DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000967	Sep 14, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* 2019-03-28

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Kritika Mahadevan, PhD	Associate Director, Product
Organization *(if applicable)*	
Clara Foods Co.	
Mailing Address *(number and street)*	
1 Tower Place Suite 800	

City	State or Province	Zip Code/Postal Code	Country
South San Francisco	California	94080	United States of America

Telephone Number	Fax Number	E-Mail Address
904-414-6295		kritika@clarafoods.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
KEVIN GILLIES	MEMBER
Organization *(if applicable)*	
KEVIN O. GILLIES CONSULTING SERVICES, LLC	
Mailing Address *(number and street)*	
1759 GRAPE ST.	

City	State or Province	Zip Code/Postal Code	Country
Denver	CO	80220	US

Telephone Number	Fax Number	E-Mail Address
001-816-590-9836		info@kogilliesconsultingservices.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Non-Animal Soluble Egg White Protein produced by Pichia pastoris

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Non-animal soluble egg white protein produced microbially by P. pastoris DFB-003 is intended as a source of dietary protein when manufactured according to cGMP. It can be used as a replacement for plant and animal-derived proteins currently used in foods. It's intended uses include but are not limited to protein fortification of conventional foods such as sports drinks, protein supplement powders, and nutritional bars. Such uses will not increase the consumption of protein for the general population. Non-animal soluble egg white protein produced microbially by P. pastoris is not intended for use in products regulated under USDA/FSIS jurisdiction or in infant formula.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Clara Foods, Co.
(name of notifier)

has concluded that the intended use*(s)* of Non-Animal Soluble Egg White Protein produced by Pichia pastoris
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Clara Foods, Co. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

1 Tower Place, Suite 800, South San Francisco, CA 94080
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	Kevin O. Gillies, Agent	09/14/2020

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667.pdf	Administrative
	GRASNotice_Non-AnimalSolubleEggWhiteProteinproducedbyPichiapastoris_NSEWP_ClaraFoods_09142020.pdf	GRAS Notice
	AppendixIndex_NSEWP.pdf	Administrative
	Appendix1_StandardOperatingProcedureandResultsforAbsenceofencodingDNAbyPCR_NSEWP.pdf	Administrative
	Appendix2_StandardOperatingProceduretodetermineAbsenceofProductionOrganismbyPlating_NSEWP.pdf	Administrative
	Appendix3_CertificateofAnalysis_productionLots_NSEWP.pdf	Administrative
	Appendix4_ExpertPanelReport_NSEWP.pdf	Administrative

Non-Animal Soluble Egg White Protein produced by *Pichia pastoris*

Generally Recognized as Safe Notice

Submitted by:

Clara Foods Co.
1 Tower Place
Suite 800
South San Francisco, CA 94080

August 2020

Prepared by:

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

Kritika Mahadevan
Associate Director, Product
Clara Foods Co.
1 Tower Place, Suite 800
South San Francisco, CA 94080
USA

Table of Contents

21 CFR 570. 225; Part 1: Signed Statements and Certification

1.1 Exemption Claim for non-animal soluble egg white protein produced by *Pichia pastoris*

Clara Foods Co. (Clara Foods) located at 1 Tower Place, Suite 800, South San Francisco, 94080 CA, USA, in accordance with FDA's final rule of August 17, 2016 (81 FR 54960) and 21 CFR §170.225(c)(1) relating to the filing of Generally Recognized as Safe (GRAS) notices, submits the following exemption claim as it relates to the use of non-animal soluble egg white protein produced by *P. pastoris* as a protein ingredient in food at levels in accordance with current Good Manufacturing Practice.

Specifically, Clara Foods has concluded, and an independent panel of experts has agreed, that non-animal soluble egg white protein produced by *P. pastoris* is Generally Recognized as Safe (GRAS) by scientific procedures in accordance with both 21 CFR 170.30(a) and (b) and is thereby exempt from pre-market approval requirements of the Food, Drug and Cosmetic Act.

In conformity with the requirements outlined in the rule, the following information is included with this exemption claim.

1.2 Information about Notifier

Notifier:

Clara Foods Co.
1 Tower Place
Suite 800
South San Francisco, CA 94080

Contact person for this file:

See agent below

Agent who is authorized to act on behalf of the Notifier:

Kevin O. Gillies
Kevin O. Gillies Consulting Services, LLC

1.3 Basis for Safety Determination

Non-animal soluble egg white protein produced by *P. pastoris* DFB-003 is GRAS under the conditions of the intended use by scientific procedures and is, thereby, not subject to pre-market approval under the Food, Drug, and Cosmetic Act.

1.4 Intended Use

Non-animal soluble egg white protein produced microbially by *P. pastoris* DFB-003 is intended as a source of dietary protein when manufactured according to cGMP. It can be used as a replacement for plant and animal-derived proteins currently used in foods. It's intended uses include but are not limited to protein fortification of conventional foods such as sports drinks, protein supplement powders, and nutritional bars. Such uses will not increase the consumption of protein for the general population. Non-animal soluble egg white protein produced microbially by *P. pastoris* is not intended for use in products regulated under USDA/FSIS jurisdiction or in infant formula.

1.5 Availability of Information

Data and information relevant to this GRAS notice is available to FDA during customary Clara Foods Co. business hours upon request.

1.6 Confidential Commercial Information

None of the information in the GRAS Notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.7 Certification Statement

Clara Foods certifies in accordance with 21CFR570.225(c)(9) that, to the best of its knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to Clara Foods and pertinent to the evaluation of the safety and GRAS status of the use of non-animal soluble egg white protein produced by *P. pastoris* DFB-003.

1.8 Signature of Responsible Party or Agent

(b) (4)

Kevin O. Gillies
Kevin O. Gillies Consulting Services, LLC
September 12, 2020

21CFR570.230; Part 2: Identity, Manufacturing, Specifications, Use

2.1 Identity of the Substance

2.1.1 Common and Usual Name of the Substance:

Non-animal soluble egg white protein
Non-animal soluble egg white protein from yeast
Fermentation-derived soluble egg white protein
Microflora-derived soluble egg white protein

2.1.2 Chemical name: Glycoprotein

In 2019, approximately 9.9 x 10_{10} eggs were sold in the US which is an egg consumption of approximately 287.1 per capita/year, representing 6.2 x 10_{11} grams of egg protein introduced into the US market per year. In addition to consumption of whole eggs, eggs are used in prepared foods to provide flavor, color and texture in addition to processing advantages such as emulsifying, stabilizing, increasing volume and machineability (American Egg Board (2020).

Hen egg ovomucoid (OVD) is located in the egg white, comprises approximately 11% of the egg white protein, and has been consumed for as long as eggs have been used as a food source. In addition, the protein has a safe history of consumption as a food ingredient and as a component of whole eggs.

The native OVD protein is a glycoprotein with bovine trypsin inhibitor activity (Travis 1971), a molecular mass of approximately 28.0 kDa, and an isoelectric point of 4.1 (Benedé, et al. 2013). Its polypeptide chain consists of 186 amino acids.

2.1.3 Substantial Equivalence to native hen egg OVD

Clara Foods has undertaken a robust evaluation of the non-animal soluble egg white protein (NSEWP) preparation and determined that it is substantially equivalent to the native hen egg OVD by:

- Glycosylation analysis
- Amino acid analysis
- Molecular weight characteristics
- Immunoreactivity characteristics
- Bovine trypsin inhibition
- *In vitro* digestibility determinations

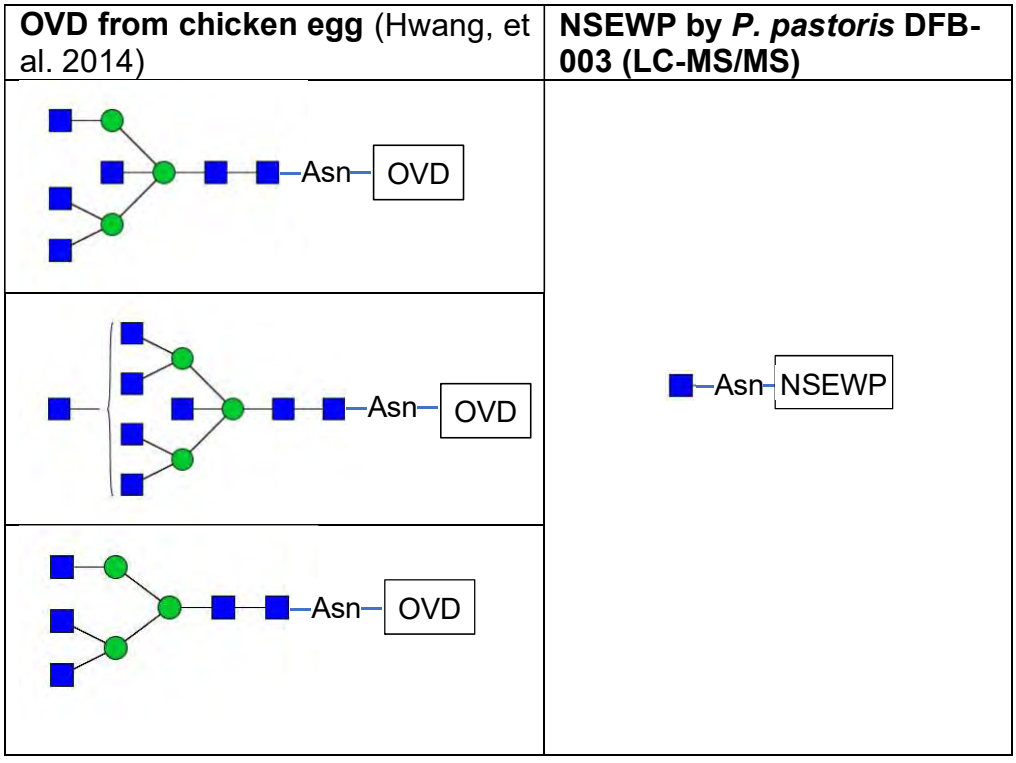
- Non-animal soluble egg white protein (NSEWP) Glycosylation Form

  LC-MS/MS analysis indicates that NSEWP produced by *P. pastoris* DFB-003 has a reduced glycan content compared to the native OVD from chicken egg (*Gallus gallus*). This is an expected result as *P. pastoris* DFB-003 produces a glycosylated form of OVD that contains a core protein/glycan moiety found in all avian OVD proteins but has branched glycoform attached to the core protein/glycan that is not found in the native OVD protein. The production organism has been modified by adding a glycohydrolase enzyme (EndoH, see Section 2.2.2.2) that cleaves the glycan form down to the core protein/glycan structure. The resulting protein as expressed in DFB-003, i.e. NSEWP, contains only the core glycosylation moiety, i.e. one N-acetylglucosamine unit, as found in all OVD molecules in nature (Figure 1).

  Figure 1. NSEWP core N-glycan structure compared to a selection of native OVD
  (adapted and altered from Hwang et al., 2014)

  Legend: ■ N-acetylglucosamine ● mannose

  –Asn– OVD protein moiety with the N-linked glycosylation site



- ‘NSEWP’ LCMS amino acid composition:

  NSEWP produced by *P. pastoris* DFB-003 was compared with OVD from chicken egg white by liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS, a widely applied and preferred method in proteomics (Switzar, Giera and Niessen 2013), was used to identify OVD and NSEWP.

  The protein samples were first digested into peptides using trypsin. Tandem mass spectrometry data was searched against *in silico* tryptic digests of a custom-built database (Clara Foods, Inc.). The database (101,326 sequences) comprised the proteins of the Uniprot-KB *Gallus gallus* database (version 2019/11) appended with the *Pichia pastoris* (version 2019/11) and additionally with a database of proteins commonly found in proteomics experiments that are present either by accident or through unavoidable contamination of protein samples (known as the common repository of adventitious proteins, “cRAP”). The search parameters were defined as: iodoacetamide modified for cysteine alkylation and trypsin as the digestion enzyme. Additional modifications and cleavages were defined previously (Colgrave, et al. 2014). The database search results were manually curated to yield the protein identifications using a 1% global False Discovery Rate (FDR) determined by the in-built FDR tool within ProteinPilot software (Tang, Shilov and Seymour 2008).

  The native form of *Gallus gallus* (chicken) OVD has 186 amino acids. NSEWP contains the mature form of OVD expressed as a fusion protein with the *Saccharomyces cerevisiae* alpha factor pre-pro sequence in *Pichia pastoris* (italics and non-italics below, respectively). Post-translation modification results in the removal of the alpha factor pre-pro sequence. Edman N terminal sequencing (Smith 2001) and LC-MS/MS analysis results showed imprecise cleavage of the N-terminal pro sequence by *Pichia* host post- translational machinery. The majority of the NSEWP starts with an N-terminal extension containing 4 extra amino acid residues (bolded below) of the *Saccharomyces cerevisiae* alpha factor pre-pro sequence.

  EAEA-AEVDC SRFPNATDKE GKDVLVCNKD LRPICGTDGV
  TYTNDCLLCA. YSIEFGTNIS KEHDGECKET. VPMNCSSYAN
  TTSEDGKVMV LCNRAFNPVC. GTDGVTYDNE CLLCAHKVEQ
  GASVDKRHDG GCRKELAAVS. VDCSEYPKPD. CTAEDRPLCG
  SDNKTYGNKC. NFCNAVVESN. GTLTLSHFGK C

There is also evidence that a minor fraction of the protein product starts with a larger extension EEGVSLEKREAEA, also from the alpha factor secretory signal sequence.

Both N-terminal extensions (EAEA and EEGVSLEKREAEA) were checked using BLAST analysis and compared to sequences of known protein allergens (Goodman, et al. 2016) and known toxins (Liu, et al. 2019) (Virulence Factor Database n.d.) and there was no significant homology to related proteins of concern (Pearson and Lipman 1988). We note specifically that the amino acid remnants of the *S. cerevisiae* pre-pro sequences are derived from an organism with a safe history of use in food. This, in conjunction with the sequence analysis, indicates that the added amino acid sequences do not present safety concerns.

- Molecular weight characteristics:

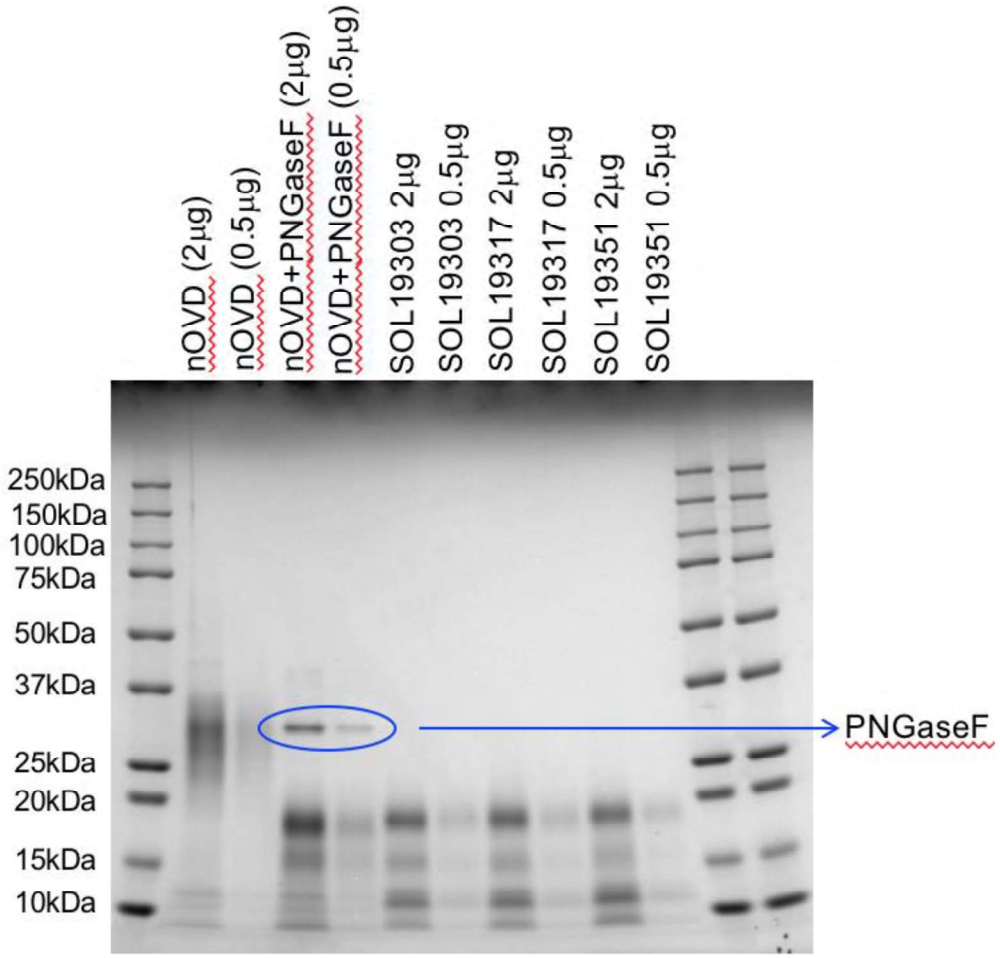
  Molecular weight of NSEWP was compared against native chicken OVD using SDS-PAGE.

  There is a difference in migration of NSEWP samples compared to the chicken OVD sample in the SDS-PAGE gel (Figure 2). This is likely due to the fact that NSEWP produced by *P. pastoris* DFB-003 has only the core N-acetylglucosamine unit attached to the Asparagine (Asn) instead of the complex branched glycosylation of native OVD from chicken egg white (see Figure 1).

  To confirm this, the deglycosylated native OVD (OVD + PNGaseF) prepared by treating native OVD with PNGase F, an enzyme that specifically deglycosylates proteins (BioLabs 2020), was also run on the same gel. The deglycosylated native OVD (nOVD + PNGaseF) displayed the same band patterns and molecular weight as the three NSEWP samples (SOL) indicating a molecular weight equivalence between deglycosylated OVD and NSEWP (Figure 2).

  A similar lowering of molecular mass and increase in electrophoretic mobility upon deglycosylation of native OVD has been reported (Benedé, et al. 2013) (Gu, et al. 1989).

Figure 2. SDS-PAGE gel of native chicken OVD (nOVD) and NSEWP produced by *P. pastoris* DFB-003 (SOL) demonstrating equivalence in molecular weight.



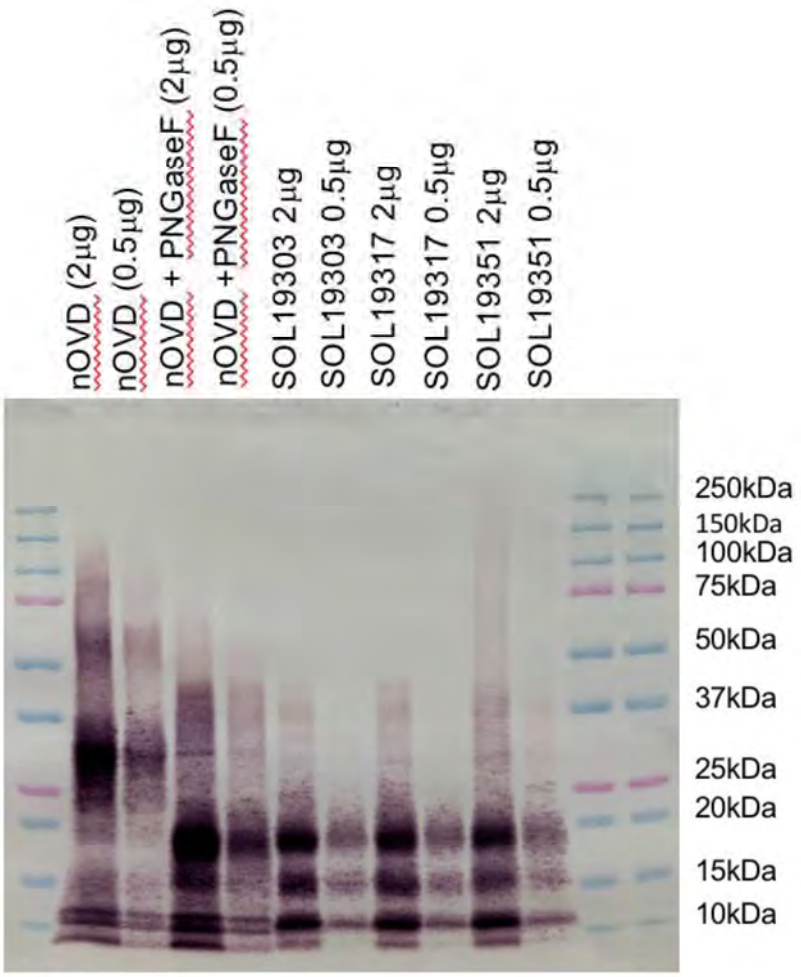
- Immunoreactivity characteristics:

  Western blotting (immunoblotting, protein blotting) is a sensitive immunological method for detecting proteins separated by electrophoresis (Towbin 1998). This technique can detect target proteins as low as 1 ng due to high-resolution capacity of gel electrophoresis and the strong sensitivity and specificity of the immunoassay.

  Western Blot was performed on three NSEWP Lots (SOL19303, SOL19317, SOL19351) using primary anti-OVD antibody from rabbit (NBP1-74676 Novus) at a 1:2500 dilution (Jensen 2012). The secondary antibody used was goat anti-rabbit IgG conjugated to alkaline phosphatase (AP ab97048 Abcam). Molecular weight marker preparation used was from Bio Rad (161-0394).

The Western blot comparison (Figure 3) shows the same immunoreactivity for the NSEWP samples (SOL), native OVD from chicken egg white (nOVD), and deglycosylated native OVD (nOVD + PNGaseF).

Figure 3. Western Blot of native chicken OVD and NSEWP produced by *P. pastoris* DFB-003 demonstrating equivalence in immunoreactivity



- Trypsin inhibition activity:

  Native OVD, from chicken egg white, has trypsin inhibitory activity (Gu, et al. 1989). It is important to note that native OVD inhibits bovine trypsin and does not inhibit human trypsin activity (Travis 1971).

  Trypsin inhibition assay (AACC #22-40.01) was conducted using bovine trypsin on samples of NSEWP and native OVD. NSEWP samples

(SOL19303, SOL19317 and SOL19351) demonstrated significant levels of trypsin inhibition activity, similar to the native OVD protein (Table 1). The slightly lower inhibition activity values reported for NSEWP may be due to their lower glycosylation. It has been shown that the carbohydrate moiety of native OVD contributes to its stability against tryptic digestion (Gu, et al. 1989).

Table 1: Comparison of trypsin inhibition activity

Product	Trypsin inhibition activity [1,2]
SOL19303	8190 TIU / g
SOL19317	8180 TIU / g
SOL19351	8469 TIU / g
Native chicken OVD	13721 TIU / g

[1] AACC 22-40.01
[2] One trypsin unit is arbitrarily defined as an increase of 0.01 absorbance unit at 410 nm per 10 ml of reaction mixture under the conditions of the assay. Trypsin inhibitor activity is expressed in terms of trypsin inhibitor units (TIU).

- *In-vitro* simulated gastric digestibility:

The *in-vitro* digestibility of samples was measured using the Protein Digestibility Assay procedure (Megazyme, Medallion Labs). The analysis demonstrated the equivalence between native chicken egg white OVD and NSEWP in terms of their *in-vitro* digestibility (Table 2).

Table 2: Comparison of OVD and NSEWP in-vitro digestibility

Product	In-vitro digestibility
SOL19303	93%
SOL19317	93%
SOL19351	93%
Native chicken OVD	92%

In summary, the results of the amino acid sequence, molecular weight, immunoreactivity, trypsin inhibition and gastric enzyme *in vitro* digestibility determinations confirm that NSEWP is substantially equivalent to the native OVD from chicken egg white.

NSEWP differs from native OVD in molecular weight due to a simplified glycosylation form. Analysis has demonstrated that the NSEWP glycosylation form is common to all avian OVD protein and this form is also equivalent to hen egg OVD. NSEWP also contains short amino acid sequences derived from *S. cerevisiae*. These sequences have been characterized and determined to be safe and suitable for use in food by

bioinformatic toxin and allergen analysis and the safe history of use in food by virtue of their *S. cerevisiae* origin.

2.2 Production Microorganism

2.2.1 Production host strain names and safe strain lineage

P. pastoris is a nonpathogenic, non-toxigenic, and well-characterized yeast with a history of safe use in the food industry.

P. pastoris strain BG08 (BioGrammatics Inc., Carlsbad; CA, USA) is a single colony isolate from the Phillips Petroleum strain NRRL Y-11430 obtained from the Agriculture Research Service culture collection (Sturmberger, et al. 2016). *P. pastoris* BG10 (BioGrammatics Inc, Carlsbad, CA, USA) was derived from BG08 using Hoechst dye selection to remove cytoplasmic killer plasmids (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.

The general taxonomy of *P. pastoris* is:

Name: *Pichia pastoris*
Kingdom: *Fungi*
Phylum: *Ascoymycota*
Class: *Hemiascomycetes*
Order: *Saccharomycetales*
Family: *Endomycetaceae*
Genus: *Pichia*
Species: *pastoris*

2.2.2 Strain construction

2.2.2.1 Production Strain

Production strain *P. pastoris* DFB-003 was constructed from recipient strain DFB-001 using transformations with different expression constructs in order to express NSEWP. In addition to the protein coding sequence for OVD, Strain DFB-003 also contains extra copies of the *P. pastoris* transcription factor HAC1, and the coding sequence for the deglycosylating enzyme Endoglycosidase H (Endo H), all expressed under strong native *P. pastoris* methanol-induced promoters. Methanol-induced gene expression is a common strategy used to produce high levels of recombinant proteins after producing biomass on glycerol and glucose and inducing with methanol (Cereghino and Cregg 2000). The genome of DFB-003 is fully sequenced and well-characterized.

The *P. pastoris* production strain background complies with the Organization for Economic Development (OECD) criteria for Good Industrial Large Scale Practice (GILSP) microorganisms (OECD 1992, 1993). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (EU Scientific Committee for Food 1992) (FAO/WHO 1996) (International Food Biotechnology Council 1990) (Jonas, et al. 1996) (OECD 1993) (Pariza and Foster 1983) (Pariza and Johnson 2001))

2.2.2.2 Construction of Production Strain DFB-003: Strain Overexpressing OVD and HAC1 and EndoH fusion protein

OVD is a protein found in egg whites. It is a trypsin inhibitor with three protein domains of the Kazal domain family. Chicken (*Gallus gallus)* OVD is also

known as "Gal d 1". OVD is expressed in animals as a pre+mature form that is secreted with removal of the "pre" sequence during the secretion process.

The *Saccharomyces cerevisiae (S. cerevisiae)* alpha mating factor pre-pro sequence ('ScPrePro) is a common secretion signal for secretion of heterologous proteins by *P. pastoris.* The protein coding sequence of the mature form of *Gallus gallus* OVD was synthesized as a fusion sequence with the ScPrePro for expression and secretion in *P. pastoris*.

HAC1 is a *P. pastoris* transcription factor that regulates genes involved in the Unfolded Protein Response (Guerfal, et al. 2010). Overexpression of HAC1 can improve production of heterologous proteins in several yeast systems, (Gasser, et al. 2006) (Guerfal, et al. 2010). The addition of a methanol inducible HAC1 leads to improved production of OVD by *P. pastoris*.

Endo-β-N-acetylglucosaminidase H (Endo H, EC3.2.1.96) is a glycohydrolase that is secreted by *Streptomyces ssp.* (Arakawa and Muramatsu 1974) (Wang, et al. 2015). *Streptomyces ssp.* that have been studied are soil-born, non-pathogenic, BioSafety Level 1 bacteria (ATCC 2020), widely used as a source of clinical antimicrobials, e.g. streptomycin. While the organism does not have a history of use in food, it does have a history of safe production of antibiotics that are consumed world-wide. A survey of the scientific literature did not yield any indication of safety concerns for the use of the Streptomyces-derived synthetic gene sequence in the strain construction[1].

EndoH cleaves the β-1, 4-glycosidic bond of the N-acetyl glucosamine core of oligosaccharides and leaves one N-acetylchitobiose attached to the asparagine residue of the glycoprotein (Trimble, et al. 1978). EndoH has been expressed in *P. pastoris* and shown to be active (Wang et al., 2015). An α-1,6-mannosyltransferase known as OCH1 catalyzes an early step in yeast glycosylation and is located in the golgi (Vervecken, et al. 2004). A fusion protein gene was designed that encodes the first 48 residues of *Pichia* OCH1 protein fused to a catalytically active version of the *Streptomyces coelicoflavus* EndoH. The OCH1 moiety anchors the EndoH enzyme to a DFB-003 cellular structure that places the glycohydrolase activity in proximity to the glycoprotein post-translation machinery to allow for deglycosylation of the pre-pro recombinant OVD protein. Anchoring the EndoH activity to a *P. pastoris* cellular organelle ensures that the enzyme is absent in the production fermentate and the final product (see Section 2.2.2.7).

Linear cassettes of methanol - inducible promoter :: ScPrePro :: OVD :: AOX1term and a linear cassette of DNA containing a HAC1 gene under the

[1] May 2020

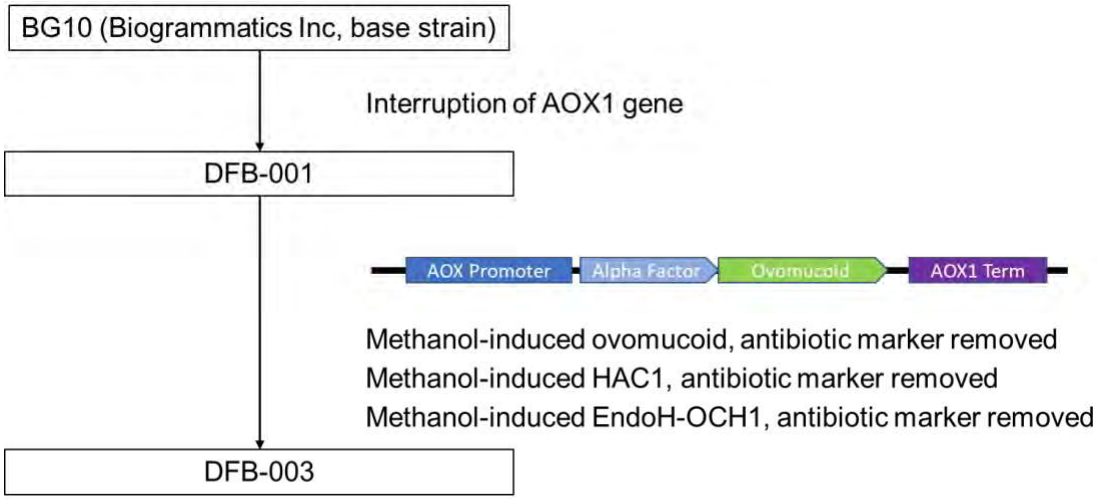
control of methanol inducible promoters such as the pAOX1 promoter and AOX1 terminator, and the EndoH-OCH1 fusion were introduced into Strain DFB-001 using standard electroporation methods (Lin-Cereghino, et al. 2005).

PCR analysis and protein expression assays identified the production strain, Strain DFB-003 which contains the ScPrePro :: OVD :: AOX1term gene, the HAC1 gene, and the EndoH-OCH1 fusion gene.

As antibiotic markers are present on the plasmid vector used to insert target genes into the DFB-001 chromosomes to produce DFB-003, loop-out techniques were used to remove the antibiotic marker prior to use of DFB-003 as a production host.

A combination of PCR, antibiotic resistance and genome sequencing analysis was used to demonstrate absence of any antibiotic resistance genes or bacterial origins of replications present in the original vector plasmid DNA in the production host DFB-003.

Figure 4: Construction of production strain using recipient strain DFB-003



2.2.2.3 Genome Sequence of the Production Strain DFB-003

The genome of production strain DFB-003 has been completely sequenced and confirmed to contain the following sequences in addition to the backbone *P. pastoris* BG10 DNA.

- 11 copies of OVD coding sequence were integrated into the genome as cassettes of methanol-inducible promoter :: ScAlphaFactor :: OVD :: AOX1term (no antibiotic resistance genes, no origin of replication)

- 3 copies of methanol-inducible promoter :: HAC1 :: AOX1term (no antibiotic resistance genes, no origin of replication)

- 1 copy of methanol-inducible promoter :: EndoH-OCH1 :: AOX1term (no antibiotic resistance genes, no origin of replication)

Sequencing and data analysis methods:

Standard Illumina library was prepared according to vendor's specification (Illumina 2020). The Illumina library was sequenced with a paired end protocol on an Illumina HiSeq 4000 instrument for a total of 300 cycles (150 bases from each end of each library insert). Total read count for Strain DFB-003 was 50553370 pairs containing roughly 24 billion base calls. Given the presence of mitochondrial DNA in the sample, Illumina sequencing data represents approximately 2,000 × coverage of chromosomal sequences.

In addition to the Illumina data, sequencing was performed on a Minion long read sequencer. There was a total of 21917 long reads in this data set, comprising approximately 350,000,000 base calls. PHRED quality scores had a distribution between 11 and 23. PHRED score is a commonly used metric to assess the accuracy of a sequencing platform. PHRED scores are on a log scale, with 10 indicating 90% probability of call being correct and 20 indicating 99% probability. A *de novo* genome assembly was performed using Unicycler (https://github.com/rrwick/Unicycler) to co-assemble Minion and Illumina data. Briefly, Unicycler is a multi–program script that uses SPAdes to assemble contigs from Illumina data and then miniasm to bridge contigs from the high-quality SPAdes assembly with Minion long reads. The assembly was then refined with cycles of Pilon. Assembly of Strain DFB-003 data resulted in 4 linear chromosomes and a circular mitochondrial genome as expected for *P. pastoris* (Sturmberger, et al. 2016).

2.2.2.4 Stability of the Production Strain

All changes introduced into production Strain DFB-003 are stably integrated in the genome and confirmed to be present after forty-five (45) generations of growth on non-selective growth media. The presence of the changes was confirmed by phenotype testing, in terms of OVD titer, of 160 yeast colonies at the end of the fermentation compared to 24 yeast colonies at the beginning of the fermentation.

No vector plasmid sequences are present in the production strain. Hence, plasmid sequences will not be transferred from the production strain to a non-related organism.

2.2.2.5 Absence of Antibiotic Resistance Genes

The production strain DFB-003 does not contain antibiotic resistance genes. Antibiotic resistance markers used in strain construction were "looped out" of the production strain. The absence of the antibiotic resistance genes was confirmed by a combination of PCR, antibiotic resistance and genome sequencing analysis.

In addition to the absence of antibiotic resistance genes, none of the NSEWP production lots contained OVD transformable DNA. The absence of transformable OVD DNA has been established by PCR analysis based on the guidelines provided by the European Food Safety Authority (EFSA Panel on Genetically Modified Organisms (GMO) 2011). The PCR analysis (Appendix 1) concludes that no encoding pieces of recombinant DNA are present in Clara Foods' NSEWP preparation. The level of detection of the PCR analysis was established to be 10 femtogram (fg) of recombinant DNA in the PCR reaction (Appendix 1, Figure B, Lane 9). Typical yeast transformations require microgram quantities of recombinant DNA for homologous integration. The PCR test protocol used is highly sensitive since it can detect ~1,000,000,000 times less transforming DNA than is required for homologous integration.

2.2.2.6 Absence of the DFB-003 Production Organism in the Final Product

The DFB-003 production organism is not detected in the NSEWP samples in accordance with the recommendation of safety evaluation by the International Food Biotechnology Committee (IFBC 1990). Procedure to ascertain absence of DFB-003 in the sample lots is presented in Appendix 1 (PCR method, demonstrating absence of organism through absence of transformable DNA) and Appendix 2 (Plating method, where no colonies of any organism were found to grow).

2.2.2.7 Absence of EndoH in the Final Product

A fusion protein gene was designed to encode the first 48 residues of *Pichia* OCH1 protein fused to a catalytically active version of the *Streptomyces coelicoflavus* EndoH. The EndoH gene is derived from the genome of an organism that does not have a safe history of use in food, but is a non-pathogenic, non-toxigenic BioSafety Level 1 organism[2]. *Streptomyces*

[2] https://bacdive.dsmz.de/strain/16204

species have a history of use in the production of drug antimicrobials (Rao, et al. 2017). Following Pariza and Johnson guidance (Pariza and Johnson 2001), Clara Foods has demonstrated the safety of the added DNA by showing that EndoH is removed from the product during the production process and thus does not pose safety concerns.

Expression of the EndoH/OCH1 fusion protein is under the control of a methanol-induced promoter and the fusion protein is postulated to be anchored in the golgi membrane of *P. pastoris* DFB-03 based on the golgi cellular location of the native OCH1. Proof of concept studies using a commercial antibody that recognizes a synthetic sequence (with a myc-tagged version of the EndoH/OCH1 fusion protein) expressed in a pre-production *P. pastoris* strain, showed no protein detected by western blot, probed with the myc epitope 9E10 (Developmental Studies Hybridoma Bank; https://dshb.biology.uiowa.edu), in either the cell lysate supernatant or the NSEWP finished product, consistent with the fusion protein being intracellular and anchored to cell components that are removed by the initial centrifugation purification step.

Even though the final protein preparation does not contain the EndoH/OCH1 fusion protein as described above, a 12 amino acid fragment of the EndoH protein has been detected at very low levels in the NSEWP final product by LC-MS/MS analysis. This LC-MS/MS analysis records the number of times a peptide is detected for each protein. In this case, there were 2293 peptides detected for OVD and 1 instance for the peptide from the EndoH/OCH1 fusion protein in the final product sample.

Because EndoH has no history of use in food, the amino acid sequence of the EndoH peptide was compared to sequences of known protein allergens (Goodman, et al. 2016) and known toxins (Liu, et al. 2019) (Virulence Factor Database n.d.) and there was no significant homology to related proteins of concern (Pearson and Lipman 1988). The absence of relationship to known toxins and allergens in addition to the very low levels found in the final product indicates that the peptide does not present a safety concern.

2.2.2.8 Absence of Potential Toxicants

The production strain and manufacturing process for NSEWP do not produce any known toxicants. The extensive purification steps, including centrifugation, ultra-filtration and drying, add an additional level of confidence that the preparations are free from potential toxicants.

The safety decision tree (Pariza and Johnson 2001) did not indicate the need for toxicological studies on NSEWP. Previous toxicology studies, as reported in GRAS Notices GRN 204 and GRN 737, conducted subacute toxicity,

repeated dose toxicity and mutagenicity/genotoxicity studies on test articles produced in the *P. pastoris* BG10 host background. No adverse test article effects were reported (See GRN 204 and GRN 737 included here by reference) (GRN 204 2006) (GRN 737 2018). Phospholipase C received FDA GRAS status in 2006 (GRN 204 2006) and has since been used for degumming vegetable oils for food use.

Biopharmaceutical Jetrea® (Ocriplasmin), produced by *P. pastoris,* was approved by FDA in 2012 after Phase 3 clinical trials. Jetrea® is used for treatment of symptomatic vitreomacular adhesion (Research Corporation Technologies. 2012). Another biopharmaceutical approved by FDA in 2009 is Kalbitor® (DX-88 ecallantide), a recombinant kallikrein inhibitor protein produced using *P. pastoris*. It is used as an injection for the treatment of acute attacks of hereditary angioedema in patients aged 16 years or older (Research Corporation Technologies 2020). Other products derived from *Pichia* include recombinant human insulin, Insugen®, produced by Biocon; and the enzyme Phytase (Phytex, USA) used as an animal feed additive to provide phosphate by cleaving plant derived phytate.

Dried *P. pastoris* is an approved food additive for use in broiler poultry feed at up to a 10% inclusion rate as a source of protein (FDA 21CFR573.750 1993).

Based on a comprehensive survey of the scientific literature[3], Clara Foods concludes that there is no publicly available information that indicates or suggests safety concerns of the use of *P. pastoris* as a production organism for food substances.

2.2.2.9 Allergenicity concern

OVD is one of the predominant allergens in egg. Since NSEWP is a fermentation-derived OVD, products containing NSEWP as an ingredient must be labeled as “Contains egg” or other appropriate ingredient descriptor that indicates that the product is derived from an egg source according to FALCPA allergen labelling requirements (FDA 2018).

P. pastoris has a history of safe usage as a food ingredient production organism. The potential allergenicity of a product produced by the *P. pastoris* BG10 host background was evaluated and reported in GRN 737, page 58. The study concluded, and FDA had no objection, that there is no evidence of a risk of allergenicity from the carryover fermentation products of the production strain, *P. pastoris*.

[3] Literature search March 2020

2.2.2.10 Safe Strain lineage

Pariza and Johnson (Pariza and Johnson 2001) recommend that microbial strains used to produce food-grade enzymes have a safe strain lineage. *P. pastoris* DFB-003 meets the following safe strain lineage characteristics:

- A well characterized nonpathogenic, nontoxigenic strain with a safe history of use in food enzyme manufacture
- Safety of all new DNA that has been introduced into the host organism
- Procedures used to modify the host organism are appropriate for food use

2.3 Intended Use

NSEWP produced by *P. pastoris* DFB-003 is intended as a source of dietary protein when manufactured according to cGMP. It can be used as a replacement for plant and animal-derived proteins currently consumed in foods.

The intended uses of NSEWP include but are not limited to protein fortification of conventional foods such as sports drinks, protein supplement powders, and nutritional bars. Such uses will not increase the consumption of protein for the general population.

NSEWP is not intended for use in products regulated under USDA/FSIS jurisdiction or in infant formula.

2.4 Manufacturing, Production and Release Specifications

The NSEWP is prepared in three (3) stages: construction of the production strain of *P. pastoris*, expression of NSEWP in fermentation, and purification of the protein.

All materials used in the production of NSEWP are standard food or pharmaceutical grade ingredients of a purity and quality suitable for the intended use, and processing conditions are appropriate for food production under cGMP as set forth in 21 CFR Part 110 and 117.

2.4.1 Raw Materials

Raw materials used in the fermentation and recovery process for the NSEWP are standard ingredients used in the food/enzyme industry. The specifications

include limits on lead and other pertinent heavy metals. The raw materials are of a purity and quality suitable for the intended use in a food product; they are food grade and GRAS or certified USP or NF or ACS grade. The raw material fermentation ingredients are not major allergens and major allergens are not used in the final product formulation.

2.4.2 Fermentation

The NSEWP was produced in a bioreactor using a *P. pastoris*-based fermentation process. The seed train for the fermentation process began with the thawing of the cryo-stored *P. pastoris* in glycerol seed vials to room temperature. The contents of the thawed seed vials were used to inoculate liquid culture media in the primary fermenter.

The primary fermenter culture was grown at process temperature for a duration long enough to achieve target cell density after which the grown *P. pastoris* primary fermenter culture was transferred to a production scale reactor.

The culture was grown in the production bioreactor at target fermentation conditions and fed a series of substrates in accordance with the developed feed algorithm. At multiple times during the process, the fermentation was analyzed for culture purity.

2.4.3 Purification

The NSEWP was purified by separating the cells from the liquid medium by centrifugation followed by micro-filtration. Further purification is accomplished using pH adjustments and ultra-filtration, before a drying step to produce the final protein product.

Figure 5: Overview of the manufacturing steps for NSEWP

2.4.4 Product release specification

The NSEWP meets the purity specifications set forth in Food Chemicals Codex (FCC) 11th edition (Pharmacopeia 2018). Final product release specifications are listed in Table 3.

The results from testing three (3) lots of the NSEWP product are presented in Table 4 (see Certificates of Analysis, Appendix 3), verifying that the product meets or exceeds FCC (Pharmacopeia 2018) specifications for proteins used as ingredients as listed in the product release specifications in Table 3.

Tables 3 and 4 are presented in the following two pages.

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Table 3. Specification for non-animal soluble egg white protein (NSEWP) produced by *P. pastoris* DFB-003

Physical properties	**Specification**
Source	Yeast fermentation-derived
Appearance	White to off-white amorphous powder
Solubility	Soluble in water

Chemical Properties (in powder as is)	**Specification**	**Method**
Protein	> 75%	AOAC 990.03[1a] AOAC 992.15[1b]
Moisture	Maximum 10.0%	AOAC 925.09[2]
Carbohydrate	Maximum 20%	Calculated
Ash	Maximum 2.0%	AOAC 942.05[3]
Fat by Acid Hydrolysis	< 0.1%	AOAC 954.02[4]
Hg	< 1 ppm	ICP-AES[5]
Pb	< 1 ppm	ICP-AES[5]
As	< 1 ppm	ICP-AES[5]
Cd	< 1 ppm	ICP-AES[5]

Microbial Properties (in powder as is)	**Specification**	**Method**
Standard Plate Count	< 10000 CFU/g	AOAC 990.12[6]
Yeast & Mold	< 100 CFU/g	AOAC 997.02[7]
Salmonella	Not Detected / 25g	AOAC 2003.09[8]
E. coli	Not Detected / 25g	AOAC 991.14[9]
Total coliform	≤ 30 CFU/g	AOAC 991.14[9]

[1a] 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.

[1b] 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.

[2] Association of Official Analytical Chemists. 1995. In Official Methods of Analysis.

[3] J AOAC Int. 2012 Sep-Oct;95(5):1392-7.

[4] 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, 2012.

[5] J. AOAC 2007. (vol. 90) 844-856.

[6] AOAC International. 2005. Aerobic plate count in foods, dry rehydratable film, method 990.12. AOAC International, 17th ed. Gaithersburg, MD.

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

[8] 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.

[9] 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.

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Table 4. Quality control results for three lots of non-animal soluble egg white protein (NSEWP) produced by *P. pastoris* DFB-003

Analysis Parameter	Specification	SOL19303	SOL19317	SOL19351
Protein	> 75%	75.31	75.06	79.94
Protein (% dry weight powder)	> 80%	82.2	82.5	87.8
Moisture and Volatiles	< 10%	8.4	9	9
Carbohydrates, Calculated	< 20%	15.53	15.28	11.06
Ash	< 2%	0.76	0.66	<0.4
Fat by Acid Hydrolysis	< 0.1%	<0.10	<0.10	<0.10
Arsenic (As)	< 1 mg/kg	<0.010	<0.010	<0.010
Mercury (Hg)	< 1 mg/kg	<0.010	<0.010	<0.010
Lead (Pb)	< 1 mg/kg	0.03	0.063	0.168
Cadmium (Cd)	< 1 mg/kg	<0.010	<0.010	<0.010
Aerobic Plate Count	< 10000 CFU/g	<10	<10	<10
Molds	< 100 CFU/g	<10	<10	<10
Yeast	< 100 CFU/g	<10	<10	<10
Salmonella	Not Detected / 25g	Not Detected	Not Detected	Not Detected
Escherichia Coli	Not Detected / 25g	Not Detected	Not Detected	Not Detected
Coliforms	< 10 CFU/g	<10	<10	<10
Absence of source organism from product	Not detected * / mg sample	Not detected	Not detected	Not detected
Absence of encoding DNA from product	Not detected ** / mg sample	Not detected	Not detected	Not detected

* Limit of detection for source organism = 11 CFU/mg sample

** Limit of detection for encoding DNA = 10 femtogram (Appendix 1)

Table 4 demonstrates that the production methods described yield a consistent product that meet the specifications listed in Table 3.

21CFR570.235; Part 3: Dietary Exposure

NSEWP is intended to be used as a source of protein in sports drinks, protein bars, fruit snacks and drinks. The estimated intake from the intended uses is outlined below.

All estimates provided herein are based on market disappearance and "eater only" data as a surrogate for consumer intake survey data and upon a worst-case (and highly unlikely) scenario that NSEWP replaces all current use of other protein sources in protein supplemented food such as milk, whey, soy protein.

It is reasonable to expect that consumers looking for protein supplementation of their diet would choose among the listed foods to supply the additional desired protein and unlikely that consumers would eat all of the protein supplemented products daily. We note specifically that it is likely that the "eater only" data represents high level consumers (90^{th} percentile) and the estimates provided below are considered to be high level consumer estimates.

3.1 Hen egg OVD

The Recommended Daily Allowance (RDA) of protein for adult males in the US is 56g/day (Institute of Medicine 2005). As part of the protein consumption in the US, consumers currently consume hen eggs as a routine component of the daily diet. In addition to consumption of whole eggs, eggs are used in prepared foods to provide flavor, color and texture in addition to processing advantages such as emulsifying, stabilizing, increasing volume and machineability (American Egg Board 2020).

In 2019, approximately 9.9×10^{10} hen eggs were sold in the US or 6.2×10^{11} grams of egg protein introduced into the US market representing an egg consumption of approximately 287 per capita (American Egg Board 2020). An average large whole egg contains 6.25g of protein. This provides 11.2% and 13.6% of the daily recommended protein intake for adult males and females respectively.

Hen egg OVD represents approximately 11% of the protein composition of hen egg white. Therefore, the dietary intake of native hen egg OVD protein is:

$$0.11 \text{ X } 6.25 \text{ g/egg x } (287 \text{ eggs/person/365d}) =$$

0.54 g/person/day

3.2 Protein Ready to Drink Sports Drinks

Protein Ready to Drink products contain 20g of protein per recommended daily serving size (Johns Hopkins Medicine 2020). Therefore, the total NSEWP intake envisioned from the consumption of one serving is:

20 g/person/day

3.3 Protein Bars

Annual per capita consumption of protein bars is estimated to be 1.4 kg or 3.8 g/person/day in 2019 (Mintel Market Sizes 2019). Protein bars can typically contain 20 g of protein per 50 g bar. Therefore, per capita NSEWP consumption resulting from a worst-case scenario where NSEWP replaces all other sources of protein would be:

3.8 g/person/day x (20 g protein / 50 g/bar) =

1.5 g/person/day

3.4 Nutritional Protein

Protein powders and protein shots are recommended to provide 25g of protein per day (Johns Hopkins Medicine 2020).

25 g/person/day

3.5 Gummies/Fruit Snacks

Consumers of gummies/fruit snacks are a small percentage of US consumers. An “eaters only” estimate indicates approximately 1.3 million Americans consumed 5 or more 14 g packs of these products in 2019 (Statistica 2019). These snacks average approximately 23% protein resulting in a protein consumption of:

(5 packs X 14g/pack) = 70 g of product per capita per year (based on eater only data on known consumers and not on the total US population). These snacks average approximately 23% protein resulting in a per capita consumption of protein from this source of:

23% X (70 g / 365 d) =
0.04 g/person/day

3.6 Estimated Maximum Total Daily Dietary Intake

20 g/person/day (sports drinks) + 1.5 g/person/day (protein bars) + 25 g/person/day (nutritional protein) + 0.04 g/person/day (fruit snacks) =

46.5 g/person/day

This dietary intake is a highly conservative and highly unlikely estimation of the intake of NSEWP given that the intake estimate is based on:

- NSEWP replacement of 100% of all protein ingredients in the envisioned foods
- disappearance estimates which are known to be highly conservative as they assume all the food is eaten and does not account for food waste
- "eater only" data which likely represent consumers in the high-level consumer category that seek out protein supplementation
- assumption that consumers will choose all of these foods daily at the recommended levels where it is much more likely that consumers will pick among the various protein supplement options in addition to other protein options in the diet

21CFR570.240; Part 4: Self-limiting Levels of Use

The use of NSEWP is limited by the functionality of the protein in the food matrix, e.g. the solubility of the protein may limit the use in Ready to Drink Sports Drink category.

21CFR570.245; Part 5: Experience Based on Common Use in Food before 1958

While it is obvious that hen egg OVD has been in human food for millennia, the substantially equivalent NSEWP produced in *P. pastoris* was not present in the food supply prior to 1958, and a GRAS determination based upon a common use in food before 1958 does not apply.

21CFR570.250; Part 6: Safety Narrative

6.1 Background

While plant and animal-derived proteins remain an important part of the food industry toolbox, today it is possible to produce such proteins, once only obtained by extraction from plant and animal sources, in higher yield and enhanced purity by virtue of fermentation processes utilizing either bacteria or yeast as production organisms. Many such "microbially-produced" food ingredients have been reviewed by the US FDA and are listed on the FDA GRAS Notice Inventory.

NSEWP produced by *P. pastoris* DFB-003 is an excellent example of a protein that is utilized as a safe and suitable protein nutrient (normally extracted from hen egg) that now may be produced in a microbial production system. The two preparations (native hen egg OVD and NSEWP) contain essentially the same protein but with different primary production processes.

The safe history of use of hen egg OVD itself is well established and is a GRAS ingredient for general use in food processing in the US at levels in accord with cGMP when extracted from hen eggs. The use of avian eggs as food predates recorded history and proteins derived from eggs in food processing have a long history of safe use and are considered GRAS (21CFR170.30(d)). In addition to consumption of whole eggs, eggs used in prepared foods provide flavor, color and texture in addition to processing advantages such as emulsification, stabilization, increasing volume and machinability (American Egg Board 2020).

6.2 Intended Use and Dietary Exposure

NSEWP produced by DFB-003 is intended as a source of dietary protein when manufactured according to cGMP. It can be used as a replacement for plant and animal-derived proteins currently consumed in foods.

In 2019, approximately 9.9 x 10^{10} eggs were sold in the US representing an egg consumption of approximately 287 per capita in 2019 (American Egg Board 2020) or approximately 1800g of egg protein per capita. Egg consumption then comprises about 9% of the 56 g/person/day RDA of protein for adult males (19-50 years old) in the US based on data from the Institute of Medicine (2005). Uses of NSEWP that are envisioned to increase the dietary exposure to NSEWP include but are not limited to protein fortification of conventional foods such as sports drinks, ready to drink protein beverages, protein bars and fruit snacks. The envisioned uses result in a worst-case total consumption of NSEWP of 46.5 g/person/day. This is well within the norms

for protein consumption in the US as represented in the RDA for protein in the diet for an adult male in the US of 56g of protein per day and 46 g/day for adult females.

While the envisioned uses of NSEWP increase the dietary exposure to OVD egg protein in high level consumers of protein fortified foods, egg white proteins are universally recognized as safe based upon consumption of eggs throughout recorded history without any reports of toxicity (see Section 6.3).

In addition, there are no known toxicological limits established for the daily intake of amino acids from protein ingested as food, including egg proteins (Institute of Medicine 2005). Thus, the consumption of NSEWP at the levels envisioned herein do not appear to present a safety concern.

6.3 Regulatory History

While regulations concerning the production of eggs and egg products are in place in a number of jurisdictions, Clara Foods knows of no regulations related to limiting the consumption of eggs or the consumption of food products containing egg components. Reinforcing the justification for the lack of regulatory limitation, FDA states "Proteins derived from egg whites do not raise toxicity concerns because egg whites have been safely consumed by humans as a source of food throughout recorded history without any reports of toxicity (FDA 1998)."

As NSEWP is a novel protein preparation, there is no direct history of regulatory oversight. However, US FDA has reviewed the use of the same *P. pastoris* host background as part of the GRN 00205 and GRN00737 review and had "no questions" concerning the safety of the use. Dry *P. pastoris* is also allowed for use in poultry food at a usage rate of up to 10% (w/w) as described in 21CFR573.750.

6.4 Safety Data

6.4.1 NSEWP substantial equivalence to the native hen egg OVD

NSEWP has been shown to be equivalent to the native hen egg OVD in amino acid sequence, immunoreactivity, digestibility in simulated gastric tests, and ability to inhibit bovine trypsin. NSEWP contains minor modifications to its structure that are not found in the native hen egg-derived preparation leading to a difference in molecular weight and n-terminal amino acid sequence but are not likely to present safety concerns for consumers.

Specifically, two *S. cerevisiae*-derived amino acid variants are added to the N-terminus of the protein as a result of post-translation processing. A protein

sequence safety evaluation of the resulting proteins has demonstrated that the NSEWP amino acid sequence has no significant similarity to known proteinaceous toxins or known allergens other than the expected amino acid sequence equivalence to the native OVD (Pearson and Lipman 1988) (Liu, et al. 2019) (Virulence Factor Database n.d.). Further, the amino acid changes to the OVD sequence are derived from the *S. cerevisiae* that has a safe history of use in food. Thus, the NSEWP structure is highly unlikely to present safety risks that are not present with consumption of hen egg.

Additionally, hen egg OVD is a highly-glycosylated protein in its native form and a variety of glycoforms have been isolated from hen egg (Hwang, et al. 2014) (Zhu, Trinidad and Clemmer 2015). All hen egg OVD glycoforms contain a core structure of the amino acid sequence and a single N-glycan moiety linked to a specific asparagine amino acid. NSEWP differs from the hen egg moiety in that NSEWP has a simplified glycoform (Figure 1) that contains only the identical core hen egg amino acid / N-glycan structure and differs from the hen egg OVD by the absence of the complex carbohydrate glycoforms appended to the core amino acid / N-glycan core. The NSEWP's amino acid / N-glycan structure in the final preparation has been demonstrated to be present in all known glycoforms of the native hen egg OVD by amino acid analysis and glycosylation identification.

Since NSEWP has essentially the same amino acid sequence, amino acid / N-glycan core, gastric digestibility profile and immunoreactivity as the native hen egg OVD, NSEWP is substantially equivalent to the native OVD and thus, enjoys the same safe history of consumption as hen egg protein.

Finally, highly conservative dietary intake estimates for the envisioned uses demonstrate that NSEWP consumption is expected to be well within current norms of protein consumption for adults in the US.

6.4.2 Safety of the production organism

Once the safety of the microbially-produced NSEWP is assured, it is appropriate that changes in the means of production should be evaluated to ensure that no increase in the likelihood of hazard to consumers is created.

Clara Foods has inserted the hen egg OVD gene into a yeast production host organism, *P. pastoris*, that has a safe history of use in the production of proteins for food use, including enzymes. It is this change in the production of OVD that requires the safety evaluation contained in this notice. The following describes the safety evaluation, following the guidelines of Pariza and Johnson (Pariza and Johnson 2001), undertaken to determine with

reasonable assurance that the *P. pastoris*-produced NSEWP is safe for use in food.

Clara Foods has determined by amino acid sequencing, immunoreactivity, glycosylation analysis, and digestibility that NSEWP produced by *P. pastoris* DFB-003 is substantially chemically and structurally equivalent to hen egg OVD. Hen egg OVD has a history of safe use in food, and therefore, the substantially equivalent protein produced by *P. pastoris* DFB-003 is presumed to be safe. Pariza and Johnson advise that if an enzyme product has a history of safe use in food that the "safety of the production strain should be the primary consideration in evaluating enzyme safety" (Pariza and Johnson 2001). While NSEWP is not an enzyme product, it is a protein, and we have followed the Pariza and Johnson decision tree and Delaney *et al* (2008) as a guide to appropriate scientific procedures for the safety determination of the protein.

Pariza and Johnson (2001) clearly state that the safety of the production strain should be the primary consideration in evaluating the safety of expressed proteins, enzymes and other microbial products. Clara Foods has fully characterized *P. pastoris* DFB-003 to ensure that it is a safe and suitable production host.

The *P. pastoris* DFB-003 production host background, *P. pastoris* BG10, is a commercially available, non-toxigenic and non-pathogenic, safe and suitable food production organism that has been reviewed by FDA in GRAS Notices GRN205 and GRN737 for the production of enzyme and protein food ingredients. *P. pastoris* BG10 background complies with the OECD criteria for Good Industrial Large Scale Practice (GILSP) microorganisms (OECD, 1992; OECD, 1993) and meets the criteria for a safe production microorganism as described by Pariza and Foster (1983), Pariza and Johnson (2001), and several expert groups, including the EU Scientific committee for Food, FAO/WHO and the International Food Biotechnology Council. *P. pastoris* BG10 has a safe strain lineage "through which improved strains may be derived via genetic modification either by using traditional/classical or rDNA strain improvement strategies" (IFBC 1990).

Pariza and Johnson (2001) also state that the elements needed to establish a safe strain lineage for the production host include characterization of the host organism, determining the safety of all new DNA introduced into the host, and ensuring that the procedures used to modify the host are appropriate for food use. Clara Foods has rigorously followed the guidance provided in Pariza and Johnson (2001) to establish the safety of the production host *P. pastoris* DFB-003 for the production of NSEWP.

The hen egg OVD gene sequence is a well characterized, contiguous gene sequence and results in the production of OVD in hen eggs. The OVD gene is inserted into the DFB-003 production host in two locations in Chromosome 3.

The microbial production host has been characterized by whole genome sequencing. Sequence analysis demonstrates that *P. pastoris* DFB-003 is identical to *P. pastoris* BG10 background with the addition of well-defined genetic elements added to generate the NSEWP production organism.

P. pastoris DBF-003 was constructed employing well-defined genetic modification techniques using only DNA that is safe for use in the production of food. DNA introduced to the production organism includes the hen egg OVD gene, copies of *P. pastoris* methanol-inducible promoters, and *Saccharomyces cerevisiae* alpha mating factor pre-pro sequence for the expression of heterologous proteins in *P. pastoris*.

An EndoH enzyme / OCH1 fusion protein gene was inserted with the intent to anchor the EndoH enzyme in the membrane of the DBF-003 golgi apparatus and to utilize the EndoH enzyme to remove glycans from *Pichia*-produced recombinant OVD in order to produce the OVD core amino acid / N-glycan structure common to all native OVD proteins. This EndoH enzyme does not have a safe history of use in food, but the gene is obtained from *Streptomyces coelicoflavus* that is a BioSafety level 1 organism. *Streptomyces species* have a safe history in producing clinical antimicrobials. In addition, because the EndoH / OCH1 protein is anchored to the cellular structure via golgi membranes, the novel protein is substantially removed from the final product. The absence of the EndoH/OCH1 fusion protein was confirmed by conducting LC-MS/MS analysis on three lots of the final protein product (NSEWP). In all the samples, LC-MS/MS analysis detected a single small peptide sequence apparently derived from the OCH1-EndoH fusion protein. This peptide sequence, with 12 amino acid residues, was present in very low abundance in the production lots and only detectable using the exquisitely sensitive LC-MS/MS method. The peptide amino acid sequence did not significantly match any known allergen (Goodman, et al. 2016) or bacterial virulence factor (Virulence Factor Database n.d.) (Liu, et al. 2019) using BLAST analysis.

Insertion of the complete hen egg OVD gene and loci of insertion in the host genome have been confirmed. Whole genome sequence analysis confirms that the native OVD gene and the production enhancing DNA cassettes were present in *P. pastoris* DFB-003 at the specific insertion locations in the production host *P. pastoris* DFB-003 and no known potentially hazardous genetic modifications were made in the construction of the production host organism.

Standard recombinant techniques were used to remove antibiotic resistance markers from the production host and standard methods, i.e. a combination of PCR, antibiotic resistance and genome sequencing analysis, were used to demonstrate the absence of antibiotic resistance genes or bacterial origins of replication present in the production host *P. pastoris* DFB-003.

The *P. pastoris* BG10 host background has been used to produce two (2) proteins that are the subject of GRN 00205 and GRN00737 (incorporated herein by reference). Toxicity testing of the preparations indicated no treatment related adverse effects, indicating the safety of the production organism and the target proteins.

Dry *Pichia pastoris* is permitted in poultry feed at an inclusion rate of up to 10% (w/w) (FDA 21CFR573.750 1993). If we assume a feed ration of 1/4 pound or 113 g of ration per broiler hen per day and a harvest weight of 3.2 kg per bird, the daily intake of *Pichia pastoris* would be 3.4 g Pichia/kg body weight/day. While we understand that chickens are not the recommended toxicity test model, the intake of *Pichia pastoris* fed to animals that are destined to be human food at these levels is further evidence of the safety of the organism. A comprehensive literature search for toxicity or pathogenicity of *P. pastoris* did not identify any references that raised safety concerns, thereby supporting the status of the organism as non-toxigenic and non-pathogenic organism that is safe for use in the production of food.

6.4.3 Summary

The non-animal soluble egg white protein (NSEWP) and the production organisms have been shown by scientific procedures to be safe and suitable for use in food production.

The NSEWP produced by *P. pastoris* is substantially chemically and structurally equivalent to hen egg OVD, which is GRAS and has a safe history of use in food. The production host *P. pastoris* DFB-003 has been fully characterized and satisfies the criteria for a safe strain lineage as recommended by Pariza and Johnson (2001). The host background has been used to produce GRAS substances. The wild type organism is also an approved food additive in poultry feed.

Toxicological testing of the host background as notified to FDA in previous GRAS Notices indicate no safety concerns for the use of the production organism and no allergen concerns.

The estimated consumption of NSEWP is well within accepted norms for protein consumption in the US as represented by current RDA. Eggs and egg proteins have been consumed for millennia without any evidence of toxicity, providing further evidence for the safety of NSEWP.

6.5 Manufacturing

Critical to the production of safe and suitable food, manufacturing facilities must meet US regulations for food production. The NSEWP produced by *P. pastoris* meets or exceeds production specifications (Table 3) and is produced in accordance with cGMP as defined in 21CFR110 and 21 CFR117 employing standard food fermentation industry practices. All ingredients used in the production of the NSEWP are GRAS ingredients, approved food additives, and other food-grade materials appropriate for food production. NSEWP is an egg protein and will be labelled as such on foods that contain it; no other major food allergens are used as fermentation raw materials or ingredients in the production of NSEWP.

6.6 Conclusion of the Pariza and Johnson Decision Tree

Pariza and Johnson (2001) provide a peer-reviewed description of the scientific procedures to be followed in determining the safety of microbially-produced proteins. Clara Foods has rigorously followed this guidance in assessing the safety of NSEWP produced by *P. pastoris*. The conclusion of the decision tree is that NSEWP produced by *P. pastoris* is accepted as a safe and suitable food ingredient. The GRAS conclusion is based on the substantial equivalence of the microbially produced NSEWP to the hen egg native OVD that has a history of safe use in food, the demonstrated safety of the production *P. pastoris* strain, and manufacturing processes that meet or exceed food cGMP requirements.

The decision tree, in question and answer format is included below:

1. Is the production strain genetically modified?
Yes. If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
Yes. If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.

3a. Do the expressed enzyme [protein] product(s) which are encoded by the introduced DNA have a history of safe use in food?
Yes. If yes, go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA?

Yes. If yes, go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

Yes. If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?

Yes. If yes, go to 5.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?

Yes. If yes, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

Yes. If yes, the test article is **ACCEPTED**.

6.7 GRAS Conclusion

Clara Foods has concluded by scientific procedures that NSEWP, produced by *P. pastoris,* is Generally Recognized as Safe (GRAS) for use in food when manufactured according to cGMP in accordance with both 21 CFR 170.30 (a) and (b) and is thereby exempt from pre-market approval requirements of the Food, Drug and Cosmetic Act.

An independent panel of experts (the GRAS Panel) was asked to critically evaluate the documentation provided herein. The GRAS Panel concluded that the proposed uses of NSEWP produced by *P. pastoris* are GRAS based on scientific procedures. The GRAS Panel opined that other equally qualified experts would concur with this conclusion (see Expert Panel Report, Appendix 4).

21CFR570.255; Part 7: List of supporting data and information

7.1 List of Figures

7.2 List of Tables

7.3 List of Appendices

Part 8. Bibliography

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Part 9. Appendices

Appendix 1: Standard Operating Procedure and Results for Absence of encoding DNA by PCR

Purpose:
This method is used to internally confirm the absence of transformable DNA in NSEWP final powder samples.

Materials:

- 2X Taq MasterMix from NEB
- Primers appropriate for cPCR and chicken OVD
- For OVD FOR: EAEA_c OVD_For: 5'GAGGCCGAAGCTGCTGAAGTAGACTGCTCAAGATTTC
- For OVD REV: c OVD_Stop_REV: 5' ACATTTACCAAAATGAGACAATGTTAATGTACCATTGCTTTC (The OVD primers have an annealing temp of 56C when using 2x Taq MasterMix)
- OVD transforming DNA (SB204-Ura3)
- PCR water
- 25mM sodium hydroxide diluted in PCR water
- PCR tubes
- Purified rOVD Product (NSEWP)
- Agarose
- SYBR green
- DNA loading dye
- Thermocycler
- Gel electrophoresis system with power supply
- Gel documentation system

Methods:

Dilute the OVD powder to 100mg powder/mL in 25mM sodium hydroxide (this may require extensive vortexing in order to get into solution. After the powder is fully dissolved, transfer 250μL to two new tubes with 250μL of 25mM sodium hydroxide (this will create two new 500μL tubes of 50mg/mL rOVD powder). To 1 of these tubes, add positive control OVD plasmid DNA to get a final concentration of 1ng/μL (this will serve as the positive control for the assay). To make a stock of your OVD plasmid positive controls, make a stock of 250ng/μL of each separately. Then add 2μL of positive control DNA to your spiked control tube of 50mg/mL rOVD powder.

In an 8-tube PCR strip tube aliquot 45uL of un-spiked 50mg/mL rOVD powder in 25mM NaOH into Tubes 2-8. In Tube 1, add 50μL of the 50mg/mL rOVD solution spiked with 1ng/μL OVD plasmid then transfer 5μL from Tube 1 to Tube 2 and mix well. Continue dilutions until the last tube. Be sure to mix very well after transferring the 5μL to each tube to ensure proper mixing. Based on the starting spiked control of 1ng/μL, you will have 1fg of control plasmid in Tube 7 which is near the detection limit of this assay. Final volumes for Tubes 1-7 will be 45μL while Tube 8 will have 50μL. Repeat this for any number of samples you may have. The samples generated in this step is simply for the limit of detection of OVD plasmid DNA in 50mg/mL rOVD product in 25mM sodium hydroxide.

Set up the master mix as seen below for the appropriate number of reactions you will need +10% for volume loss. Load the test sample of 50mg/mL rOVD without spiked DNA in duplicate. For positive control reaction load 1μL of 1ng/μL of OVD plasmid DNA. For negative control, load 1μL of 25mM sodium hydroxide. For each sample Lot of rOVD, there will be a total of 12 tubes in the cPCR test: 50mg/mL rOVD product in duplicate, the 8 dilutions to show limit of detection of plasmid DNA, one positive and one negative control. For all samples, load 1μL into the PCR reaction.

Component	x1	x 15
2X TaqMM	10	15
EAEA_c OVD_FOR 10μM	1	15
cOVD_Stop_REV 10μM	1	15
DNA	1	-
water	7	105
PCR conditions OVD ORF		
Standard cPCR Taq protocol		
95C 3' denature		
95C 30" 30 cycle		
56C 30" 30 cycle		
68C 1’10" 30 cycle		
68C 5'		
4C forever		

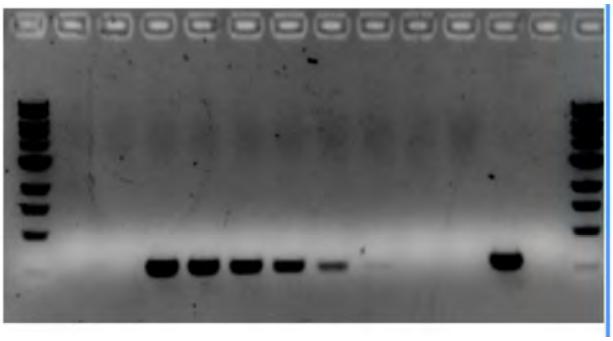
After PCR reactions are finished, add loading buffer and then load 20μL of the sample on a 1% agarose gel and run for 35 minutes at 110V. Product for OVD ORF should be 570bp.

Presence of coding DNA is determined by presence of the respective products showing up on the DNA gel. Limit of detection is around 10 femtogram (fg) of positive control plasmid.

Gel Results for SOL19303:

Lane description is provided below the gel picture (Figure A). Limit of detection is ~10fg of plasmid DNA as observed from the thin band in Lane 9. There is no detectable OVD DNA in the duplicate test samples (Lanes 2 and 3).

Figure A: Gel picture illustrating absence of OVD coding DNA in SOL19303 NSEWP sample (Lanes 2 and 3)



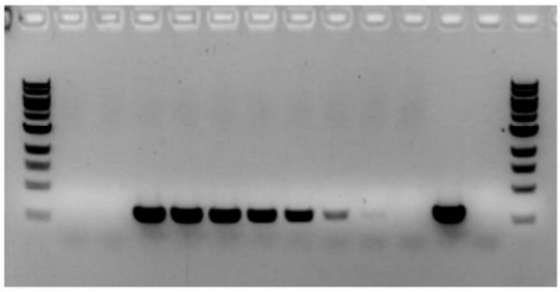
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane #	
1	1kb DNA ladder
2	50mg/mL rOVD **SOL19303**
3	50mg/mL rOVD **SOL19303**
4	1ng OVD plasmid DNA
5	0.1ng OVD plasmid DNA
6	0.01ng OVD plasmid DNA
7	1pg OVD plasmid DNA
8	0.1pg OVD plasmid DNA
9	10fg OVD plasmid DNA
10	1fg OVD plasmid DNA
11	0.1fg OVD plasmid DNA
12	positive control
13	negative control
14	1kb DNA ladder

Gel Results for SOL19317:

Lane description is provided below the gel picture (Figure B). Limit of detection is ~10 femtogram (fg) of plasmid DNA. There is no detectable OVD DNA in the duplicate test samples (Lane 2 and 3).

Figure B: Gel picture illustrating absence of OVD coding DNA in SOL19317 NSEWP sample (Lanes 2 and 3)



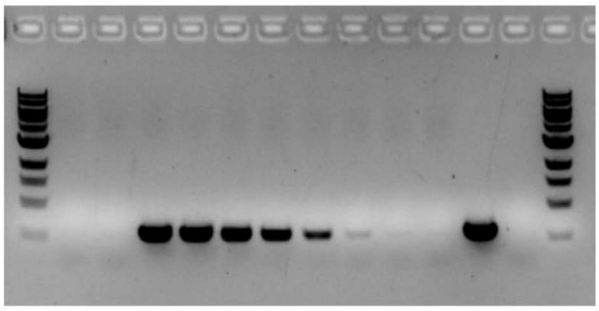
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane #	
1	1kb DNA ladder
2	50mg/mL r OVD **SOL19317**
3	50mg/mL r OVD **SOL19317**
4	1ng OVD plasmid DNA
5	0.1ng OVD plasmid DNA
6	0.01ng OVD plasmid DNA
7	1pg OVD plasmid DNA
8	0.1pg OVD plasmid DNA
9	10fg OVD plasmid DNA
10	1fg OVD plasmid DNA
11	0.1fg OVD plasmid DNA
12	positive control
13	negative control
14	1kb DNA ladder

Gel Results for SOL19351:

Lane description is provided below the gel picture (Figure C). Limit of detection is ~10fg of plasmid DNA. There is no detectable OVD DNA in the duplicate test samples (Lanes 2 and 3.

Figure C: Gel picture illustrating absence of OVD coding DNA in SOL19351 NSEWP sample (Lanes 2 and 3)



Lane# 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane #	
1	1kb DNA ladder
2	50mg/mL r OVD **SOL19351**
3	50mg/mL r OVD **SOL19351**
4	1ng OVD plasmid DNA
5	0.1ng OVD plasmid DNA
6	0.01ng OVD plasmid DNA
7	1pg OVD plasmid DNA
8	0.1pg OVD plasmid DNA
9	10fg OVD plasmid DNA
10	1fg OVD plasmid DNA
11	0.1fg OVD plasmid DNA
12	positive control
13	negative control
14	1kb DNA ladder

Appendix 2: Standard Operating Procedure to determine Absence of Production Organism by Plating

Purpose

- This method internally confirms the presence of the recombinant *P. pastoris* species used to manufacture the Clara Foods product of interest.
- This protocol is only necessary if the internal bioburden check assay resulted in colonies on the PGA plates.
- This protocol should be done for each colony type present on the PGA plate. If there are numerous colonies of each type, select at least 5 of each type to complete the protocol.

Materials

- *Minimal methanol (MM) agar plates*
- *Potato Glucose Agar (PGA) plates*
- *Sterile deionized water*
- *Incubating cabinet*
- *Biosafety cabinet*
- *Inoculum spreading loops*

Procedure

1) Collect partial sample of the colony in question with inoculation loop and streak onto minimal media plate in 2 quadrants.
2) Collect remaining colony in question with inoculation loop and streak onto PGA plate in 2 quadrants.
3) Incubate PGA plates for 48 hours at 30 °C.
4) Incubate *Minimal methanol* plates for 120 hours at 30 °C.
5) If colonies grow on *Minimal methanol* plates within 120 hours at 30C, select single colonies and run colony PCR with cassette specific primers (see PCR method, Appendix 1). If colony PCR confirms presence of production cassette, it can be concluded that the manufacturing organism is present.

Appendix 3: Certificates of Analysis

Certificate of Analysis

Product Name: Non-animal soluble egg white protein (Powder)

Source: Yeast fermentation-derived

Lot #: SOL19303

Physical properties	**Specification**
Appearance	White to off-white amorphous powder
Solubility	Soluble in water

Characteristic (in powder as is)	**Specification**	**Method**	**Result**
Protein	> 75%	AOAC 990.03; AOAC 992.15	75.31
Moisture and Volatiles	< 10%	AOAC 925.09	8.4
Carbohydrates, Calculated	< 20%	Calculated	15.53
Ash	< 2%	AOAC 942.05	0.76
Fat by Acid Hydrolysis	< 0.1%	AOAC 954.02	< 0.10
Arsenic (As)	< 1 mg/kg	ICP-AES	< 0.010
Mercury (Hg)	< 1 mg/kg	ICP-AES	< 0.010
Lead (Pb)	< 1 mg/kg	ICP-AES	0.03
Cadmium (Cd)	< 1 mg/kg	ICP-AES	< 0.010
Aerobic Plate Count	< 10000 CFU/g	AOAC 990.12	< 10
Molds	< 100 CFU/g	AOAC 997.02	< 10
Yeast	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not Detected
Escherichia Coli	Not Detected / 25g	AOAC 991.14	Not Detected
Coliforms	< 10 CFU/g	AOAC 991.14	< 10

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Certificate of Analysis

Product Name: Non-animal soluble egg white protein (Powder)

Source: Yeast fermentation-derived

Lot #: SOL19317

Physical properties	**Specification**
Appearance	White to off-white amorphous powder
Solubility	Soluble in water

Characteristic (in powder as is)	**Specification**	**Method**	**Result**
Protein	> 75%	AOAC 990.03; AOAC 992.15	75.06
Moisture and Volatiles	< 10%	AOAC 925.09	9
Carbohydrates, Calculated	< 20%	Calculated	15.28
Ash	< 2%	AOAC 942.05	0.66
Fat by Acid Hydrolysis	< 0.1%	AOAC 954.02	< 0.10
Arsenic (As)	< 1 mg/kg	ICP-AES	< 0.010
Mercury (Hg)	< 1 mg/kg	ICP-AES	< 0.010
Lead (Pb)	< 1 mg/kg	ICP-AES	0.063
Cadmium (Cd)	< 1 mg/kg	ICP-AES	< 0.010
Aerobic Plate Count	< 10000 CFU/g	AOAC 990.12	< 10
Molds	< 100 CFU/g	AOAC 997.02	< 10
Yeast	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not Detected
Escherichia Coli	Not Detected / 25g	AOAC 991.14	Not Detected
Coliforms	< 10 CFU/g	AOAC 991.14	< 10

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Certificate of Analysis

Product Name: Non-animal soluble egg white protein (Powder)

Source: Yeast fermentation-derived

Lot #: SOL19351

Physical properties	**Specification**
Appearance	White to off-white amorphous powder
Solubility	Soluble in water

Characteristic (in powder as is)	**Specification**	**Method**	**Result**
Protein	> 75%	AOAC 990.03; AOAC 992.15	79.94
Moisture and Volatiles	< 10%	AOAC 925.09	9
Carbohydrates, Calculated	< 20%	Calculated	11.06
Ash	< 2%	AOAC 942.05	< 0.4
Fat by Acid Hydrolysis	< 0.1%	AOAC 954.02	< 0.10
Arsenic (As)	< 1 mg/kg	ICP-AES	< 0.010
Mercury (Hg)	< 1 mg/kg	ICP-AES	< 0.010
Lead (Pb)	< 1 mg/kg	ICP-AES	0.168
Cadmium (Cd)	< 1 mg/kg	ICP-AES	< 0.010
Aerobic Plate Count	< 10000 CFU/g	AOAC 990.12	< 10
Molds	< 100 CFU/g	AOAC 997.02	< 10
Yeast	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not Detected
Escherichia Coli	Not Detected / 25g	AOAC 991.14	Not Detected
Coliforms	< 10 CFU/g	AOAC 991.14	< 10

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Appendix 4: Expert Panel Report

REPORT OF THE GRAS PANEL ON THE *GENERALLY RECOGNIZED AS SAFE* (GRAS) STATUS OF THE INTENDED USES OF NON-ANIMAL SOLUBLE EGG WHITE PROTEIN PRODUCED BY PICHIA PASTORIS

September 2020

GRAS Panel Members

Joseph F. Borzelleca, Ph.D.

Michael W. Pariza, Ph.D.

Advisor to the GRAS Panel

Kevin O. Gillies

Introduction

Clara Food Company ("the company") proposes to use non-animal soluble egg white protein, produced by a genetically-modified strain of *Pichia pastoris*, as a direct replacement for all current food uses of hen egg ovomucoid, including, but not limited to, protein fortification of conventional foods such as sports drinks, protein supplement powders, and nutritional bars. The company convened a panel of independent scientists (the "GRAS Panel"), qualified by their scientific training and national and international experience to evaluate the safety of food ingredients, to conduct an independent and critical evaluation of the available information on the safety of its genetically-modified *P. pastoris* production strain for non-animal soluble egg white protein, and to determine whether the proposed uses of the non-animal soluble egg white protein manufactured using this strain are safe and suitable and *Generally Recognized As Safe* (GRAS) based on scientific procedures. The members of the GRAS Panel were Professors Michael W. Pariza and Joseph F. Borzelleca, with Kevin O. Gilles serving as advisor to the GRAS Panel.

Summary and Basis for GRAS

The GRAS Panel, individually and collectively, critically evaluated a dossier prepared by Clara Foods entitled, "Non-animal soluble egg white protein produced by *Pichia pastoris* Generally Recognized as Safe Notice," dated May 2020 and amended in September 2020. This dossier described (1) the history of safe use of non-animal soluble egg white protein as a food ingredient; (2) the biology of *P. pastoris* and its history of safe use in food manufacture; (3) the non-animal soluble egg white protein production strain DFB-003 and its fully sequenced genome; (4) the cloning methodology that was utilized to create *P. pastoris* DFB-003; (5) protein sequence data showing that the non-animal soluble egg white protein produced by *P. pastoris* DFB-003 is chemically identical to naturally-occurring non-animal soluble egg white protein (i.e., hen egg ovomucoid); (6) the manufacturing process; (7) product specifications; and (8) projected consumer intake. The GRAS Panel participated in a teleconference on May 12, 2020, with Clara Food Company Senior Manager Dr. Kritika Mahadevan and Mr. Gilles, who served as technical advisor to the GRAS Panel.

Following its independent and collective critical evaluation of the publicly available information, the GRAS Panel unanimously concluded that the proposed uses of Clara Food's non-animal soluble egg white protein preparation are safe and suitable and *Generally Recognized As Safe* (GRAS) based on scientific procedures.

Conclusions

We, the GRAS Panel, independently and collectively critically evaluated the information and data summarized above and unanimously conclude that Clara Food's *Pichia pastoris* production strain DFB-003 is safe and suitable for the manufacture of food-grade non-animal soluble egg white protein.

We further conclude that Clara Food's non-animal soluble egg white protein preparation, produced using *Pichia pastoris* DFB-003, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is safe and suitable and *Generally Recognized as Safe* (GRAS) based on scientific procedures for use as a direct replacement for all current food uses of hen egg ovomucoid, including, but not limited to, protein fortification of conventional foods such as sports drinks, protein supplement powders, and nutritional bars.

It is our opinion that other experts qualified to assess the safety of food ingredients would concur with these conclusions.

[redacted]

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

September 4, 2020
Date

[redacted]

Joseph F. Borzelleca, Ph.D.
Emeritus Professor, Pharmacology and Toxicology
Virginia Commonwealth University School of Medicine
Richmond, VA

September 4, 2020
Date

[redacted]

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September 5, 2020
Date