003.09) is designed and validated for use on various protein-containing food matrices and is fit for the purpose of detecting Salmonella serovars in NSEWP preparation.

17. Please provide complete and appropriate citations for the analytical methods used to analyze for the specification parameters and indicate that the methods are validated for their intended purpose. If an internally developed method is used, please indicate that it has been validated for the intended purpose.

The analytical methods used by the ISO 17025 accredited external lab to analyze the sample Lots for the specification parameters are cited below. These methods are validated for their intended purpose.

Protein analysis: AOAC 990.03, AOAC 992.15
AOAC 2006. Protein (crude) in animal feed, combustion method, 990.03. In: *Official methods of analysis of AOAC International*. 18th ed. Gaithersburg: ASA-SSA Inc.

AOAC 2006. Proximate Analysis and Calculations Crude Protein Meat and Meat Products Including Pet Foods - item 80. In: *Official methods of analysis Association of Analytical Communities*, Gaithersburg, MD, 17th edition, Reference data: Method 992.15 (39.1.16); NFNAP; NITR; NT.

Moisture analysis: AOAC 925.09
AOAC 2005. Solids (total) and moisture in flour. In: *Official Methods of Analysis of AOAC International*, Methods 925.09: 18th Ed., AOAC International, Gaithersburg, MD

Ash analysis: AOAC 942.05
AOAC 2005. Official Method 942.05, Ash of Animal Feed. In: *Official Methods of Analysis of AOAC International*, 18th edition, Chapter 4, p. 8, AOAC International, Gaithersburg, MD.

Fat by Acid Hydrolysis: **AOAC 954.02**
AOAC International. 2012. Official Method Fat (crude) or ether extraction in pet food. Gravimetric method, 954.02. *In*: *Official Methods of Analysis of AOAC International*, 19th ed., AOAC International, Gaithersburg, MD, USA.

Heavy metals analysis: ICP-AES
Julshamn, K., Maage, A., Norli, H. S., Grobecker, K. H., Jorhem, L., Fecher, P. 2007. "Determination of Arsenic, Cadmium, Mercury, and Lead by Inductively Coupled Plasma/Mass Spectrometry in Foods after Pressure Digestion: NMKL Interlaboratory Study" *Journal of AOAC International*, Volume 90, Issue 3, 844–856

Standard Plate Count: AOAC 990.12
AOAC International. 2005. Aerobic plate count in foods, dry rehydratable film, method 990.12. AOAC International, 17th ed. Gaithersburg, MD.

Yeast and Mold: AOAC 997.02
AOAC Official Method 997.02. Yeast and Mold Counts in Foods Dry Rehydratable Film Method (Petrifilm™ Method) First Action 1997 Final Action 2000.

Salmonella: AOAC 2003.09
AOAC International. 2005. *Salmonella* in selected foods, BAX automated system, method 2003.09. In: *Official methods of analysis of AOAC International*, 17th ed. AOAC International, Gaithersburg, MD.

E. coli and total coliforms: AOAC 991.14
AOAC International. 2005. *E. coli* count in foods, dry rehydratable film, method 991.14. In: *Official methods of analysis of AOAC International,* 17th ed. AOAC International, Gaithersburg, MD.

18. In Section 3.6, the notifier estimates a maximum total dietary exposure of NSEWP to be 46.5 g/p/d. However, we note that the notifier just combines the recommend amounts of protein (drinks, nutritional protein) and *per capita* consumption of protein (nutritional bars, and fruits snacks) together to get this value. This is not the appropriate method for the estimation of potential dietary exposure. The notifier should provide a dietary exposure estimate for NSEWP at the mean and 90th percentile for the U.S. population aged 2 years and older based on the intended use in foods using available U.S. food-consumption surveys.

    Please see the cumulative 2-day average dietary exposure estimate (provided by Exponent, Inc.) in Addendum A for NSEWP preparation at the mean and 90th percentile for the U.S. population aged 2 years and older based on the intended use in food using available U.S. food consumption surveys, as requested.

    We note that the "user only" EDI for NSEWP based on disappearance data on egg consumption and market usage data for the identified food uses stated in Section 3.6 of 46. 5 g/p/d, where we stated that the "user only" data was likely to represent the 90th percentile of consumers, is supported by the cumulative 2-day average U.S. 2 years and older 90th percentile EDI calculated using publicly available U.S. food-consumption surveys (NHANES 2015-2018 consumption data) of 47.1 g/p/d with a mean value of 21.1 g/p/d (see below). Both estimates are "cumulative" EDIs in that the totals are the sum of the estimated intake from substitutional uses and use for the supplemental intake of protein in the described uses.

    Given the approximate agreement of the EDIs calculated by two independent methods, i.e., the original method described in GRN 000967 and the method described in Addendum A, we are amending the total EDI calculated in Section 3.6 to include the calculation based upon the NHANES 2015-2018 data as supporting documentation as follows:

Intake from use as a substitute for egg protein (nOVD)

Total egg consumption data were used to estimate intake of NSEWP used as a substitute for egg protein (nOVD). This approach provides a conservative estimate of nOVD intake that may be replaced by NSEWP, as the estimate reflects total egg intake and NSEWP will not replace all egg consumption. Based on US Department of Agriculture (USDA) intake estimates using the What We Eat In America (WWEIA) 2013-2016 dietary component of the National Health and Nutrition Examination Survey (NHANES), mean usual egg intake by the US population age 2 years and older is 0.6 egg/day (as egg without the shell)[22]. Estimates of mean NSEWP intake were developed as indicated below. Estimates of the pseudo 90th percentile[23] of NSEWP intake were developed as two times the mean intake.

- $Mean\ NSEWP\ intake = \frac{0.6\ egg}{day}\ x\ \frac{50\ g\ egg}{egg}\ x\ \frac{11\ g\ \ nOVD}{100\ g\ EWP}\ x\ \frac{9.3\ g\ EWP}{100\ g\ egg}\ x\ \frac{100\ g\ NSEWP}{80\ g\ protein}$

  $Mean\ NSEWP\ intake = \ 0.38\ g/day$

- $Psuedo\ 90th\ percentile\ NSEWP\ intake = \ 2 \times Mean\ NSEWP\ intake\ = 0.38\ g/day\ \times 2$

- $Psuedo\ 90th\ percentile\ NSEWP\ intake = \ 0.76\ g/day$

Intake from use as a supplemental source of protein

Intake of NSEWP was developed from reported intakes of foods representative of the intended uses. The full intake assessment report and the list of food codes identified as representative of the intended uses and used in the analysis of intakes is provide in Addendum A.

The per capita cumulative estimates of intake for the U.S. population 2 years and older were developed by adding the estimated intake from substitutional uses (0.38 g/p/d at the mean and 0.76 g/p/day at the 90th percentile of intake) to the estimated intakes from the intended use as a supplemental source of protein (2.9 g/p/d at the mean and 6.2 g/p/d a the 90th percentile) to yield a cumulative per capita estimate of intake mean of 3.3 g/p/day and 7.0 g/p/d at the 90th percentile.

We note specifically that the cumulative EDI presented in GRN 000967 Section 3.6 of 46. 5 g/p/d is a "per user" estimate of the 90th percentile intake based upon disappearance and market data. This EDI is strongly supported by the "per user" cumulative dietary intake based upon NHANES 2015-2018 consumption data. The cumulative 2-day average estimated daily intake of NSEWP for the U.S.

[22] 1 What We Eat in America, NHANES 2007-2008, individuals 2 years and over (excluding breast-fed children), day 1 dietary intake data, weighted. Food Intakes Converted to Retail Commodities Database 2007-2008.

[23] Guidance for Industry: Estimating Dietary Intake of Substances in Food; AUGUST 2006

population 2 years and older is a mean of 21.5 g/p/day and 47.9 g/p/d. The EDIs presented here are highly conservative in that they assume:

- NSEWP replaces all nOVD in conventional foods.
- All Protein and nutritional powders, protein and nutritional drinks and protein-supplemented snacks and chips contain only NSEWP.

19. Part 3 only address exposure to NSEWP from use in sports drinks, protein bars, protein powders, and fruit snacks. However, in other places in the notice (pages 5, 21, 32, 56), the notifier indicates that the intended use is in all conventional foods. Please clarify if the intended use is in all conventional foods or just the listed food categories. If the intended use is in all conventional foods or in food categories that are in addition to those listed, the dietary exposure estimate should be revised to be reflective of all the intended uses of NSEWP.

    The intended uses are all conventional foods containing eggs and the listed food categories. Thus, the exposure to NSEWP is the cumulative exposure as described in Part 3 addresses the exposure of U.S. consumers to native(n) OVD (a surrogate estimate for the use of NSEWP) based upon U.S. egg consumption data, Sections 3.2-3.5 EDIs of the listed food categories and Part 3.6 the cumulative EDI or the sum on the EDIs of the intended uses including use as a substitute for nOVD in conventional foods and the use in the listed food categories.

    Please see Clara Foods' response to Question 18, which amends the dietary exposure estimate to NSEWP.

20. The notifier proposes to use NSEWP as a direct replacement for all current food uses of hen egg OVD (page 56). If the notified use could be considered as substitutional for existing uses of egg white protein, please provide a narrative indicating that there would be no appreciable increase in the cumulative exposure to protein.

    We note specifically that NSEWP may be used as a replacement for all current uses of hen egg OVD, but not all uses of egg white protein which is comprised predominantly of ovalbumin (OVA), ovomucoid (OVD) and ovomucin (OVM) proteins. Clara Foods does not anticipate the substitution of NSEWP for all egg white proteins but in applications where the single protein NSWEP with defined characteristics may be appropriate. As the use of NSEWP would be a subset of such current uses, Clara Foods does not anticipate that such uses would increase the cumulative exposure to OVD-related protein.

Sincerely,

Kevin O. Gillies

Addendum A

Dietary exposure estimate (provided by Exponent, Inc.) for non-animal soluble egg white protein (NSEWP) preparation

Exponent®

EXTERNAL MEMORANDUM

To:	Dr. Kritika Mahadevan, Clara Foods
From:	Exponent, Inc.
Date:	March 31, 2021
PROJECT:	2101843.000
SUBJECT:	Estimated Daily Intake of Non-animal Soluble Egg White Protein (NSEWP) from Intended Uses in Food

Introduction

Exponent Inc. (Exponent) was engaged by Clara Foods, Inc. (Clara Foods) to develop estimates of intake of non-animal soluble egg white protein (NSEWP) from intended uses in select foods. Clara Foods notes that NSEWP is a fermentation-derived protein that is substantially equivalent to chicken egg protein ovomucoid (nOVD).

The intended uses of NSEWP include (1) use as a substitute for egg protein (nOVD) in all processed foods, and (2) use as a source of supplemental protein in products including protein and nutritional powders and drinks, protein bars, and snacks. Estimates of intake are presented below.

Methods

Intake from use as a substitute for egg protein (nOVD)

Total egg consumption data were used to estimate intake of NSEWP. This approach provides a conservative estimate of nOVD intake that may be replaced by NSEWP, as the estimate reflects total egg intake and NSEWP will not replace all egg consumption. For example, the estimate of egg intake include eggs consumed as whole eggs (e.g., scrambled, fried) and eggs in mixtures (e.g., egg salad sandwich, eggs in breakfast sandwiches) and these uses will not be replaced with NSEWP.

Mean usual egg intake by the US population age 2 years and older is 0.6 egg/day (as egg without the shell) as reported by USDA. This estimate of egg intake is based on data collected in the What We Eat in America (WWEIA), the dietary recall component of the National Health and Nutrition Examination Survey (NHANES).

Estimates of the pseudo 90th percentile of NSEWP intake were developed as two times the mean intake. Estimates of mean NSEWP intake were developed using data on USDA's mean egg intake of 0.6 egg/day, USDA's portion weight for an egg, and the concentration of nOVD and EWP in egg and the formula below:

$$Mean\ NSEWP\ intake = \frac{0.6\ egg}{day}\ x\ \frac{50.3\ g\ egg}{egg}\ x\ \frac{11\ g\ \ nOVD}{100\ g\ EWP}\ x\ \frac{9.3\ g\ EWP}{100\ g\ egg}\ x\ \frac{100\ g\ NSEWP}{80\ g\ protein}$$

Where:

- 0.6 egg/day is from WWEIA 2013-2016
- 50.3 g/egg is from USDA FoodData Central (NDB Number 1123)[1]
- 11 g nOVD/100 g egg white protein (EWP), 9.3 g EWP/100 g (egg liquid), and 80 g protein/100 g NSEWP were provided by Clara Foods

This approach provides a conservative estimate of nOVD intake that may be replaced by NSEWP, as the estimate reflects total egg intake and NSEWP will not replace consumption of all eggs.

Intake from use as a supplemental source of protein

Estimates of NSEWP intake from the proposed use as a supplemental source of protein were developed by Exponent from food consumption records collected in the WWEIA, NHANES conducted in 2015-2016 and 2017-2018 (NHANES 2015-2018).[2] The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the U.S.

As part of the examination, trained dietary interviewers collected detailed information on all foods and beverages consumed by respondents in the previous 24-hour time period (midnight to midnight). A second dietary recall was administered by telephone three-to-ten days after the first dietary interview, but not on the same day of the week as the first interview. A total of 13,666 individuals in the survey period 2015-2018 provided two complete days of dietary recalls.

The intended use categories and intended use of NSEWP per category are shown in Table 1. The list of all food codes reported consumed in NHANES 2015-2018 was reviewed, and food codes corresponding to or foods containing ingredients corresponding to the proposed foods were identified.

The list of food reported consumed by NHANES respondents did not include protein supplemented forms of foods for all intended use categories (i.e., protein supplemented cookies, fruit snacks, and chips), therefore it was necessary to identify representative surrogates for these categories. The rationale for the representative food codes selected for use in the assessment is provide in Table 1. The list of food codes identified as representative of the intended uses and used in the analysis of intakes is provide in Appendix A.

[1] https://fdc.nal.usda.gov/fdc-app.html#/?query=egg%20whold
[2] https://wwwn.cdc.gov/nchs/nhanes/Default.aspx

Table 1. Intended use and use level of NSEWP as a source of protein

Category	Maximum NSEWP Use Level (g/serving)	Serving Size RACC (g)	Representative Food Codes Used for EDIs
Protein and nutritional powders	25	-[a]	Powder mixes in the "Protein and nutritional powders" WWEIA category.
Protein and nutritional drinks	30	240[b]	Beverages in the "Nutritional beverages" WWEIA category.
Protein cookie	20	40	Bars in the "Nutrition bars" WWEIA category, excluding bars with <10 g protein per 100 g bar, which corresponded to all bars other than bars for children. Also included niche cookies (gluten free or high fiber). These food codes are representative of the intended use in protein bars or cookies.
Protein-supplemented bars	20	40	
Protein-supplemented fruit snacks	3.2	30	Fruit snacks, with or without high vitamin C.
Protein supplemented chips	19	30	All chips other than potato, corn, tortilla or shrimp chips were used as a surrogate for protein-supplemented chips.

Abbreviations: EDI – Estimated Daily Intake, RACC – reference amount customarily consumed, WWEIA – What We Eat In America.
[a] There is not a RACC for protein powders in 21 CFR 101.12. In this analysis, the amount of powder used to prepare 1 serving as noted in the USDA data files (range of 8-87 g) was used to estimate use of the maximum intended use of NSEWP.
[b] The RACC is 240 mL as consumed, which is assumed to weigh 240 g.

Using the NHANES 2015-2018 consumption data, Exponent estimated the 2-day average daily intake on a *per capita* and *per user* basis. *Per capita* estimates refer to the consumption based on the entire population of interest, whereas *per user* estimates refer to the consumption among those who reported consuming any of the foods of interest on either of the survey days. For each subject with a complete 2-day dietary recall, a 2-day average intake estimate was derived by multiplying the reported intake of foods from the 24-hour recall with the NSEWP use level and the cumulative sum over the two 24-hr recalls was divided by two. The mean and 90th percentile of the 2-day average NSEWP intake were calculated for the total U.S. population two years and older (2+ y) and subpopulations including children ages 2-12 years, teenagers ages 13-18 years, and adults ages 19 years and older (19+ y).

The analysis was limited to individuals who provided two complete and reliable dietary recalls as determined by NCHS. The 2-day average intakes by each individual were estimated using Exponent's Foods Analysis and Residues Evaluation Program (FARE® version 14.05) software. Exponent uses the statistically weighted values from the survey in its analyses. The statistical weights compensate for variable probabilities of selection, adjusted for non-response, and provide intake estimates that are representative of the U.S. population.

Results

Intake from use as a substitute for egg protein (nOVD)

Using the inputs of mean per capita total egg intake, the concentration of nOVD in egg, and the concentration of protein in NSEWP comparable to nOVD, the intake of NSEWP from

substitutional uses was calculated as 0.39 g/day at the mean, and 0.78 g/day at the pseudo 90th percentile of NSEWP intake (i.e., 0.39 g/day x 2).

Intake from use as a supplemental source of protein

Estimated daily intakes (EDI) of NSEWP from the proposed uses are presented in Table 2, and cumulative estimates of intake are presented in Table 3. The cumulative estimates of intake were developed by adding the estimated intake from substitutional uses (0.39 g/day at the mean and 0.78 g/day at the 90th percentile of intake) to the estimated intakes from the intended use as a supplemental source of protein. Estimating the cumulative 90th percentile of intake as the sum of 90th percentile intakes from substitutional uses and 90th percentile intakes from use as a supplemental source of protein provides a highly conservative estimate of the 90th percentile of intake.

Table 2. 2-day average estimated daily intake of NSEWP from proposed food uses by the U.S. population 2 years and older; NHANES 2015-2018

Population	Total Sample, n	Users, n	% User	Per Capita (g/day)		Per User (g/day)	
				Mean	90th Percentile	Mean	90th Percentile
US 2+ y	12717	1474	13.9	2.9	6.2	21.1	47.1
Children 2-12 y	2743	434	18.1	1.0	2.1	5.5	15.5
Adolescents 13-18 y	1433	185	12.4	1.9	2.4	15.5	33.2
Adults 19+ y	8541	855	13.2	3.4	8.8	25.9	54.7

Proposed uses as shown in Table 1.

Table 3. Cumulative 2-day average estimated daily intake of NSEWP from substitutional and proposed food uses by the U.S. population 2 years and older; NHANES 2015-2018

Population	Total Sample, n	Users, n	% User	Per Capita (g/day)		Per User (g/day)	
				Mean	90th Percentile	Mean	90th Percentile
US 2+ y	12717	1474	13.9	3.3	7.0	21.5	47.9
Children 2-12 y	2743	434	18.1	1.4	2.9	5.8	16.3
Adolescents 13-18 y	1433	185	12.4	2.3	3.2	15.9	34.0
Adults 19+ y	8541	855	13.2	3.8	9.6	26.3	55.5

Substitutional use assumes mean intake of 0.6 egg per day by the US population[3] and a pseudo 90th percentile intake of 1.2 egg per day which results in mean and 90th percentile intakes of NSEWP of 0.39 and 0.78 g/day, respectively; proposed uses as shown in Table 1.

[3] USDA. 2020. Food Group and Nutrient Distribution: All Life Stages. Available at: https://www.dietaryguidelines.gov/sites/default/files/2020-07/DA_Supplement_FoodGroup_NutrientDistribution.pdf.

Appendix A. Food Codes Used in Analysis

Food code	Food description
Protein and nutritional powders	
95201000	Nutritional powder mix (Carnation Instant Breakfast)
95201010	Nutritional powder mix, sugar free (Carnation Instant Breakfast)
95201200	Nutritional powder mix (EAS Whey Protein Powder)
95201300	Nutritional powder mix (EAS Soy Protein Powder)
95201500	Nutritional powder mix, high protein (Herbalife)
95201600	Nutritional powder mix (Isopure)
95201700	Nutritional powder mix (Kellogg's Special K20 Protein Water)
95202000	Nutritional powder mix (Muscle Milk)
95210000	Nutritional powder mix (Slim Fast)
95210020	Nutritional powder mix, high protein (Slim Fast)
95220000	Nutritional powder mix, NFS
95220010	Nutritional powder mix, high protein, NFS
95230000	Nutritional powder mix, whey based, NFS
95230010	Nutritional powder mix, protein, soy based, NFS
95230020	Nutritional powder mix, protein, light, NFS
95230030	Nutritional powder mix, protein, NFS
Protein and nutritional drinks	
95101000	Nutritional drink or shake, ready-to-drink (Boost)
95101010	Nutritional drink or shake, ready-to-drink (Boost Plus)
95102000	Nutritional drink or shake, ready-to-drink (Carnation Instant Breakfast)
95103000	Nutritional drink or shake, ready-to-drink (Ensure)
95103010	Nutritional drink or shake, ready-to-drink (Ensure Plus)
95104000	Nutritional drink or shake, ready-to-drink, sugar free (Glucerna)
95105000	Nutritional drink or shake, ready-to-drink (Kellogg's Special K Protein)
95106000	Nutritional drink or shake, ready-to-drink (Muscle Milk)
95106010	Nutritional drink or shake, ready-to-drink, light (Muscle Milk)
95110000	Nutritional drink or shake, ready-to-drink (Slim Fast)
95110010	Nutritional drink or shake, ready-to-drink, sugar free (Slim Fast)
95110020	Nutritional drink or shake, high protein, ready-to-drink (Slim Fast)
95120000	Nutritional drink or shake, ready-to-drink, NFS
95120010	Nutritional drink or shake, high protein, ready-to-drink, NFS
95120020	Nutritional drink or shake, high protein, light, ready-to-drink, NFS
95120050	Nutritional drink or shake, liquid, soy-based
95101000	Nutritional drink or shake, ready-to-drink (Boost)
53231400	Cookie, multigrain, high fiber
53261000	Cookie, gluten free
53710800	Cereal or granola bar (Kashi Chewy)
53710802	Cereal or granola bar (Kashi Crunchy)

Food code	Food description
53720100	Nutrition bar (Balance Original Bar)
53720200	Nutrition bar (Clif Bar)
53720300	Nutrition bar (PowerBar)
53720400	Nutrition bar (Slim Fast Original Meal Bar)
53720500	Nutrition bar (Snickers Marathon Protein Bar)
53720600	Nutrition bar (South Beach Living Meal Bar)
53720610	Nutrition bar (South Beach Living High Protein Bar)
53720700	Nutrition bar (Tiger's Milk)
53720800	Nutrition bar (Zone Perfect Classic Crunch)
53729000	Nutrition bar or meal replacement bar, NFS
Protein-supplemented fruit snacks	
91708030	Fruit leather and fruit snacks candy
91708100	Fruit snacks candy, with high vitamin C
Protein supplemented chips	
41310900	Bean chips
54318000	Chips, rice
54420210	Multigrain chips (Sun Chips)
54440020	Cracker chips
71220000	Vegetable chips
71905410water	Plantain chips
71980200	Taro chips
73410210	Sweet potato chips