From: Kevin Gillies
To: Kampmeyer, Christopher
Subject: [EXTERNAL] Re: Your submission to the FDA GRAS Notification Program
Date: Saturday, July 31, 2021 5:15:33 PM
Attachments: image002.png
image004.png
image006.png
image008.png
image010.png
image012.png
Replacement_GRAS Notice_Pepsin A Produced by Pichia pastoris final.pdf

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Dear Mr. Kampmeyer:
Thank you for your email and attached FDA letter. We would like to thank you for calling this inconsistency to our attention as we state, erroneously, that the use includes infant formula in some sections and not in others. It was not Clara Foods' intention to include a use in infant formula, which is reflected in the document not mentioning the use in the dietary intake assessmnet and in the safety narrative. My client has made the corrections to the GRAS notice for pepsin A produced by *Pichia pastoris* (attached) to the effect that the intended uses do not include use in infant formula as suggested in your e-mail.

Again, we thank you for the opportunity to correct this error and to move the notice to review once you have verified that the suggested changes have been made. We look forward to your confirmation that the changes to the notice have met the requirements noted.

Should you have further questions or comments, please don't hesitate to contact me.

Best,
Kevin Gilllies

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

Tel:+1 816 590 9836

On Fri, Jul 30, 2021 at 2:53 PM Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Dear Mr. Gillies:
>
> Attached please find a letter regarding the filing status of your submission on behalf of Clara Foods, Inc. to the GRAS notification program for pepsin A produced by *Pichia pastoris*. Unfortunately, we were not able to file the submission as a GRAS notice (GRN) for reasons that are explained in the attached letter. In addition, we noted that the type of infant formula

was not specified and there was a lack of safety discussion related to infants. However, given that the filing team is already familiar with this submission, if we receive a revised version that excludes the intended use in infant formula, it would be a straightforward process for us to simply confirm that the issue with the present submission was fixed and then move the notice on to a review team.

Best regards,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

Pepsin A produced by *Pichia pastoris*

Generally Recognized as Safe Notice

Submitted by:

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

November 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.
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South San Francisco, CA 94080
USA

Table of Contents

21 CFR 170. 225; Part 1: Signed Statements and Certification

1.1 Exemption Claim for Pepsin A 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 pepsin A produced by *P. pastoris* as an enzyme processing aid 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 Pepsin A 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
Suit 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

Pepsin A produced by *P. pastoris* DFB-002 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

Pepsin A produced by *P. pastoris* DFB-002 is intended as a direct replacement for processing aid uses of porcine Pepsin A (EC 3.4.23.1) as an enzyme for use in food in accordance with GMP as described in 21CFR184.1595. The estimated dietary intake from the use is 8 mg TOS/kg bw/day. Fermentation-derived Pepsin produced by *P. pastoris* uses do not include infant formula or products regulated under USDA/FSIS jurisdiction.

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 further 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 Pepsin A produced by *P. pastoris* DFB-002.

1.8 Signature of Responsible Party or Agent

Kevin O. Gillies
Kevin O. Gillies Consulting Services, LLC
October 20, 2020

21 CFR 170.230; Part 2: Identity, Manufacturing, Specifications, Use

2.1 Identity of the Substance

- Common and Usual Name of the Substance:

  Pepsin A;
  Pepsin from yeast;
  Fermentation-derived Pepsin

  For the purpose of this document, the microbially derived Pepsin A produced by *P. pastoris* DFB-002 will be called Fermentation-derived Pepsin.

- Chemical name: Peptide hydrolase, aspartic protease

- CAS number: 9001-75-6

- IUB number: 3.4.23.1

- LCMS amino acid composition equivalence to porcine Pepsin A:

  Pepsin A produced by *P. pastoris* DFB-002 (Fermentation-derived Pepsin) was compared to porcine Pepsin A 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 Fermentation-derived Pepsin and porcine Pepsin. The protein samples were first digested into peptides using endoproteinase GluC and chymotrypsin, in parallel, to get improved cleavage of Pepsin A. The peptides produced were analyzed through LC-MS/MS. The resulting spectra were matched to peptide sequences using the software tool, X!tandem (https://proteomics.ucdavis.edu/protein-identification/). Fermentation-derived Pepsin was an exact match to the mature form of *Sus scrofa* (Porcine) Pepsin A, consisting of 326 amino acids as detailed below.

  Sus scrofa Pepsin A (P00791|PEPA_Pig)

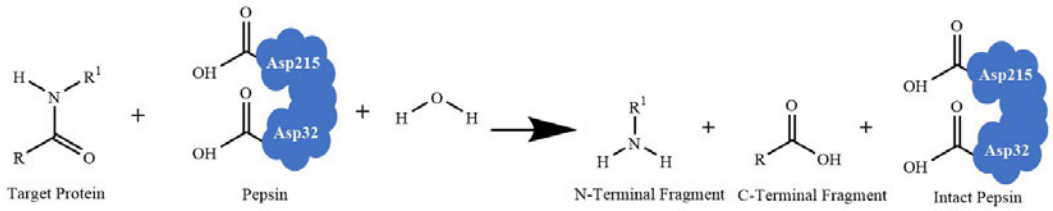
  IGDEPLENYL DTEYFGTIGI GTPAQDFTVI FDTGSSNLWV
  PSVYCSSLAC SDHNQFNPDD SSTFEATSQE LSITYGTGSM
  TGILGYDTVQ VGGISDTNQI FGLSETEPGS FLYYAPFDGI
  LGLAYPSISA SGATPVFDNL WDQGLVSQDL FSVYLSSNDD

SGSVVLLGGI DSSYYTGSLN WVPVSVEGYW QITLDSITMD
GETIACSGGC QAIVDTGTSL LTGPTSAIAN IQSDIGASEN
SDGEMVISCS SIDSLPDIVF TINGVQYPLS PSAYILQDDD
SCTSGFEGMD VPTSSGELWI LGDVFIRQYY TVFDRANNKV GLAPVA

- Enzyme Activity:

  Pepsin A catalyzes the acid hydrolysis of peptide bonds between hydrophobic or aromatic residues of a protein substrate. Bonds such as Phe-Phe, Phe-Trp, and Phe-Tyr are commonly hydrolyzed (Kageyama, et al. 2009).

Figure 1: Reaction mechanism of Pepsin A

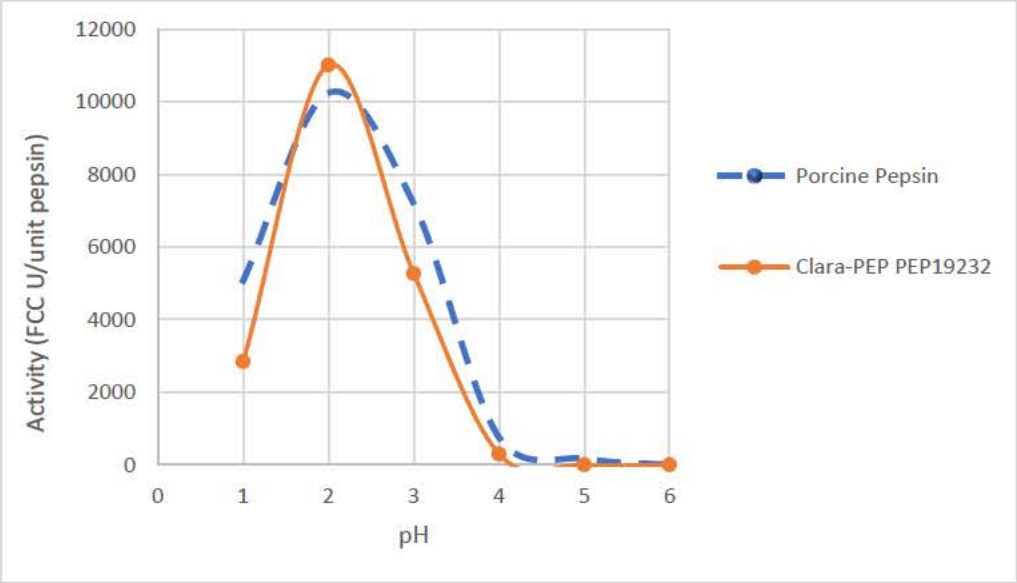


In order to ensure that Fermentation-derived Pepsin produced by *P. pastoris* DFB-002 is substantially equivalent to native porcine Pepsin A, the two protein preparations were compared by molecular weight, immunoreactivity and enzyme activity profile.

Fermentation-derived Pepsin was tested for activity against a range of pH and compared against the activity of porcine pepsin using the FCC (9th Edition) Pepsin assay (Pharmacopeial Convention. 2014). The optimum activity was at pH 2 for both porcine pepsin and Fermentation-derived Pepsin.

Both pepsin enzymes tested had a similar activity profile (Figure 2). The pepsin activity in each sample was presented as FCC units / unit pepsin, wherein each Pepsin unit is defined as the amount of pepsin present in the sample, derived from its peak area determined through HPLC.

Figure 2: Pepsin activity (FCC U / unit pepsin) profile based on pH

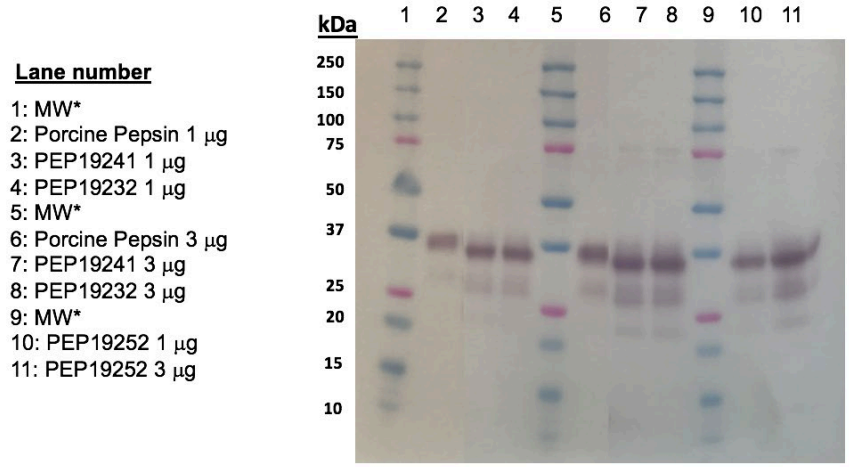


- Immunoreactivity characteristics:

  Fermentation-derived Pepsin from three product Lots (PEP19232, PEP19241, PEP19252) showed the same immunoreactivity as the native porcine pepsin (Figure 3). Western Blot was performed on the samples using primary pepsin antibody from rabbit (Abcam (ab182945)) at a 1:5000 dilution (Jensen 2012). The secondary antibody used was goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 dilution).

  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.

Figure 3: Western Blot of porcine Pepsin and Pepsin A produced by *P. pastoris* DFB-002 demonstrating equivalence in molecular weight and immunoreactivity



* Lane 1, 5 and 9 are Precision Plus Standard (dual color) from BioRad (#161-0374).

- Molecular weight characteristics:

  Although there is a slight difference in migration of Fermentation-derived Pepsin compared to a commercial preparation of Pepsin A in the Western Blot gel, because the LCMS analysis indicates that the Fermentation-derived Pepsin enzyme has the identical amino acid composition as porcine Pepsin A, it is likely that the slight difference in migration observed is an artifact due to protein concentration and buffer salt differences. Thus, the gel data supports the conclusion that Fermentation-derived Pepsin has the same molecular weight as porcine Pepsin A (Figure 3).

  The results of the molecular weight, immunoreactivity and enzyme activity determinations confirm that Fermentation-derived Pepsin is substantially equivalent to the GRAS affirmed ingredient (21CFR184.1595) porcine Pepsin A.

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-002 was constructed from recipient strain DFB-001 using transformations with different expression constructs in order to express porcine Pepsinogen A. Subsequent process steps convert Pepsinogen A to Pepsin A. In addition to the protein coding sequence for pepsinogen, Strain DFB-002 also contains three extra copies of the *P. pastoris* transcription factor HAC1, 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-002 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) (OECD 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-002: Strain Overexpressing Pepsinogen and HAC1

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 by several yeast (Guerfal, et al. 2010), (Gasser, et al. 2006). The addition of a methanol-inducible HAC1 leads to improved production of Pepsinogen by *P. pastoris*. Pepsin A, a gastric protease, is initially expressed in animals as a Pre-pro-mature protein where the pre-sequence directs the pro-mature form of the protein to be secreted out of the cell. The pro-mature form is inactive until it reaches the low pH of the stomach where it then self-processes, removing the pro sequence and becomes an active protease. The pro-mature form is referred to as Pepsinogen. The *Saccharomyces cerevisiae* (*S. cerevisiae*) alpha mating factor pre-pro sequence ('ScPrePro') is a common secretion signal for directing secretion of heterologous proteins in *P. pastoris.*

The protein coding sequence for the *Sus scrofa* (domestic pig) pepsinogen was synthesized as a fusion sequence with the *S. cerevisiae* alpha factor pre-pro sequence for expression in *P. pastoris*.

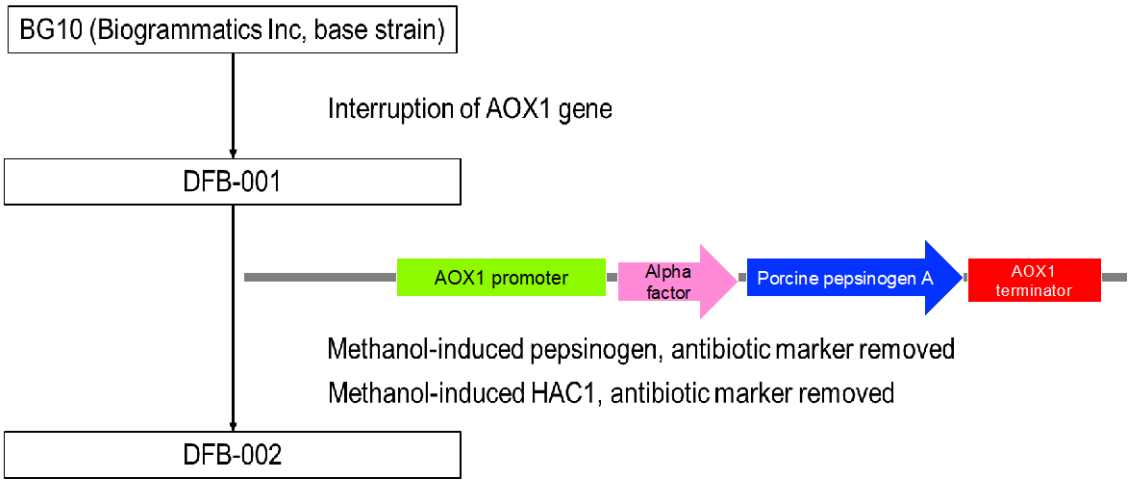
Linear cassettes of methanol - inducible promoter :: ScPrePro :: Pepsinogen :: AOX1term and a linear cassette of DNA containing a HAC1 gene under the control of methanol inducible promoters such as the pAOX1 promoter and AOX1 terminator was introduced into Strain DFB-001 using standard electroporation methods (Lin-Cereghino, et al. 2005).

PCR analysis and protein expression assays identified the production strain, Strain DFB-002 which contains five copies of the ScPrePro :: Pepsinogen :: AOX1term gene and three additional copies of the HAC1 gene.

As antibiotic markers are present on the plasmid vector used to insert target genes into the DFB-001 chromosome to produce DFB-002, loop-out techniques were used to remove the antibiotic marker prior to use of DFB-002 as a production host.

A combination of PCR, antibiotic resistance and genome sequencing analysis were 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-002.

Figure 4: Construction of production strain using recipient strain DFB-001



2.2.2.3 Genome Sequence of the Production Strain DFB-002

The genome of production strain DFB-002 has been completely sequenced and confirmed to contain the following sequences in addition to the backbone *P. pastoris BG10* DNA.

- 5 copies of pepsinogen coding sequence were integrated into the genome as cassettes of methanol-inducible promoter :: ScAlphaFactor :: Pep :: 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)

Sequencing and data analysis methods:

Standard Illumina library was prepared according to vendor's specification (https://www.illumina.com/techniques/sequencing/ngs-library-prep.html). 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-002 was 79,346,263 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 29,129 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-002 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-002 are stably integrated in the genome and confirmed to be present after forty-five (45) generations of growth on non-selective growth media. 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-002 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 Fermentation-derived Pepsin product lots contained Pepsinogen A transformable DNA. The absence of transformable Pepsinogen A 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' Pepsin A preparation. The level of detection of the PCR analysis was established to be ~1 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-002 Production Organism in the Final Product

The DFB-002 production organism is not detected in Fermentation-derived Pepsin samples in accordance with the recommendation of safety evaluation by the International Food Biotechnology Committee (Coulston, Kolbye and Carr 1990). Procedure to ascertain absence of DFB-002 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 Potential Toxicants

The Fermentation-derived Pepsin production strain and manufacturing process 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 Fermentation-derived Pepsin. 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, produced by *Pichia pastoris*, received a FDA "no questions" letter 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[1], 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 and no publicly available information that suggests safety concerns related to the ingestion of Pepsin A when used as an enzymeprocessing aid in food manufacturing.[2]

2.2.2.8 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-002 meets the following safe strain lineage characteristics:

[1] Literature search March 2020
[2] Literature search July 2020

- 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

2.2.2.9 Allergenicity concern

Clara Foods knows of no issues of allergenicity related to the consumption of Pepsin A used as an enzyme in food production. In addition, *P. pastoris* has a history of safe usage as a food ingredient production organism. Extensive studies have been carried out on a product produced by the BG10 host background to identify potential allergenicity (GRN 737, page 58). The study concluded that there is no evidence of a risk of allergenicity from the carryover fermentation products of the production strain, *P. pastoris*.

Even though the Pepsin A preparation does not present allergenicity risks to consumers when ingested, there are well known and understood issues of sensitization when individuals are exposed to high concentration of protease powders such as Pepsin A in the manufacturing environment. Adequate precautions should be exercised by the manufacturer and individuals handling these protease powders to prevent dermal and inhalation exposure during large scale production (Pariza and Foster 1983).

2.3 Intended Use

Pepsin A produced by *P. pastoris* DFB-002 is intended as a direct replacement for processing aid uses of porcine pepsin A (EC 3.4.23.1) as an enzyme as defined in 21CFR 170.3(o)(9) for use in food in accordance with GMP as described in 21CFR184.1595 and 21CFR184.1(b)(1).

Such uses include but are not limited to the modification of food proteins to enhance their texture and organoleptic properties as well as addition to enzyme preparations such as rennet to aid in the production of cheese. Fermentation-derived Pepsin produced by *P. pastoris* uses do not include infant formula or products regulated under USDA/FSIS jurisdiction.

2.4 Manufacturing, Production and Release Specifications

The Fermentation-derived Pepsin is prepared in four (4) stages: construction of the production strain of *P. pastoris*, expression of pepsinogen A in fermentation, conversion of Pepsinogen A to Pepsin A and purification of the protein.

All materials used in the production of pepsin 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 Fermentation-derived Pepsin 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

Recombinant Pepsin A was produced in a bioreactor using a *P. pastoris*-based fermentation process. The seed train for the fermentation process began with the thaw 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.

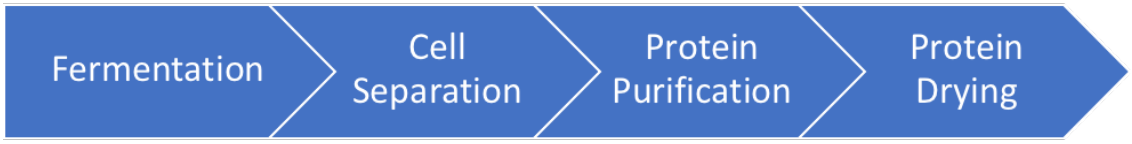
The primary fermenter culture was grown at process temperature for a duration long enough to achieve target cell density after which the grown *P. pastoris* primary fermenter culture was transferred to a production scale reactor.

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

Figure 5: Overview of the manufacturing steps for recombinant pepsin



2.4.4 Product release specification

The Fermentation-derived Pepsin meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex (FCC) 10th edition (Pharmacopeia 2016). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives i.e. JECFA (FAO/WHO 2006). Final product release specification is listed in Table 1.

The results from testing three (3) lots of Fermentation-derived Pepsin product is given in Table 2 (see Certificates of Analysis, Appendix 3), verifying that the product meets or exceeds FCC (Pharmacopeia 2016) and JECFA (FAO/WHO 2006) specifications for enzyme preparations as listed in the product release specifications in Table 1.

Tables 1 and 2 are presented in the following two pages.

Table 1. Specification for Pepsin A produced by *P. pastoris* DFB-002

Physical properties	**Specification**
Source	Yeast fermentation-derived
Appearance	White to off-white amorphous powder
Solubility	Mostly soluble in water with slight opalescence. Practically insoluble in alcohol, chloroform and ether.

Enzyme Activity	**Specification**	**Method**
Activity in Units/mg powder	1:30000 FCC Units	FCC Assay[1]

Chemical Properties (in powder as is)	**Specification**	**Method**
Moisture	Maximum 10.0%	AOAC 925.09[2]
Ash	Maximum 5.0%	AOAC 942.05[3]
Hg	< 1 ppm	ICP-AES[4]
Pb	< 1 ppm	ICP-AES[4]
As	< 1 ppm	ICP-AES[4]
Cd	< 1 ppm	ICP-AES[4]

Microbial Properties (in powder as is)	**Specification**	**Method**
Standard Plate Count	< 10000 CFU/g	AOAC 990.12[5]
Yeast & Mold	< 100 CFU/g	AOAC 997.02[6]
Salmonella	Not Detected / 25g	AOAC 2003.09[7]
E. coli	< 10 CFU / g	AOAC 991.14[8]
Total coliform	≤ 30 CFU/g	AOAC 991.14[8]

[1] Food Chemical Codex, 9th ed. (Pharmacopeial Convention. 2014)

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

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

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

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

[6] 17.2.09 AOAC Official Method 997.02. Yeast and Mold Counts in Foods Dry Rehydratable Film Method (Petrifilm™ Method) First Action 1997 Final Action 2000

[7] 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.

[8] 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.

Table 2. Quality control results for three lots of Pepsin A produced by *P. pastoris* DFB-002

Parameter	Specification *	PEP19232	PEP19241	PEP19252
Activity (FCC Units / mg powder)	1:30000	1:31440	1:31000	1:32200
Moisture	< 10%	9.4	9.1	9.6
Ash %	< 5%	3.54	3.79	3.61
TOS %	Not specified	87.1	87.1	86.8
Hg	< 1 mg/kg	< 0.01	< 0.01	< 0.01
Pb	< 1 mg/kg	< 0.01	< 0.01	< 0.01
As	< 1 mg/kg	< 0.01	< 0.01	< 0.01
Cd	< 1 mg/kg	< 0.01	< 0.01	< 0.01
Aerobic plate count	< 10000 CFU/g	< 10	< 10	< 10
Yeast & Mold	< 100 CFU/g	< 10	< 10	< 10
Salmonella	Not Detected/25g	Not detected	Not detected	Not detected
E. coli	< 10 CFU/g	< 10	< 10	< 10
Total coliforms	≤ 30 CFU/g	Not detected	Not detected	Not detected
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

* Specification for purity based on FCC (2016) and JECFA (FAO/WHO 2006)

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

Total organic solids (TOS) values were calculated using the formula:
% TOS = 100 % - (% Ash + % Moisture + % Diluents) as recommended by JECFA. The 3 samples do not contain any diluents.

21 CFR 170.235; Part 3: Dietary Exposure

Pepsin A produced by *P. pastoris* DFB-002 is intended as a direct replacement for porcine-derived pepsin A currently in the US food supply with the exceptions that fermentation-derived Pepsin is not intended for use in infant formula or products regulated under USDA/FSIS jurisdiction. Thus, the Estimated Dietary Intake (EDI) of fermentation-derived pepsin is based upon the EDI for porcine Pepsin A as described below. As such, Clara Foods does not envision an increase in the dietary exposure to US consumers as a result of the change of production technology.

Dietary exposure to porcine Pepsin A data is not readily available since the use as a processing aid does not lead to labeling of the use on foods and the processes that employ Pepsin A are, for the most part, proprietary. Because of the lack of publicly available information on the current usage of porcine Pepsin A, Clara Foods Co. has relied upon application technology publications to provide estimates of the usage rates in known applications.

Table 3. Estimated application rates of porcine Pepsin A in food.

Application	Raw Material (RM)	Typical Pepsin usage levels	Maximum Recommended Use Level (mg TOS/kg Raw Material)
Cheese	Milk	1.56mg / 100g milk[3]	14[#]
Vegetable Protein Hydrolysis	Wheat, pea protein	1.2 g / 100 g protein[4]	10
Beer Stabilization	Beer	5g in 31 Gallon Beer i.e. 4mg/100mL[5]	34

[#] usage rate was normalized to 10,000 USP/mg powder activity, 85% TOS.

The estimates for consumption of foods which have been subjected to porcine Pepsin A hydrolysis are likely to be very high compared to current exposure because the application data for the use of porcine Pepsin A predates the development of microbially produced proteases that have come to dominate the protease food enzyme market. From a technology perspective, the usage rate as a coagulant in cheese production assumes that 100% of the coagulant is pepsin, which is not the case, as most often the dominant protease in cheese

[3] Merker, H.M. (1919). J. Dairy Sc. Vol. 2 (6), p. 482-486
[4] Crévieu-Gabriel et al. (1999). Reprod. Nutr. Dev. Vol 39, p. 443-453
[5] Wallerstein, L. (1937) Process of chill proofing and stabilizing beers and ales. United States Patent 2077448

manufacture is chymosin to which pepsin may be added for use in traditional or artisanal cheeses. The removal of whey from the curd in cheese making is commonly known to remove the majority of water-soluble proteins including coagulants, thus further limiting consumer exposure to the enzyme.

Clara Foods has chosen the usage rate for vegetable protein hydrolysis for the calculation of the EDI as this use is most likely to result in consumer exposure to the enzyme. The use as a coagulant in cheese production leads to the loss of coagulant and other soluble proteins in the cheese whey with very little carry over into the final cheese product. Similarly, the use of proteases for chill proofing beer often involves the addition of the protease to the wort for breaking down large proteins that may influence the clarity (chill hazing) of the final product. As the wort is cooked and filtered to remove solids, including precipitated proteins coagulated by cooking, pepsin is not expected to be carried over into the final product.

In order to calculate the EDI for fermentation-derived pepsin and recognizing the difficulty in determining a meaningful estimate of consumption of pepsin from the food supply today, it is assumed that the food consumed is hydrolyzed vegetable protein and the protein hydrolysate is consumed at the average Recommended Daily Allowance (RDA) value for consumption of protein by adult males of 0.8g/kg bw/day[6]. Thus, the inclusion rate of pepsin in all protein is 1.0 g TOS / 100 g of protein or (0.01 g TOS/g protein).[7] Thus, with these assumptions, the EDI on a TOS basis for Clara Foods fermentation-derived pepsin is calculated to be:

0.8 g protein/kg bw/day x 0.01g TOS/g protein = **0.008g TOS/kg bw/day**

It is obvious that this EDI calculation is highly exaggerated based upon the following assumptions:

- All protein consumed in the US is enzyme hydrolyzed protein;
- All protein consumed in the US is hydrolyzed by fermentation-derived pepsin;
- All producers of hydrolyzed protein use the highest usage rate of pepsin in the production of hydrolyzed protein;
- The amount of TOS does not decrease as a result of the food production process.

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[6]. Institute of Medicine. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. Washington, DC: National Academies Press; 2002

[7] (1.0 g/g raw material) X 85% TOS

21 CFR 170.240; Part 4: Self-limiting Levels of Use

The usage levels of Pepsin A produced by *P. pastoris* DFB-002 is self-limiting as there may be a negative impact on product quality as use levels increase above those required for its catalytic function.

21 CFR 170.245; Part 5: Experience Based on Common Use in Food before 1958

The conclusion that Pepsin A produced by *P. pastoris* DFB-002 is GRAS under the conditions of use is based upon scientific procedures and not on experience based on common use in food before 1958.

21 CFR 170.250; Part 6: Safety Narrative

6.1 Background

The use of enzymes in food processing has a long history pre-dating the industrial age when calf stomachs were used to make cheese, taking advantage of the proteases and lipases associated with the stomach lining to coagulate the milk. Once the reactive agents (enzymes) were identified in the various animal and plant sources, the enzymes could be extracted from the sources and used in their more purified form, e.g. rennet extracted from calf stomachs. While plant and animal extracted enzymes remain an important part of the enzyme industry, today it is possible to produce enzymes, 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 so-called "microbial-produced enzymes" have been reviewed by the US FDA and are listed on the FDA GRAS Notice Inventory.

Pepsin A produced by *P. pastoris* DFB-002 is an excellent example of an enzyme that was once utilized as an enzyme extracted from animal tissue and now produced in a microbial production system. The two preparations produce the same enzyme but with different enzyme production processes.

The safe history of use of Pepsin A itself is well established and is a GRAS affirmed ingredient for general use in food processing in the US at levels in accord with cGMP when extracted from porcine stomachs. Given the general recognition of the safety of Pepsin A, once the identity of the fermentation-derived Pepsin enzyme to its porcine counterpart is assured, it is appropriate that changes in the production of the enzyme should be evaluated to ensure that no increase in the likelihood of hazard to consumers is created.

Clara Foods has inserted the porcine Pepsinogen A 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 Pepsin A 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 Pepsin A is safe for use in food.

6.2 Intended Use and Dietary Intake

Pepsin A produced by DFB-002 is intended as a direct replacement for uses of porcine pepsin A (EC 3.4.23.1) as an enzyme as defined in 21CFR 170.3(o)(9) for use in food in accordance with GMP as described in 21CFR184.1595 and 21CFR184.1(b)(1).

Such uses include but are not limited to the modification of food proteins to enhance their texture and organoleptic properties as well as addition to enzyme preparations such as rennet to aid in the production of cheese. The uses do not include infant formula or USDA regulated products.

The Estimated Dietary Intake (EDI) of the enzyme under the intended use is 8 mg TOS/kg bw/day. As described below (Section 6.3.1), the EDI, even though it is understood to be a highly exaggerated estimate, is in accordance with 21 CRF 184.1595.

6.3 Regulatory History

Porcine Pepsin A has a substantial history of safe use in food as an enzyme preparation. The following are examples of regulatory approvals for the use in food including dietary supplements:

6.3.1 US

Porcine pepsin preparation EC 3.4.23.1 is a GRAS affirmed ingredient (21 CFR 184.1595) obtained from pig stomach. In accordance with 21CFR184.1(b)(1), the ingredient is used in food with no limitation other than cGMP so long as the ingredient is used as an enzyme as defined in 21CFR170.3(o)(9).

In addition to the uses in food production as an enzyme preparation, porcine pepsin A is commonly sold in the US as a dietary ingredient digestive enzyme preparation at various recommended intake levels per serving, for example, 42 mg Pepsin per serving, at 15000 U/mg activity[8] (approximately 63 mg per serving, if normalized to 1:10000 U/mg activity).

6.3.2 Canada

Canadian food regulations allow for the use of porcine pepsin A in a variety of food applications as shown in Table 4(below). In addition, porcine Pepsin A is approved for use as a digestive enzyme (meeting FCC edition 9 specifications) for oral administration in adults eighteen years and older in Natural Health Products alone or in combination with other approved

[8] https://vitalnutrients.net

ingredients (Health Canada 2019). The Licensed Natural Health Products database lists 114 products containing pepsin either as a single ingredient or in combination with other approved ingredients (Health Canada 2018).

Table 4. List of Permitted Food Enzymes (List of Permitted Food Additives)[#]

Additive	Permitted source	Permitted in or upon	Maximum level of use and other conditions
Pepsin	Glandular layer of porcine stomach	(1) Ale; Beer; Light beer; Malt liquor; Porter; Stout	(1) Good Manufacturing Practice
		(2) Cheddar cheese; Cottage cheese; Cream cheese; Cream cheese spread; Cream cheese spread with (naming the added ingredients); Cream cheese with (naming the added ingredients); (naming the variety) Cheese	(2) Good Manufacturing Practice
		(3) Defatted soya flour	(3) Good Manufacturing Practice
		(4) Pre-cooked (instant) breakfast cereals	(4) Good Manufacturing Practice
		(5) Hydrolyzed animal, milk and vegetable proteins	(5) Good Manufacturing Practice

[#] (Health Canada, List of Permitted Food Enzymes (Lists of Permitted Food Additives) 2019)

6.4 Safety Data

FDA has affirmed the safety of porcine-produced Pepsin A in GRAS Regulation 21CFR184.1595 for use as an enzyme in food production. Pepsin A produced by *P. pastoris*, as described in this GRAS Notice, is produced from the safe and suitable yeast production strain *P. pastoris* DFB-002 expressing the un-modified porcine Pepsin A gene.

Clara Foods has determined by molecular weight, amino acid composition, immunoreactivity and enzyme activity that Pepsin A produced by *P. pastoris* DFB-002 is substantially equivalent to porcine-Pepsin A and, as porcine-derived Pepsin A has a safe history of use in food, the identical enzyme Pepsin A produced by *P. pastoris* DFB-002 is also presumed to be safe. Pariza and Johnson advise that if the enzyme product has a safe history of use in food that the "safety of the production strain should be the primary consideration in evaluating enzyme safety" (Pariza and Johnson 2001).

Following the guidance of Pariza and Johnson that the safety of the production strain should be the primary consideration in evaluating the safety of Fermentation-derived Pepsin once the safety of the expressed enzyme is assured, Clara Foods has fully characterized *P. pastoris* DFB-002 to ensure that it is a safe and suitable production host.

The *P. pastoris* DFB-002 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. In addition, *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, Pariza and Johnson, and several expert groups, including the EU Scientific committee for Food, FAO/WHO and the International Food Biotechnology Council. As such, *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 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 to establish the safety of the production host *P. pastoris* DFB-002 for the production of Pepsin A preparation.

The porcine Pepsinogen A gene sequence is a well characterized, contiguous gene sequence and produces Pepsin A in the porcine stomach. The porcine Pepsinogen A gene is inserted into the DFB-002 production host in one location in Chromosome 1.

The microbial production host has been characterized by whole genome sequence. Sequence analysis demonstrates that *P. pastoris* DFB-002 is identical to *P. pastoris* BG10 background with the addition of well-defined genetic elements added to generate the pepsin A production organism.

P. pastoris DBF-002 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 porcine Pepsinogen A 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*. All gene cassettes have a safe history as they are derived from safe and suitable food production organisms.

Insertion of the complete porcine pepsinogen A gene and loci of insertion in the host genome have been confirmed. Whole genome sequence analysis confirms that Pepsinogen A and the production enhancing DNA cassettes were present in *P. pastoris* DFB-002 at the specific insertion locations in the production host *P. pastoris* DFB-002 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-002.

In summary, Pepsin A produced in *P. pastoris* is identical to porcine-derived Pepsin A, which is GRAS and has a safe history of use in food. Clara Foods knows of no publicly available information that indicates any safety concerns related to the uses of microbial-produced Pepsin A in food processing under the anticipated conditions of use. Further, *P. pastoris* DFB-002 has been fully characterized and satisfies the criteria for a safe strain lineage as recommended by Pariza and Johnson (2001) and is thereby a safe and suitable host for the production of Pepsin A.

6.5 Manufacturing

Critical to the production of safe and suitable food, manufacturing facilities must meet US regulations for food production. Pepsin A produced by *P. pastoris* meets or exceeds FCC specifications for pepsin activity and is produced in accordance with cGMP as defined in 21CFR110 and 21 CFR117 employing standard enzyme fermentation industry practices. Further, all ingredients used in the production of the enzyme preparation are GRAS ingredients, approved food additives and other food-grade materials appropriate for food production. No major food allergens are used as fermentation raw materials or ingredients in the final product formulation.

6.6 Conclusion of the Pariza and Johnson Decision Tree

Clara Foods had rigorously followed the guidance of Pariza and Johnson (Pariza and Johnson 2001) in assessing the safety of Pepsin A produced by *P. pastoris*. The conclusion of the decision tree is that Pepsin A produced by *P. pastoris* is accepted as a safe and suitable food ingredient. In general, the GRAS conclusion is supported by the equivalence of the microbially produced Pepsin A enzyme to the porcine Pepsin A that has a safe history of use in food, 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 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 Pepsin A produced by *P. pastoris* is Generally Recognized as Safe (GRAS) for use in food as an enzyme processing aid 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 was asked to review the documentation provided herein, and the panel concurred that Pepsin A produced by *P. pastoris* is GRAS for the intended uses and that other experts in the field were likely to agree with the panel's conclusion (see Expert Panel Report, Appendix 4).

21 CFR 170.255; 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 Fermentation-derived Pepsin enzyme preparation.

Materials:

- 2X Taq MasterMix from NEB
- Primers appropriate for cPCR and the POI (here pepsinogen A ORF):
- For PEP FOR: Pep_EAEA_For1: 5'GAAGCTGAAGCTCTAGTAAAGGTGCCTCTAG
- For PEP REV: Pep_Stop_Rev1: 5' TGCAACAGGTGCTAGACCCACCTTGTTGTTAG (The PEP primers have an annealing temp of 58C when using 2x Taq MasterMix)
- Pepsinogen transforming DNA (PGA401-Z)
- PCR water
- 25mM sodium hydroxide diluted in PCR water
- PCR tubes
- Purified Pepsin Product
- Agarose
- SYBR green
- DNA loading dye
- Thermocycler
- Gel electrophoresis system with power supply
- Gel documentation system

Methods:

Dilute the pepsin 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 pepsin). To 1 of these tubes, add positive control pepsinogen 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 pepsinogen 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 pepsin.

In an 8-tube PCR strip tube aliquot 45uL of un-spiked 50mg/mL pepsin in 25mM NaOH into Tubes 2-8. In Tube 1, add 50μL of the 50mg/mL pepsin solution spiked with 1ng/μL pepsinogen 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 pepsinogen plasmid DNA in 50mg/mL pepsin 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 pepsin without spiked DNA in duplicate. For positive control reaction load 1μL of 1ng/μL of pepsinogen A plasmid DNA. For negative control, load 1μL of 25mM sodium hydroxide. For each sample Lot of pepsin, there will be a total of 12 tubes in the cPCR test: 50mg/mL 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
Pep_EAEA_For1 10uM	1	15
Pep_Stop_Rev1 10uM	1	15
DNA	1	-
water	7	105
PCR conditions OVD ORF		
Standard cPCR Taq protocol		
95C 3' denature		
95C 30" 30 cycle		
60C 30" 30 cycle		
68C 1’10" 30 cycle		
68C 5'		
4C forever		

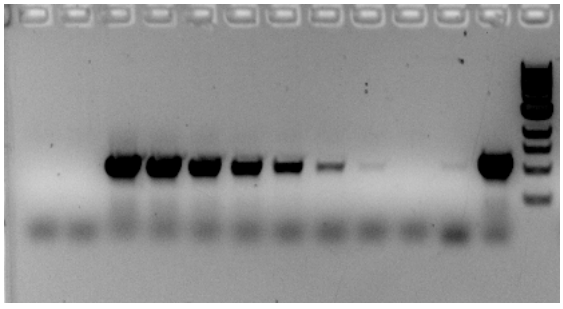
After PCR reactions are finished, add loading buffer and then load 20μL of the sample on a 1% agarose gel and run for 35 minutes at 110V. Product for pepsin ORF should be 1122bp.

Presence of coding DNA is determined by presence of the respective products showing up on the DNA gel. Limit of detection is around 1 femtogram (fg) of positive control plasmid.

Gel Results for PEP19232:

Lane description is provided below the gel picture (Figure A). Limit of detection is ~1fg of plasmid DNA as observed from the thin band in Lane 9. There is no detectable pepsinogen A DNA in the duplicate test samples (Lanes 1 and 2).

Figure A: Gel picture illustrating absence of pepsinogen coding DNA in PEP19232 Fermentation-derived Pepsin sample (Lanes 1 and 2)



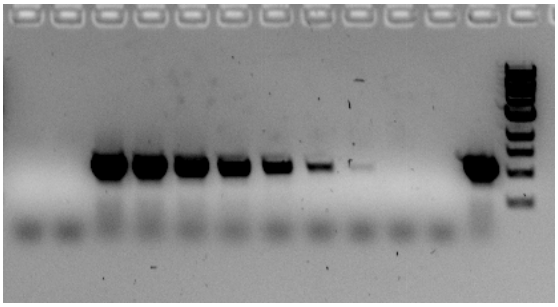
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13

Lane #	
1	50mg/mL Pepsin **PEP19232**
2	50mg/mL Pepsin **PEP19232**
3	1ng pepsinogen plasmid DNA
4	0.1ng pepsinogen plasmid DNA
5	0.01ng pepsinogen plasmid DNA
6	1000fg pepsinogen plasmid DNA
7	100fg pepsinogen plasmid DNA
8	10fg pepsinogen plasmid DNA
9	1fg pepsinogen plasmid DNA
10	0.1fg pepsinogen plasmid DNA
11	negative control
12	positive control
13	1kb DNA ladder

Gel Results for PEP19241:

Lane description is provided below the gel picture (Figure B). Limit of detection is ~1femtogram (fg) of plasmid DNA. There is no detectable pepsinogen A DNA in the duplicate test samples (Lane 1 and 2).

Figure B: Gel picture illustrating absence of pepsinogen coding DNA in PEP19241 Fermentation-derived Pepsin sample (Lanes 1 and 2)



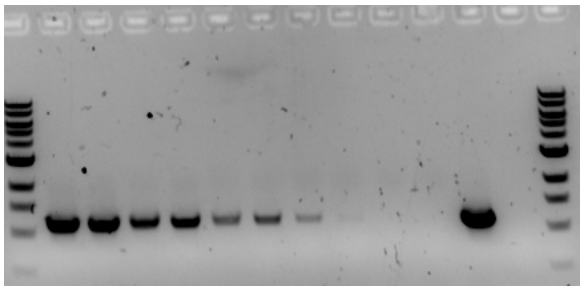
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13

Lane #	
1	50mg/mL Pepsin **PEP19241**
2	50mg/mL Pepsin **PEP19241**
3	1ng pepsinogen plasmid DNA
4	0.1ng pepsinogen plasmid DNA
5	.01ng pepsinogen plasmid DNA
6	1000fg pepsinogen plasmid DNA
7	100fg pepsinogen plasmid DNA
8	10fg pepsinogen plasmid DNA
9	1fg pepsinogen plasmid DNA
10	0.1fg pepsinogen plasmid DNA
11	negative control
12	positive control
13	1kb DNA ladder

Gel Results for PEP19252:

Lane description is provided below the gel picture (Figure C). Limit of detection is ~1fg of plasmid DNA. There is no detectable pepsinogen A DNA in the duplicate test samples (Lanes 10 and 11).

Figure C: Gel picture illustrating absence of pepsinogen coding DNA in PEP19252 Fermentation-derived Pepsin sample (Lanes 1 and 2)



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

Lane #	
1	1kb DNA ladder
2	1ng pepsinogen plasmid DNA
3	0.1ng pepsinogen plasmid DNA
4	.01ng pepsinogen plasmid DNA
5	1000fg pepsinogen plasmid DNA
6	100fg pepsinogen plasmid DNA
7	10fg pepsinogen plasmid DNA
8	1fg pepsinogen plasmid DNA
9	0.1fg pepsinogen plasmid DNA
10	50mg/mL Pepsin **PEP19252**
11	50mg/mL Pepsin **PEP19252**
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: Fermentation-derived Pepsin (Powder)

Source: Fermentation derived Pepsin

Lot #: PEP19232

Characteristic	Specification	Result
Appearance	White to Off-white amorphous powder	Complies
Solubility	Mostly soluble in water with slight opalescence. Practically insoluble in alcohol, chloroform and ether.	Complies

Characteristic (in powder as is)	Specification	Method	Result
Enzyme activity in FCC Units/mg	1:30000	FCC Assay[1]	1:31440
Moisture	Maximum 10.0%	AOAC 925.09	9.4
Ash	Maximum 5.0%	AOAC 942.05	3.54
Hg	< 1 ppm	ICP-AES	< 0.01
Pb	< 1 ppm	ICP-AES	< 0.01
As	< 1 ppm	ICP-AES	< 0.01
Cd	< 1 ppm	ICP-AES	< 0.01
Standard Plate Count	< 10000 CFU/g	AOAC 966.23	< 10
Yeast & Mold	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not detected
E. coli	< 10 CFU / g	AOAC 991.14	< 10
Total Coliforms	≤ 30 CFU/g	AOAC 991.14	Not detected

[1]Food Chemical Codex, 9th edition

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes

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

Certificate of Analysis

Product Name: Fermentation-derived Pepsin (Powder)

Source: Fermentation derived Pepsin

Lot #: PEP19241

Characteristic	Specification	Result
Appearance	White to Off-white amorphous powder	Complies
Solubility	Mostly soluble in water with slight opalescence. Practically insoluble in alcohol, chloroform and ether.	Complies

Characteristic (in powder as is)	Specification	Method	Result
Enzyme activity in FCC Units/mg	1:30000	FCC Assay[1]	1:31000
Moisture	Maximum 10.0%	AOAC 925.09	9.1
Ash	Maximum 5.0%	AOAC 942.05	3.79
Hg	< 1 ppm	ICP-AES	< 0.01
Pb	< 1 ppm	ICP-AES	< 0.01
As	< 1 ppm	ICP-AES	< 0.01
Cd	< 1 ppm	ICP-AES	< 0.01
Standard Plate Count	< 10000 CFU/g	AOAC 966.23	< 10
Yeast & Mold	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not detected
E. coli	< 10 CFU / g	AOAC 991.14	< 10
Total Coliforms	≤ 30 CFU / g	AOAC 991.14	Not detected

[1]Food Chemical Codex, 9th edition

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes

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

Certificate of Analysis

Product Name: Fermentation-derived Pepsin (Powder)

Source: Fermentation derived Pepsin

Lot #: PEP19252

Characteristic	Specification	Result
Appearance	White to Off-white amorphous powder	Complies
Solubility	Mostly soluble in water with slight opalescence. Practically insoluble in alcohol, chloroform and ether.	Complies

Characteristic (in powder as is)	Specification	Method	Result
Enzyme activity in FCC Units/mg	1:30000	FCC Assay[1]	1:32200
Moisture	Maximum 10.0%	AOAC 925.09	9.6
Ash	Maximum 5.0%	AOAC 942.05	3.61
Hg	< 1 ppm	ICP-AES	< 0.01
Pb	< 1 ppm	ICP-AES	< 0.01
As	< 1 ppm	ICP-AES	< 0.01
Cd	< 1 ppm	ICP-AES	< 0.01
Standard Plate Count	< 10000 CFU/g	AOAC 966.23	< 10
Yeast & Mold	< 100 CFU/g	AOAC 997.02	< 10
Salmonella	Not Detected / 25g	AOAC 2003.09	Not detected
E. coli	< 10 CFU / g	AOAC 991.14	< 10
Total Coliforms	≤ 30 CFU/g	AOAC 991.14	Not detected

[1]Food Chemical Codex, 9th edition

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes

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

Appendix 4: Expert Panel Report

REPORT OF THE GRAS PANEL ON THE *GENERALLY RECOGNIZED AS SAFE* (GRAS) STATUS OF PEPSIN A FROM GENETICALLY MODIFIED *PICHIA PASTORIS* FOR USE AS A PROCESSING AID IN FOOD MANUFACTURE

November 2019

GRAS Panel Members

Joseph F. Borzelleca, Ph.D.

Michael W. Pariza, Ph.D.

Advisor to the Panel

Kevin O. Gillies

Introduction

Clara Food Company proposes to use Pepsin A produced by a genetically-modified strain of *Pichia pastoris* as a processing aid in food manufacture. 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 ingredients added to foods, to conduct an independent and critical evaluation of the available information on the safety of *P. pastoris* and Pepsin A and to determine whether the proposed uses of this enzyme preparation 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 panel.

Summary and Basis for GRAS

The GRAS Panel, individually and collectively, critically evaluated a dossier prepared by Clara Foods entitled, "Pepsin A produced by *Pichia pastoris* Generally Recognized as Safe Notice," dated October 2019. This dossier described (1) the history of safe use of porcine Pepsin A as a food processing aid; (2) the biology of *P. pastoris* and its history of safe use in food manufacture; (3) the porcine Pepsinogen A gene that was inserted into the production strain; (4) the cloning methodology that was utilized; (5) sequencing data showing that the Pepsin A produced by the production strain is identical to porcine-derived Pepsin A; (6) the manufacturing process; (7) the enzyme product specifications; (8) dietary exposure; and (9) safety considerations including the Pariza-Johnson decision tree (MW Pariza and EA Johnson. *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology 33: 173-186, 2001).

The GRAS Panel participated in a teleconference on November 5, 2019 with Clara Food Company senior manager Kritika Mahadevan and Mr. Gilles, who served as technical advisor to the GRAS Panel. A critical consideration was that Clara Food's Pepsin A enzyme preparation complies fully with the Pariza-Johnson decision tree.

Following its independent and collective critical evaluation of the available information as part of this call, the GRAS Panel unanimously concluded that the proposed uses of Clara Food's Pepsin A enzyme 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 is safe for the manufacture of food-grade Pepsin A.

We further conclude that Clara Food's Pepsin A enzyme preparation, produced using its *Pichia pastoris* production strain, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is safe and *Generally Recognized as Safe* (GRAS) based on scientific procedures for use as a processing aid in food manufacture.

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

[redacted]

November 11, 2019

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

Date

[redacted]

19 November 2019

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

Date

[redacted]

November 19, 2019

Kevin O. Gillies (Advisor to the panel)
Kevin O. Gillies Consulting Services, LLC
1759 Grape St.
Denver, CO

Date

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

1.1 Exemption Claim for Pepsin A 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 pepsin A produced by *P. pastoris* as an enzyme 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 Pepsin A 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. DBA The EVERY Company
1 Tower Place
Suit 800
South San Francisco, CA 94080

Contact person for this file:

Dr. Kritika Mahadevan
Director Quality Assurance and Regulatory Affairs
Clara Foods Co. DBA The EVERY Company

1.3 Basis for Safety Determination

Pepsin A produced by *P. pastoris* DFB-002 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

Pepsin A produced by *P. pastoris* DFB-002 is intended as a direct replacement for processing aid uses of porcine Pepsin A (EC 3.4.23.1) as an enzyme for use in food in accordance with GMP as described in 21CFR184.1595. The estimated dietary intake from the use is 8 mg TOS/kg bw/day. Fermentation-derived Pepsin produced by *P. pastoris* uses include infant formula but excludes products regulated under USDA/FSIS jurisdiction.

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 further 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 Pepsin A produced by *P. pastoris* DFB-002.

1.8 Signature of Responsible Party or Agent

November 14, 2021

Dr. Kritika Mahadevan
Director Quality Assurance and Regulatory Affairs
Clara Foods Co. DBA The EVERY Company

EVERY
by Clara Foods

Dr Kritika Mahadevan
Director Quality Assurance and Regulatory Affairs
Clara Foods Co. DBA The EVERY Company
1 Tower Place, Suite 800
South San Francisco
California 94080
USA

Dr. Katie Overbey
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

5th October 2022

Dear Dr Overbey,

Re: FDA Questions for GRN 1025

Thank you for the opportunity to respond to the review team's questions sent via email dated 12th July 2022. Please find below our responses to the questions. We first set forth the FDA question in Bold Font, followed by our response in normal font.

1. **Please clarify whether the pepsin enzyme is secreted or is lysed.**

   Pepsin enzyme is secreted as Pepsinogen A into the fermentation liquid. It is subsequently activated to produce Pepsin A.

2. **Please correct the method for analysis in Table 1 for the heavy metals to reflect the reference in the footnote, i.e. ICP-MS instead of ICP-AES.**

   The appropriate method of analysis used for heavy metals in Table 1 was ICP-MS.

3. **Please consider reducing the specifications for arsenic, cadmium, mercury and lead to reflect the results from the batch analyses presented in the notice.**

   We note that there is a limited data set of quality testing results during the product development stage of Pepsin A enzyme preparation and we believe it is premature to lower the heavy metals specifications below the currently accepted QC limits for enzymes in the industry.

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.

4. Please clarify what the units in Table 3 are for the maximum recommended use level for the vegetable protein hydrolysis use.

The maximum recommended use level of Pepsin for the vegetable protein hydrolysis in Table 3 is 10 mg TOS / kg of vegetable protein.

5. The dietary exposure to pepsin is based on the IOM RDI of 56 g protein per day (IOM, 2010). The notifier should provide dietary exposure using results from a more recent intake assessment such as WWEIA (2013-16), budget method for estimating the EDI of enzyme preparation of NHANES consumer intake surveys.

Clara Foods clarifying question: Here, it is mentioned using budget method with NHANES, but we are aware that other GRNs have used the budget method without NHANES data, or it's possible to use NHANES alone to estimate intake. Do you have a preference? For instance, can we use NHANES data and estimate the EDI?

Further correspondence from FDA dated 13th Sep 2022:

The notifier can use (1) the budget method for estimation of the EDI, (2) a recent NHANES consumption survey (2015-2016/2017-2018), or (3) base their EDI on the protein consumption from a more recent assessment such as WWEIA (2013-2016).

Clara Foods Response:
As noted in GRN 1025 (p. 22), Clara Foods calculated potential consumer exposure to fermentation-derived pepsin based on the usage rate for vegetable protein hydrolysis and intake of total dietary protein. Clara Foods based the estimated exposure to fermentation-derived pepsin on use from vegetable protein hydrolysis, as this use is most likely to result in consumer exposure to the enzyme. The use of pepsin in cheese production results in very little carry over of pepsin to the final cheese product. Likewise, the use of pepsin in chill proofing beer does not result in carry over of pepsin to the final beer product as the enzyme is precipitated and filtered from the beer.

In calculating potential consumer exposure to fermentation-derived pepsin from use in vegetable protein hydrolysis, Clara Foods assumed that consumption of vegetable protein hydrolysate is represented by the concentration of fermentation-derived pepsin in protein (1.0 g TOS/100 g of protein) multiplied by the intake of total dietary protein on a bodyweight (kg bw) basis. The calculation is as follows:

$$1 \frac{g\ TOS}{100\ g\ protein} \times X \frac{g\ protein}{kg\ bw/day} = Y \frac{g\ TOS}{kg\ bw/day}$$

Clara Foods assumed that intake of total dietary protein is represented by the Recommended Dietary Allowance (RDA) for intake of protein by adults, which is 0.8 g/kg bw/day. The value of 0.8 g/kg bw/day represents a recommended intake rather than an actual intake.

In response to FDA's request, Clara Foods is providing a revised estimate of fermentation-derived pepsin intake based on more recent estimates of protein intake. USDA has developed distributions of usual protein intake for the U.S. population using dietary recalls collected in the What We Eat in America (WWEIA) dietary recall component of the National Health and Nutrition Examination Survey (NHANES). Currently, the most recent estimates of usual protein intake on a bodyweight basis available from WWEIA/NHANES are for the time period 2015-2018. Based on these data, the estimated usual intake of total protein by the U.S. population ages 1 year and older is 1.42 g/kg bw/day at the mean and 2.24 g/kg bw/day at the 90th percentile of intake (Table 1).[1] In developing the estimates of protein intake on a bodyweight basis, USDA adjusted bodyweights to values within normal weight ranges. Specifically, for individuals 4-19 years of age, body weights outside of the normal range were set to the normal boundary; for individuals aged 19 years and older, body weights were set to Body Mass Index (BMI) cutoffs; and for children ages 1-3 years, reference weights were used.

Total protein intake reflects consumption of protein from animal and plant sources. Animal sources of dietary protein include meat, poultry, fish, eggs, and milk, while plant sources of dietary protein include grains (e.g., wheat, oats, rice), nuts and seeds, and fruits and vegetables.

The fermentation-derived pepsin is used to hydrolyze select proteins (e.g., wheat, pea) within the category of plant proteins. The fermentation-derived pepsin is not used on animal proteins. It is therefore reasonable to calculate intake of fermentation-derived pepsin based on the fraction of total protein consumed as vegetable protein.

USDA reports that the majority of total protein intake by adults is from animal sources (66%), with only one-third (33%) of total protein intake from plant sources.[2] The usual intake of plant protein by the U.S. population can therefore be approximated as 0.33 times the intake of total protein, or 0.47 g/kg bw/day at the mean and 0.74 g/kg bw/day at the 90th percentile of intake for the U.S. population ages 1 year and older.

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[1] USDA, Agricultural Research Service, 2021. Usual Nutrient Intake from Food and Beverages, by Gender and Age, What We Eat in America, NHANES 2015-2018. Available at https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/usual/Usual_Intake_gender_WWEIA_2015_2018.pdf

[2] Hoy MK, Clemens JC, Moshfegh AJ. Protein Intake of Adults in the U.S.: What We Eat in America, NHANES 2015-2016. Food Surveys Research Group Dietary Data Brief No. 29. January 2021, revised slightly from July 2020. Available at https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/DBrief/29_Protein_Intake_of_Adults_1516.pdf

Assuming pepsin is present in all plant protein at a concentration of 1.0 g TOS/100 g protein, the intake of fermentation-derived pepsin based on these levels of protein intake are 0.005 g TOS/kg bw/day at the mean and 0.007 g TOS/kg bw/day at the 90th percentile of intake.

Table 1. Estimated daily intake of protein and fermentation-derived pepsin by the U.S. population ages 1 year and older, WWEIA/NHANES 2015-2018

Parameter	**Mean Intake**	**90th Percentile Intake**
Total protein, g/kg bw/day [a]	1.42	2.24
Plant protein, g/kg bw/day [b]	0.47	0.74
Fermentation-derived pepsin, g TOS/kg bw/day	0.005	0.007

[a] Estimates of usual total protein intake per kg bw/day as reported by USDA.
[b] Calculated as 0.33 times usual total protein intake.

As noted in the discussion of GRN 1025, estimates of fermentation-derived pepsin intake exaggerate intake based on several conservative assumptions:

- All plant protein consumed in the U.S. is enzyme hydrolyzed protein
- All producers of hydrolyzed protein use the highest usage rate of pepsin in the production of hydrolyzed protein
- The amount of TOS does not decrease as a result of the food production process.

6. Please provide a margin of exposure (MOE) for the pepsin enzyme preparation based on the NOAEL and the updated EDI.

Clara Foods clarifying response:
Can you please clarify your request on the NOAEL? Porcine pepsin has been deemed as a safe ingredient for human consumption as affirmed in 21 CFR 184.1595. There is no indication of a safety concern by virtue of decades of Pepsin usage as a processing aid. Therefore, we have not come across any reported NOAEL for Porcine Pepsin in the literature.

Our recombinant Porcine Pepsin A enzyme is identical in amino acid sequence to the native Porcine Pepsin A. Our safety narrative is based on the similarity of our recombinant Pepsin A enzyme to Porcine Pepsin A. We propose the same use levels of our enzyme as that of Porcine Pepsin and anticipate no increase in the exposure levels. Since there is no reported NOAEL for Porcine Pepsin in the literature, and our enzyme is identical to Porcine Pepsin, we believe it translates to no NOAEL for our enzyme preparation.

Further correspondence from FDA dated 13th Sep 2022:

Thank you for the clarification on question 6 and confirming that the pepsin enzyme in GRN 1025 is identical to porcine pepsin. In the dietary exposure section, you indicated that the pepsin enzyme will be used at a maximum level of 1g TOS per 100 g of hydrolyzed protein. Please provide a brief narrative using scientifically accepted biochemical pathways that are well-documented, such as textbooks or published literature, to show that any residual enzyme, if consumed as part of normal diet, would be absorbed and metabolized by the human body.

The human body digests proteins through a series of hydrolytic enzymes and changes in pH conditions (B.E. Goodman, 2010)[3]. The first instance of digestion is exposure to Pepsin itself at low pH in the stomach that helps open proteins and facilitates their cleavage into large peptides by Pepsin. That material is moved out of the stomach into the small intestine where the pH is much higher and a new set of proteases and peptidases attack the large peptides. Examples of the pancreatic proteases include trypsin, chymotrypsin and elastase that reduce the size of the peptides. There are also a series of exopeptidases, such as Carboxypeptidase A and B, that then convert the small peptides into single amino acids. If any residual recombinant Pepsin is ingested, it will be broken down eventually into single amino acids by the time it enters the small intestine. These amino acids will then be absorbed and metabolized.

7. On page 15, you reference studies discussed in GRN 737 (pg. 58) to support your conclusion that "there is no evidence of a risk of allergenicity from the carryover fermentation products of the production strain, *P. pastoris*." The notifier for GRN 737 on pg. 58 appears to discuss studies that are unpublished. Please provide and discuss peer-reviewed publication(s) that support this conclusion.

A recent study, Frazer et al., 2018, demonstrated the safety of a recombinant protein preparation containing 66% target protein (soy leghemoglobin, LegH) and 34% *K. phaffii* host proteins in 28 day feeding studies using rats. In these studies, they fed up to 750 mg/kg/d LegH, and approximately 375 mg *K. phaffii* host protein/kg/day and the authors concluded "the results of the studies presented raise no issues of toxicological concern". This publication shows that *K. phaffii* host proteins were well tolerated when included in a daily diet.

Fraser et al., 2018. Safety Evaluation of Soy Leghemoglobin Protein Preparation Derived From Pichia pastoris, Intended for Use as a Flavor Catalyst in Plant-Based Meat. Int J Toxicol. 2018 May; 37(3): 241–262.

[3] B.E. Goodman (2010). Insights into digestion and absorption of major nutrients in humans. Adv Physiol Educ 34: 44–53, 2010

8. 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). 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 citation for Section 1.7 for certification statement is 21 CFR 170.225(c)(9).

9. Please explicitly state the molecular weight of the pepsin A produced by *K. phaffii*

EVERY's Pepsin A is 326 amino acids long and has a molecular weight of 34.5 kDa. The pro form, Pepsinogen, is 370 amino acids long and 39.5 kDa.

10. Please state whether *K. phaffii* (previously classified as *Pichia pastoris*) strain "DFB-002" has been deposited in a recognized culture collection.

Yes, our *K. phaffii* strain DFB-002 has been deposited at ATCC with accession number GSD-1197.

11. Large sections of the description of the production microorganism in Section 2.2.1 (page 8-9) 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; last accessed May 13, 2022). These sections should be re- written and appropriately cited.

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

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.[4] 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 and Knapp, 1956)[5]. 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

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

[5] 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.

development of *Komagataella phaffii* into a protein production platform (Cregg, et al. 1985)[6].

Recent phylogenetic work, using molecular information such as 26S RNA sequence information (C. Kurtzman 2005)[7], 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)[8]. *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)[9]. Clara Foods further modified BG10 to have a phenotype that reduces the strain's ability to consume methanol. This base strain is called DFB-001A.

12. In section 2.2.2.1 the notifier states "Subsequent process steps convert Pepsinogen A to Pepsin A." Please specify these subsequent process steps.

The pH is reduced to at least 3.5 acidic environment and the temperature of the liquid system is raised to between 45 and 50C. At this point, Pepsinogen A is converted to Pepsin A.

13. The notifier states that the method used to detect *Salmonella* serovars is AOAC 2003.09 (page 18), 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 Pepsin A powder.

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[6] 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.

[7] 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.

[8] 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.

[9] 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.

14. For the administrative record, please state if the analytical methods used for batch analyses are validated for their intended purpose.

Yes, analytical methods used for batch analyses are validated for their intended purpose. Additionally, ISO 17025 accredited laboratory is used to carry out the analyses on the samples.

15. Please state if the pepsin A enzyme is expected to remain active in the final food products or if the enzyme will be inactivated or denatured during food production.

Pepsin A enzyme is generally used as a processing aid and is removed from the final food product. In the event there are small amounts carried over in the food matrix, it is expected to be inactive in the final food product.

16. On pg. 15, you state that "Clara Foods knows of no issues of allergenicity related to the consumption of Pepsin A used as an enzyme in food production." It is not clear whether this statement is based on anecdotal evidence or through a literature or case-report searches. Please clarify your statement and confirm that given your enzyme's identical amino acid sequence to porcine pepsin A that has a history of safe use, you do not expect the allergenicity potential of your enzyme to be different from that of porcine pepsin A that is currently used in food processing.

Clara Foods Pepsin A enzyme's amino acid sequence is identical to the amino acid sequence for Porcine Pepsin A that has a history of safe use. As such, we do not expect the allergenicity potential of our enzyme to be different from that of Porcine Pepsin A that is currently used in food processing.

Sincerely,

Kritika Mahadevan