motif FOOD WORKS
An ingredient innovation company

April 15, 2021

Dr. Paulette Gaynor
OFFICE OF FOOD ADDITIVE SAFETY (HFS-200)
CENTER FOR FOOD SAFETY AND APPLIED NUTRITION (CFSAN)
FOOD AND DRUG ADMINISTRATION
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740
USA

Dear Dr. Gaynor:

Re: GRAS Notice for Bovine Myoglobin

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Motif FoodWorks Inc., as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that bovine myoglobin produced by fermentation using a modified strain of *Pichia pastoris,* is GRAS on the basis of scientific procedures, for use meat alternative products; these food uses of myoglobin are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*.

Information setting forth the basis for Motif's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the Agency.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Yours sincerely,

DocuSigned by:
Janet E. Collins
2F33C163C3DE422...

Janet E. Collins, Ph.D., R.D.
Vice President, Regulatory Government and Industry Affairs
Motif FoodWorks, Inc.
jcollins@motiffoodworks.com

GRAS NOTICE FOR MYOGLOBIN PREPARATION

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Motif FoodWorks, Inc.
27 Drydock Avenue, 2nd Floor
Boston, Massachusetts
02210 USA

DATE:
April 14, 2021

GRAS Notice for Myoglobin Preparation

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GRAS Notice for Myoglobin Preparation

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Motif FoodWorks, Inc. (Motif FoodWorks) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that a Myoglobin Preparation, as manufactured by Motif FoodWorks, is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Motif FoodWorks' view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Motif FoodWorks the undersigned hereby certifies that all data and information presented in this Notice represents a complete, representative, and balanced submission, and considered all unfavorable as well as favorable information known to Motif FoodWorks and pertinent to the evaluation of the safety and GRAS status of the Myoglobin Preparation as a food ingredient for use in a variety of food products, as described herein.

DocuSigned by:
Janet E. Collins
2F33C163C3DE422...

4/15/2021

Janet E. Collins, Ph.D., R.D.
Vice President, Regulatory Government and Industry Affairs
Motif FoodWorks, Inc.
jcollins@motiffoodworks.com

Date

1.1 Name and Address of Notifier

Motif FoodWorks, Inc.
27 Drydock Avenue, 2nd Floor
Boston, Massachusetts
02210 USA

1.2 Common Name of Notified Substance

Yeast-derived heme protein (non-animal)

1.3 Conditions of Use

Motif FoodWorks' Myoglobin Preparation is intended for use in plant-based ground meat analogue products at levels providing ≤2% myoglobin protein to contribute to the flavor and aroma in ground meat analogues to mimic flavors associated with cooked ground meat. Examples of meat analogue products include burgers, patties, sausages, and other plant-based meat analogues, including fresh and/or frozen entrées or meals, where ground meat or poultry is typically the principal ingredient.

The use of Myoglobin Preparation in ground meat analogues is self-limiting based on acceptable organoleptic (flavor and aroma) properties of the final food products.

The Myoglobin Preparation is intended for use in food products consumed by the general population. As Myoglobin Preparation will be used in meat alternative products, substituting 1:1 for conventional meat and poultry products, consumption patterns for food products containing Myoglobin Preparation as an ingredient are anticipated to be similar to those for meat and poultry in a typical American diet. The Myoglobin Preparation is not intended for use in infant formula and is not intended for addition to meat and poultry products regulated by the United States Department of Agriculture (USDA). Motif notes that myoglobin imparts a red coloration when exposed to oxygen and simulated ready-to-cook meat products that incorporate myoglobin will typically have a red to pink coloration similar to meat. Although the primary function of myoglobin in food is for flavor, a secondary effect of the ingredient on the coloring of some food applications is recognized and a Color Additive Petition will be submitted to the Agency to support such uses.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020a), Motif FoodWorks has concluded that the intended uses of Myoglobin Preparation as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Motif FoodWorks, Inc.
27 Drydock Avenue, 2nd Floor
Boston, Massachusetts
02210 USA

Should the U.S. FDA have any questions or additional information requests regarding this Notification, Motif FoodWorks will supply these data and information upon request.

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

It is Motif FoodWorks' view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, 5 U.S.C. 552.

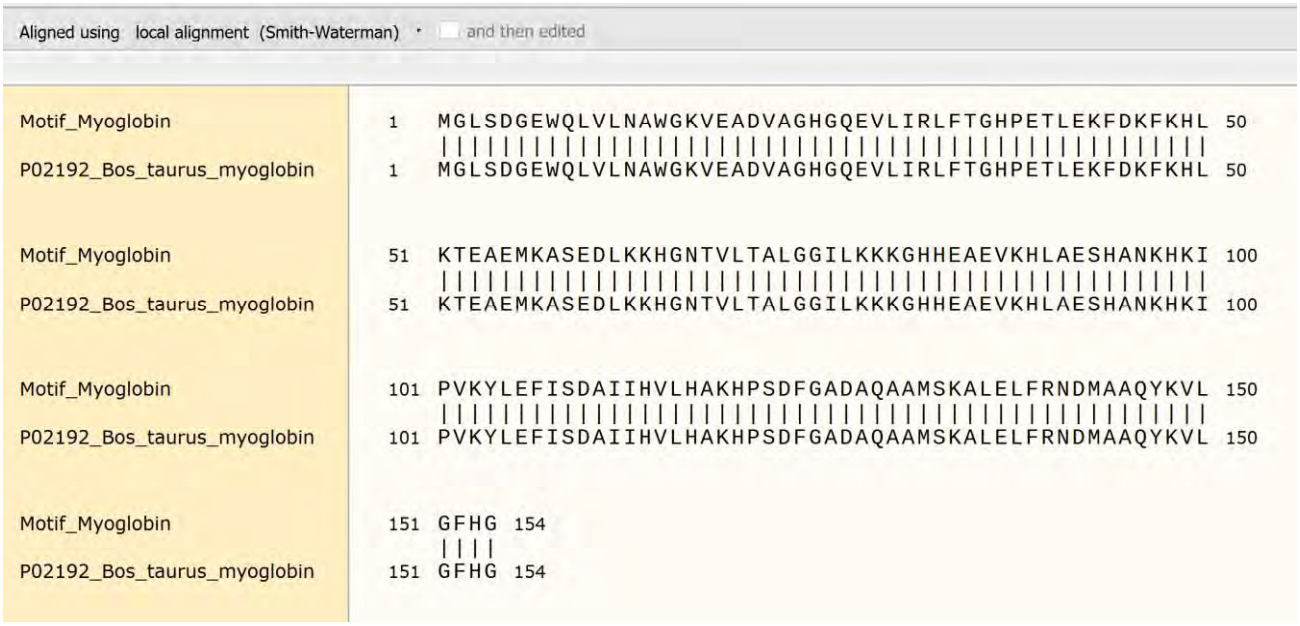
Part 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity of the Ingredient

Motif FoodWorks' myoglobin ingredient is a liquid flavoring preparation (herein referred to as Myoglobin Preparation) containing myoglobin produced by fermentation from a modified strain of *Pichia pastoris* expressing the myoglobin gene from *Bos taurus*. The ingredient has a moisture content of ≥92.5%, a myoglobin content of ≥3%, and a myoglobin protein purity of ≥65%. The remaining components of Myoglobin Preparation include water, ash (≤1.5% w/w), fat (≤1% w/w), and carbohydrate (≤0.5% w/w) and has a total organic solids (TOC) content of ≤7.5%[1]. Myoglobin Preparation is formulated with food-grade excipients, stabilizers, preservatives (*e.g.*, sodium phosphate, sodium ascorbate, sodium chloride), and antimicrobial agents, depending on storage conditions.

Myoglobin[2] (UniProtKB/Swiss-Prot No. P02192, GeneID: 280695, VGNC Symbol: MB) is the characterizing component of the Myoglobin Preparation. As shown in Figure 2.1-1 below, the myoglobin protein in the Myoglobin Preparation has 100% sequence homology to myoglobin protein from *Bos taurus*. Bovine myoglobin has a high level of homology to hemoglobin proteins from porcine and ovine species, as well as from birds.

Figure 2.1-1 Amino Acid Sequence Homology of Motif FoodWorks' Myoglobin to Bovine Myoglobin



[1] The Myoglobin Preparation may contain ≤0.2 mg/L *Pichia* protein.
[2] UniProtKB/Swiss-Prot No. P02192. GeneID No. 280695. VGNC Symbol: MB.

The myoglobin present in Motif FoodWorks' Myoglobin Preparation has been characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC), and peptide mass spectrometry. As shown in Figure 2.1-2, myoglobin obtained from fermentation of *P. pastoris* strain t838417 (Lane 2) has a molecular weight of approximately 17 kDa, corresponding to its predicted molecular weight, and displays a similar gel migration pattern to a commercial bovine muscle-derived myoglobin standard (Innovative Research; Catalog # IBOMBLY250MG). The slight differences in the migration patterns are explained by the apparent low-level O-glycosylation within the bovine standard identified during proteomic analyses. Similar findings were observed in the SEC profiles when Motif FoodWorks' Myoglobin Preparation was compared to a commercial standard demonstrating a high purity of the ingredient and slight differences in retention times due to the absence of glycosylation in myoglobin from *P. pastoris* (Figure 2.1-3). The results of the proteomics analyses corroborate the identity of the protein as bovine myoglobin relative to a commercial standard and demonstrate that the material contains negligible glycosylation compared to 3 potential O-glycosylation sites identified in the bovine myoglobin standard (Table 2.1-4). Low level residues (≤0.2 mg/L) of native proteins from the fermentation organism are expected to be present in the myoglobin preparation. *Pichia* yeast has a long history of safe use in food biotechnology for production of food enzymes (EFSA, 2017, Spohner *et al*., 2015) and the production strain has been used previously for the manufacture of soybean leghemoglobin, a similar protein used for flavoring (FDA, 2018a).

A discussion of the historical use and safety of *Pichia* yeast as a food processing organism is presented in Sections 2.2.1 and 6.1. Based on the long history of safe use of *Pichia pastoris* in food production and recent characterization of native proteins from the same production strain as reported by Jin *et al*., (2018), further characterization of the residual *Pichia* proteins was not considered necessary for the GRAS evaluation.

Figure 2.1-2 **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Results of Myoglobin from Myoglobin Preparation with Lane 2 Showing Motif FoodWorks' Myoglobin Preparation and Lane 3 Showing Bovine Myoglobin Standard**

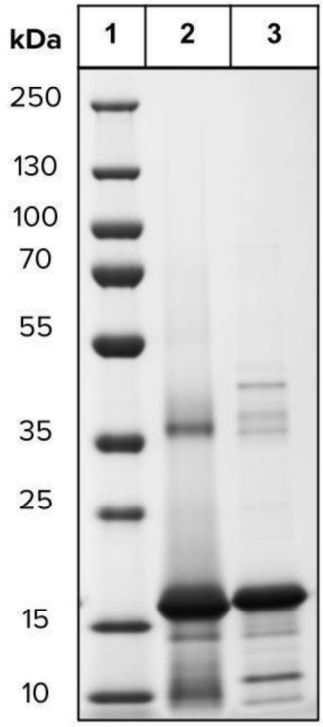
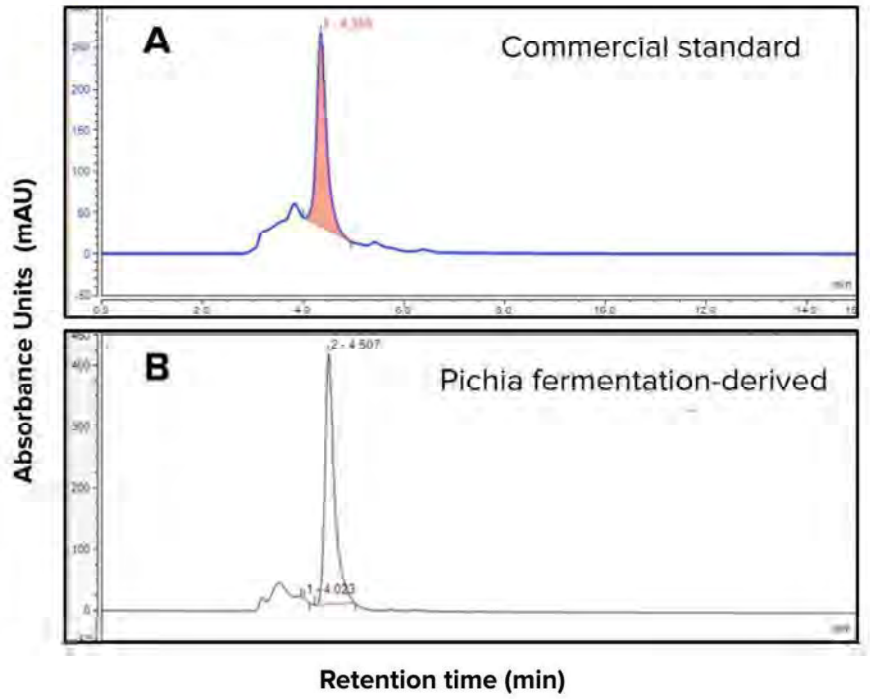


Figure 2.1-3 **Characterization of Myoglobin from Myoglobin Preparation and Bovine Muscle-Derived Myoglobin Standard by Size Exclusion Chromatography (A and B) and Ultraviolet-Visible Spectroscopy (C and D)**



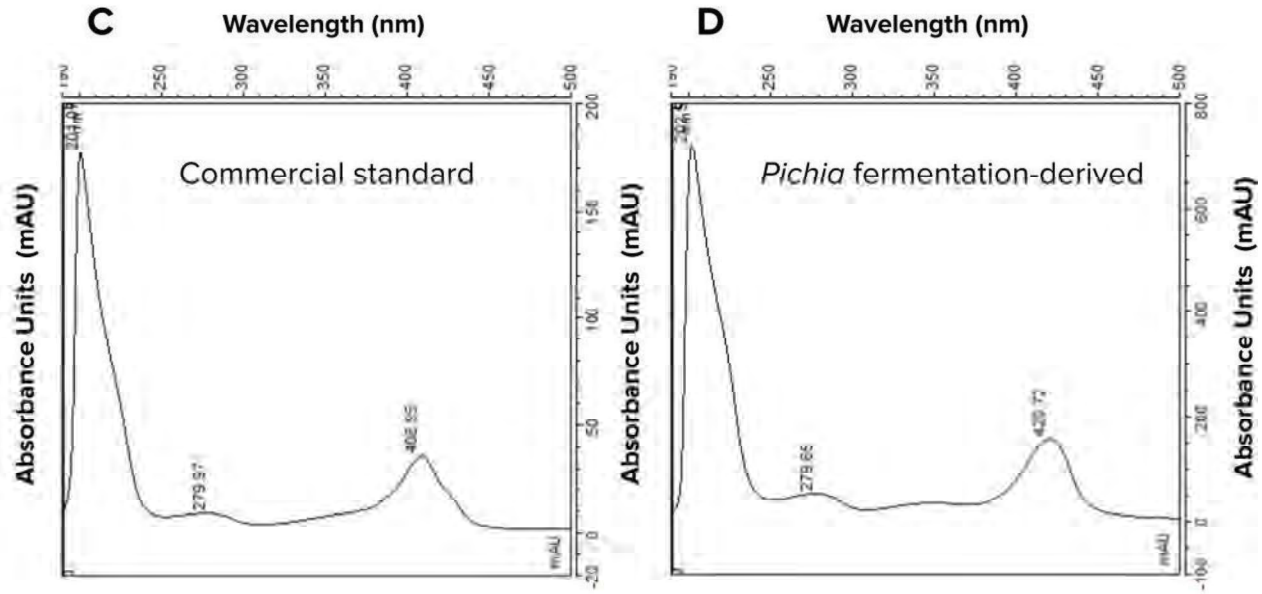
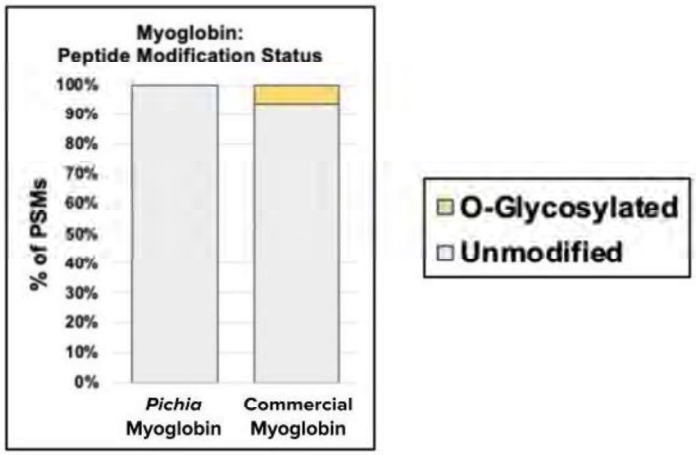


Figure 2.1-4 **Predicted Glycosylation Pattern Using Liquid Chromatography Mass Spectroscopy (LC-MS/MS) Analyses of Commercial Bovine Myoglobin Standard (Right) Compared to Myoglobin from *Pichia* (Left)**



2.2 Method of Manufacture

2.2.1 Description of the Production Microorganism

2.2.1.1 Host (Parental) Organism

Pichia pastoris[3] is a eukaryotic, methylotrophic, non-pathogenic, and non-toxigenic microorganism widely used by the biotechnology industry for production of recombinant proteins and food enzymes (Balamurugan *et al.*, 2007; Kurtzman, 2009). The genome of *P. pastoris* was sequenced in 2009 (De Schutter *et al*., 2009). *P. pastoris* was first used in the commercial preparation of a single cell protein for use in animal feed, and since then has been extensively used in food production and human pharmaceutical products (Ahmad *et al*., 2014; Brady *et al*., 2020). According to Brady *et al.* (2020), since 2003, *P. pastoris* has been used as a host organism in over 7,000 research articles and accounted for approximately 17% of the total recombinant genes produced in 2009 (Sørensen, 2010). *P. pastoris* is a well characterized microorganism and has an established history of safe use in food production. A detailed description of *P. pastoris* was discussed in GRAS Notice (GRN) 737. In the European Union (EU), *P. pastoris* (*K. phaffiii*) was granted qualified presumption of safety (QPS) status by the European Food Safety Authority (EFSA) Panel on Biological Hazards for use in enzyme production (EFSA, 2017).

[3] *Pichia pastoris* was reassigned to the genus *Komagataella* following phylogenetic analysis of gene sequences, and important strains of '*Pichia pastoris*' commonly used in biotechnology are members of *Komagatealla phaffii*. (Kurtzman *et al*., 2020).

The taxonomic identity of *P. pastoris* is presented in Table 2.2.1.1-1.

Table 2.2.1.1-1 Taxonomic Identity of *Pichia pastoris*

Kingdom	Fungi
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Phaffomycetaceae
Genus	*Komagataella*
Species	*phaffi (pseudonym = Pichia pastoris)*

The host organism, *P. pastoris* NRRL Y-7556, used for construction of the production strain (t303048), is a methylotrophic yeast capable of using methanol as the sole carbon source. The lineage of this organism was discussed by Brady *et al.* (2020). Genetic typing of the strain has resulted in re-naming of the strain from *Pichia pastoris* to *Komagatella phaffii* (Kurtzman, 2009); however, the strain is still often referred to as *Pichia pastoris,* and for simplicity the name *P. pastoris* will be used throughout the Notification.

As reported by Braun-Galleani *et al*. (2019) almost all research on *K. phaffii* (*P. pastoris*) has been conducted using the genetic background of strain CBS7435 (synonymous with NRRL Y-11430). The origin of this strain was previously unclear since the strain was first deposited in the CBS and NRRL culture collections in connection with a US patent granted to Phillips Petroleum; however, Braun-Galleani *et al*. (2019) have demonstrated that CBS7435 (NRRL Y-11430) is identical to the type strain of *K. phaffii* (NRRL Y-7556), which was isolated from an oak tree. Corroborating these conclusions, genotypic analyses by Brady *et al*., (2020) have demonstrated that strain NRRL Y-11430 and NRRL Y-7556 differ by a single nucleotide polymorphism (SNP). Motif FoodWorks has therefore concluded that the *P. pastoris* NRRL Y-7556 host strain is from the same lineage as *P. pastoris* NRRL Y-11430, which served as the host organism for production of soybean leghemoglobin (Impossible Foods, Inc., 2017; U.S. FDA, 2018a).

2.2.1.2 Construction of the Production Organism

The production strain, *P. pastoris* t838417, was constructed obtained from a genetically modified strain of *P. pastoris*, using the principles described by the Organisation for Economic Co-operation and Development (OECD) criteria for Good Industrial Large-Scale Practice (GILSP) microorganisms (OECD, 1992, 1993), as well as criteria for safe production microorganisms (Pariza and Foster, 1983; Pariza and Johnson, 2001).

The parental organism, *P. pastoris* t303048, is genetically modified to overexpress the proteins of the native heme biosynthetic pathway of *P. pastoris*. The heme biosynthetic pathway consists of 8 steps, each catalyzed by an enzyme that is highly conserved across plant, animal, and fungal species. Genes encoding all 8 enzymes were generated by DNA synthesis and transformed into *P. pastoris* t303048 using antibiotic resistance cassettes. The antibiotic resistance cassettes were removed from the strains after each round of transformation. This process yielded a stable intermediate strain, *P. pastoris* t486367, containing extra copies of each of the native *Pichia* heme biosynthesis enzymes.

P. pastoris t486367 was then modified to express *Bos taurus* (bovine myoglobin) protein. The *Bos taurus* myoglobin gene was codon-optimized for expression in *P. pastoris* and generated by DNA synthesis; multiple copies of the gene were stably integrated, along with an antibiotic resistance cassette, into *P. pastoris* t486367, using standard biotechnology practices. Subsequently, the antibiotic resistance cassette was removed. The resulting strain was identified as *P. pastoris* t830652. The gene encoding for bovine myoglobin is the only recombinant protein-encoding DNA inserted into the host organism.

To support optimal expression of the promoters utilized in the previous steps, *P. pastoris* t830652 was modified by inserting an additional copy of the gene encoding a transcription factor native to *P. pastoris*, along with an antibiotic resistance cassette. Following introduction of the transcription factor gene and removal of the antibiotic resistance gene, the production strain *P. pastoris* t838417 was obtained.

The production strain does not contain any antibiotic resistance genes or plasmid sequences, and therefore, does not pose any risk of transferring antibiotic resistance to non-related organisms. Similarly, no antibiotic resistance genes/DNA are present in the Myoglobin Preparation. Removal of all antibiotic resistance genes introduced during construction of the production strain was confirmed phenotypically and by whole genome sequencing. Myoglobin Preparation does not contain viable cells of the production strain, as they are lysed during the manufacturing process and removed by centrifugation and microfiltration. The Myoglobin Preparation may contain residual *Pichia* proteins at levels ≤0.2 mg/L.

All changes introduced into the production strain *P. pastoris* t838417 are stably integrated in the genome and confirmed to be present after growth on non-selective fermentation media during and after a round of fermentation. No plasmid sequences are present in the production strain, and therefore no plasmid sequences are expected to be capable of being transferred from the production strain to non-related organisms.

2.2.2 Description of the Manufacturing Process

2.2.2.1 Raw Materials and Processing Aids

All raw materials and processing aids comply with food-grade specifications, as established in the *Food Chemicals Codex* (FCC) or equivalent international food or pharmacopeia standard (*e.g*., United States Pharmacopeia), and are permitted for use in food by U.S. federal regulations or are GRAS for their respective uses. All filtration aids are those commonly used by the food industry in the purification of food ingredients.

2.2.2.2 Production Process

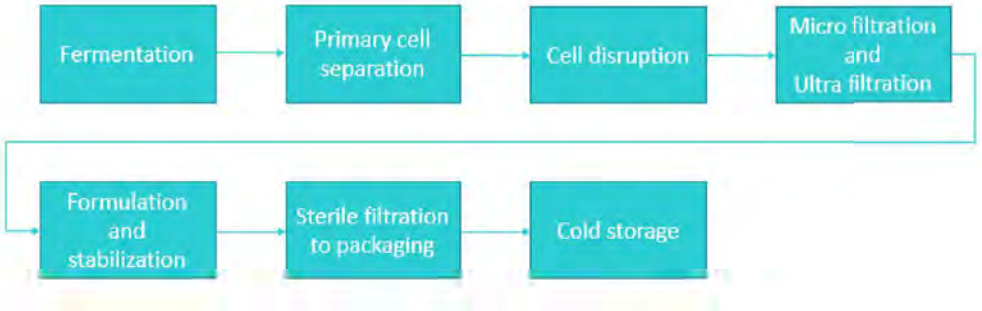
The myoglobin protein is prepared in stages: expression of myoglobin protein by the production organism following submerged fermentation (Fermentation Process), then enrichment and stabilization of the expressed myoglobin protein (Recovery Process). The Myoglobin Preparation is standardized to a concentration of about 3% myoglobin protein (purity ≥65%). The production process of the Myoglobin Preparation is discussed in further detail below.

In the Fermentation Process, the production strain *P. pastoris* t838417 is fermented by submerged fed-batch fermentation for the expression of myoglobin protein. The cells of the production strain are kept at -80°C in 20% (v/v) glycerol as the source inoculum. Working cell banks are prepared from the master cell bank after testing for microbial purity, specific growth rate, and yield prior to production fermentation. The fermentation broth is periodically analyzed microscopically to ensure culture purity. Process parameters including pH, temperature, agitation, dissolved oxygen, methanol concentration, and glycerol concentration are routinely monitored through fermentation following methods consistent with GRN 737 (Impossible Foods, Inc., 2017; U.S. FDA, 2018a). If microbial contamination is detected or other process deviations impacting safety and/or quality of the final product are identified, the fermentation broth is sterilized by steam in place and discarded.

Following the fermentation process, the production strain cells in the fermentation broth are washed and lysed. Insoluble material within the lysate is removed by centrifugation and microfiltration. Ultrafiltration is used to concentrate the myoglobin protein. The resulting concentrate is formulated with sodium chloride, sodium phosphate, sodium ascorbate, and may include other food-grade antimicrobials and antioxidants to stabilize the formulation, which is stored as a frozen liquid (-20°C).

A schematic overview of the recovery process is provided in Figure 2.2.2.2-1.

Figure 2.2.2.2-1 Schematic Overview of the Recovery Process Steps to Obtain the Myoglobin Protein



2.3 Product Specifications and Batch Analysis

Food-grade specifications for physical, chemical, and microbiological parameters have been established for the Myoglobin Preparation. All methods of analysis are internationally recognized (*e.g.*, Association of Official Analytical Chemists, U.S. FDA Bacteriological Analytical Manual). The chemical, physical, and microbiological specifications of the product are presented in Table 2.3-1. Pathogen presence or greater than 10^4 CFU/mL aerobic count, or failure to comply with the specifications would result in batch discard; execution of additional sanitization standard operating procedures in compliance with internal food-safety standards, and a root cause analysis.

Table 2.3-1 Product Specifications and Analysis of 3 Production Batches of Myoglobin Preparation

Specification Parameter	Specification	Method of Analysis	Manufacturing Lot No.		
Physico-Chemical Parameters					
Myoglobin Protein (% w/w)[a]	≥3	SEC HPLC	3	3	3
Myoglobin Protein Purity (% w/w)[b]	≥65	Chromatographic purity (at 280 nM)	98	98	97
Protein (% w/w)[c]	≤4.62	Calculated	3.06	3.06	3.09
Fat (% w/w)	≤1	AOAC 933.05	<0.08	<0.08	0.29
Moisture (% w/w)	≥92.5	AOAC 925.40	95.60	96.04	95.93
Total Organic Solids (% w/w)[d]	≤7.5	Difference	4.40	3.96	4.07
Ash (% w/w)	≤1.5	AOAC 945.46	1.27	1.36	1.6
Carbohydrate (% w/w)[e]	≤0.5	Difference	0.01	0.00	0.00
pH	6.5 to 8.5	AOAC 981.12	6.88	7.11	7.32
Heavy Metals					
Lead (ppm)	<0.4	AOAC 2015.01 Mod 2232	<0.01	<0.01	<0.01
Arsenic (ppm)	<0.05	AOAC 934.03	<0.01	<0.01	<0.01
Mercury (ppm)	<0.05	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)	<0.005	<0.005	<0.005
Cadmium (ppm)	<0.2	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)	<0.001	<0.001	<0.001
Microbiological Parameters					
Aerobic plate count (CFU/g)	$<10^4$	AOAC 966.23	<100	<100	<100
Yeast (per g)	<10	FDA-BAM, 7th ed.	<10	<10	<10
Mold (per g)	<10	FDA-BAM, 7th ed.	<10	<10	<10
Escherichia coli (3 tubes MPN) (per g)	<3	AOAC 966.24	<3	<3	<3
Salmonella spp. (per 25g)	Negative	AOAC RI 100201	Negative	Negative	Negative
Listeria monocytogenes (per 25 g)	Negative	AOAC 2003.12	Negative	Negative	Negative

AOAC = Association of Official Analytical Chemists; BAM = Bacteriological Analytical Manual; CFU = colony forming units; FDA = Food and Drug Administration; HPLC = high-performance liquid chromatography; ICP-MS = inductively coupled plasma-mass spectrometry; MPN = most probable number; ppm = parts per million; SEC = size exclusion chromatography.

[a] Myoglobin protein may exceed 3%, if additional moisture is removed during the concentration step of the manufacturing process (Figure 2.2.2.2-1).

[b] The balance of protein in the Myoglobin Preparation is residual *Pichia* protein.

[c] Protein (% w/w) content calculated as follows: Protein (% w/w) = Myoglobin Protein (% w/w)/Myoglobin Protein Purity (%).

[d] Solids (% w/w) calculated by difference as follows, Solids (% w/w) = 100-Moisture (% w/w).

[e] Carbohydrates (% w/w) calculated by difference as follows, Carbohydrate (% w/w) = Solids (% w/w) – Fat (% w/w) – Ash (% w/w) – Protein (% w/w).

Part 3. § 170.235 Dietary Exposure

3.1 Background Intake of Myoglobin

No federal regulations permitting the addition of myoglobin to the U.S. food supply have been promulgated, and Motif FoodWorks is not aware of other GRAS sources of myoglobin in the U.S. marketplace. Current background intakes of bovine myoglobin are solely contributed from the consumption of beef; however, myoglobin isoforms are also present in pork products and poultry. Reported concentrations of myoglobin in various meat products consumed in the diet vary based on species, muscle tissue and animal age. Myoglobin imparts a characteristic red coloration to raw meat products and myoglobin levels are accordingly highest in red meats such as beef and lowest in white meats such as poultry (see Table 3.1-1 below). The variability of myoglobin concentrations in meat products complicates estimation of dietary intakes from background foods; conservative estimates of 0.5% myoglobin have been reported for "meat" (Yip and Dallman, 1996).

Table 3.1-1 Concentrations of Myoglobin in Meat and Poultry Products

Meat Source	Myoglobin Concentration	Reference
Beef	0.02 to 0.18%	Texas A&M (2021)
	0.243%	Fleming *et al.* (1960)
	0.4 to 1%	Clydesdale and Francis (1971)
	0.199 to 0.364%	Rickansrud and Hendrickson (1967)
Pork	0.062% to 0.095%	Newcom *et al*. (2004)
	0.1 to 0.3%	Clydesdale and Francis (1971)
	0.079% to 0.16%	Lawrie (1950)
Chicken	0 to 0.582%	Kranen *et al*. (1999)

Dietary intakes of meat in the U.S. population have been estimated by the Economic Research Service (ERS) of the USDA. *Per capita* intakes of meat are reported as part of the ERS Food Availability Data System (FADS), which includes 3 data series on food and nutrient availability for consumption: food availability data, loss-adjusted food availability data, and nutrient availability data. The ERS considers these data to serve as proxies for actual consumption of food commodities at the national level. The food availability data series includes estimates for loss-adjusted food availability data (LAFA) to adjust for food spoilage, plate waste, and other losses thereby more closely approximating actual consumption (USDA-ERS, 2021). Data for loss-adjusted food availability data for red meat, poultry and fish are shown in Table 3.1-2 below. *Per capita* total intake estimates for red meat, poultry and fish were 180g/person per day. Using a mean estimated myoglobin concentration of 0.5% as reported by Yip and Dallman (1996), the total estimated dietary intake of myoglobin is *ca*. 1 g/person per day.

Table 3.1-2 USDA-ERS *Per Capita* Consumption Estimates for Various Meat Sources Adjusted for Loss[a]

Meat Type	*Per Capita* Intake (g/person/day)	Myoglobin Concentration (% wt/wt)[b]	*Per Capita* Myoglobin Intake (g/person/day)
Red Meat	94.4	0.5%	0.472
Poultry	77.6	0.5%	0.388
Total Fish	8.3	0.5%	0.06
Total Meat	180.3	0.5%	0.90

Conc. = concentration; ERS = Economic Research Service; USDA = United States Department of Agriculture.
[a] USDA, Economic Research Service - based on data from various sources as documented on the Food Availability Data System home page. Data last updated June 1, 2020.
[b] Mean concentration of myoglobin based on reported estimates from Yip and Dallman (1996).

Assuming that the proposed food uses of myoglobin in meat alternative products would substitute for various meat products on a 1:1 basis, the introduction of Motif FoodWorks' Myoglobin Preparation to the U.S. marketplace would not change background intakes of myoglobin in the U.S. population. Motif FoodWorks notes that the intended use levels provided here, of up to 2%, are higher than anticipated concentrations naturally occurring in meat products; however, typical use levels in foods are expected to be closer to 1.0 to 1.25% for most food categories. Motif FoodWorks also notes that estimated dietary intakes of Myoglobin Preparation from the proposed food uses will be limited to a large extent by the current market availability of meat alternative products. Although there is limited information available on the current food supply of meat alternative products, it has been estimated that meat alternative products may capture up to 20% of the market for conventional protein sources from animals in North America (Gourévitch *et al.,* 2021). It can be concluded that a maximum use level of up to 2% myoglobin in the diet and 1:1 substitution of meat alternative products for conventional meat products will not increase total dietary intake of myoglobin in the U.S. population.

3.2 Estimated Dietary Intake of Myoglobin Preparation Using NHANES

In addition to the 1:1 substitutional approach described above using *per capita* estimates, Motif FoodWorks has also conducted dietary estimates for the company's Myoglobin Preparation *via* dietary intake modeling using survey data provided by the National Health and Nutrition Examination Surveys (NHANES). As discussed, Motif FoodWorks' Myoglobin Preparation is intended to be used in meat analogue products that will substitute for conventional meat-based products currently in the marketplace. Currently, the majority of such foods in the U.S. marketplace are plant-based products that simulate ground meat (*e.g*., plant-based burgers, sausages and "meat" snacks, frozen entrees).

Using the NHANES Data Derivation [2013-2018 (CDC, 2019)], the U.S. dietary exposure to plant-based meat and poultry analogues was estimated. One-day dietary intake data were analyzed from a population of 22,818 individuals over the age of 2 years, excluding incomplete data and individuals pregnant or lactating, in the data set, using SAS 9.4[4]. The population of individuals in the survey reporting consumption of plant-based meat analogues was extremely small in the survey population [116 participants in the subgroup of a population of 22,818 (NHANES, 2013-2018)]. Therefore, exposure data was limited to consumers only to understand the current consumption patterns of frequent consumers of these products.

Food codes for various meat analogue products (soy-based burger, grain-based sausages, vegetarian hot dog) were selected and dietary intake estimates for total consumers were obtained for myoglobin based on

[4] Fulgoni V (2021) [Personal communication. RE: NHANES data: Dietary exposure to myoglobin added at various levels to plant-based meat analogues. NHANES (2013-2018) data].

incorporation levels of 1%, 1.5%, and 2% for consumers only (N=116). Estimated daily intakes of myoglobin from proposed food uses of the Myoglobin Preparation in meat alternative products is presented in Table 3.2-1. Due to the small sample size, data are limited to mean intakes for consumers only as the 90th percentile estimates were considered unreliable. Mean intakes for consumers aged 2+ years was 0.714 g per person per day based on a myoglobin use level of 1%. At the highest use level of 2% the mean dietary intake of myoglobin was 1.43 g.

Table 3.2-1 Estimated Mean Daily Intake of Myoglobin from Meat Alternative Products in the U.S. for "Consumers Only" Aged 2+ Years (2013-2018 NHANES Data)

Population	Myoglobin Inclusion Level (% wt/wt basis)		
	1%	1.5%	2%
	Mean Intakes of Myoglobin (g/person/day)		
Consumers (2+ years) (N=116)	0.71	1.07	1.43
Male Consumers	0.69	1.03	1.38
Female Consumers	0.73	1.09	1.46

NHANES = National Health and Nutrition Examination Surveys; U.S. = United States.
Due to the small sample size data 90th percentile data was statistically unreliable and therefore is not reported .

As discussed, typical food use applications will incorporate a use level of between 1 to 1.25%, and therefore the estimated dietary intakes of *ca*. 1 g per person per day are largely in-line with background consumption of myoglobin in the diet from conventional meat sources (see Section 3.2). As food uses of the Myoglobin Preparation will be substitutional for conventional meat on a 1:1 basis, no change in total population intakes of myoglobin is expected from the introduction of the ingredient to the U.S. marketplace.

Motif FoodWorks notes that the dietary intake estimates reported in Table 3.2-1 below will over-estimate dietary intakes as it assumes that all potential foods to which Myoglobin Preparation may be added are consumed in a given day. Motif FoodWorks also notes that the general category of meat alternative products is a rapidly growing area of food technology and that current food codes represented within the NHANES databases are unlikely to be inclusive of the broad variety of foods now and soon-to-be available to U.S. consumers. Consumer demand for such products also is growing and therefore limitations in extrapolating the small sample size of consumers (N=116) to the U.S. population of meat analogue consumers should be recognized. Accordingly, Motif FoodWorks placed an emphasis on the dietary intake calculations presented in Section 3.2 where a 1:1 substitution for conventional meat products is assumed relative to the anticipated market share for such products in the immediate and foreseeable future.

Part 4. § 170.240 Self-Limiting Levels of Use

The use of myoglobin in plant-based meat analogues has self-limiting levels of use due to changes in sensory characteristics associated with cooked meat that appear to peak at an inclusion level of around 1.0 to 1.25% of the formulation and may negatively impact flavor and aroma at an inclusion level close to 2% of the formulation. The amount of myoglobin added above the inclusion level of 1.25% in a food formulation limits the sensory acceptability of the plant-based meat analogues.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

The subject of this GRAS Notice is Myoglobin Preparation containing myoglobin, a heme protein obtained through fermentation of a genetically modified strain of *P. pastoris*. The Myoglobin Preparation is a liquid mixture containing bovine myoglobin (≥3% w/w) with a purity of at least 65%. Residual proteins from *Pichia* are <0.2%. The safety assessment of the Myoglobin Preparation therefore focused on hazard characterization of the production organism, and hazard characterization of the protein expression product (*i.e*., myoglobin) under its conditions of intended use. Motif FoodWorks has applied the safety assessment practices used for biotechnology-derived food enzymes outlined by Pariza and Johnson (2001) for the Myoglobin Preparation. Under this safety assessment paradigm, the need for toxicological investigations of enzyme preparations produced using biotechnology is determined on the basis of 2 primary considerations: (1) the availability of data and information substantiating that the production organism is from a safe lineage that has been the subject of previous toxicological evaluations; and (2) that there is evidence to support the safety of the introduced protein expression product(s) (*i.e*., myoglobin). Motif FoodWorks also considered the science-based 2-tiered, weight-of-evidence strategy to assess the safety of novel proteins used in the context of agricultural biotechnology developed by the International Life Sciences Institute (ILSI) International Food Biotechnology Committee (Delaney *et al*., 2008). Under this paradigm, the safety assessment draws upon knowledge of the biological and chemical characteristics of the protein for analyses of hazard at the Tier I level and includes an assessment of the biological function or mode of action and intended application of the protein, history of safe use, comparison of the amino acid sequence of the protein to other proteins, as well as the biochemical and physico-chemical properties of the proteins. Only proteins that cannot be adequately characterized under the Tier I evaluation would proceed to toxicological evaluation under Tier II.

With respect to the safety of the production organism, as discussed in further detail in Section 6.2, the production strain, *P. pastoris* t838417, is a non-pathogenic and non-toxigenic yeast species with a long history of safe use in food production (U.S. FDA, 2018b). The production strain has been genetically modified to express a synthetic gene encoding for bovine myoglobin. Other than the gene encoding for bovine myoglobin, the production strain does not contain any other exogenous DNA, and the final Myoglobin Preparation is absent of detectable levels of the production strain. Successful integration of the myoglobin gene has been confirmed using whole genome sequencing. The introduced nucleotide sequences are codon optimized for expression in *Pichia* are confirmed to encode for a protein sequence that is identical to bovine myoglobin. Motif FoodWorks has demonstrated that the production strain (NRRL Y-7556) is genetically identical to the strain host (NRRL Y-11430) used for the manufacture of soybean leghemoglobin described by Impossible Foods in GRN 737 and therefore is from a safe strain lineage with a history of food use. Bioinformatic evaluations conducted on the production strain by Jin *et al*., (2018) and Reyes *et al*., (2021), have demonstrated that residual proteins from the production strain are non-toxigenic and of low allergenic potential for cross-reactivity to major food allergens.

For safety evaluation of the myoglobin protein, Tier I evaluation leveraged the history of safe consumption of myoglobin from meat, and therefore emphasis was placed on demonstrating qualitative equivalence of myoglobin in Motif FoodWorks' flavor preparation to myoglobin from meat. If it could be demonstrated that Motif FoodWorks' myoglobin is qualitatively equivalent to myoglobin in meat, then it could be concluded that a 1:1 substitutional use of the Myoglobin Preparation in meat analogue products would be as safe as dietary intake of myoglobin from current food consumption patterns of meat and other myoglobin containing foods. In this regard, Motif FoodWorks has presented analytical data confirming the identity of the myoglobin synthesized by the production strain using qualitative comparisons of the myoglobin preparation relative to a commercial bovine myoglobin standard using SDS-PAGE, SEC, and proteomic mass spectrometry. The only qualitative difference between myoglobin expressed by *Pichia* and bovine derived myoglobin is the relative absence of glycosylation in *Pichia* expressed myoglobin, which compares to an observed low-level O-glycosylation of bovine myoglobin in at least 3 residues. Motif FoodWorks has therefore concluded that there are no qualitative differences between myoglobin expressed by *Pichia* relative to native bovine myoglobin that is present in meat. Accordingly, the long history of safe consumption of myoglobin from consumption of meat products can be extended to Motif FoodWorks' myoglobin ingredient. As the outcome the Tier I hazard assessment was sufficient to conclude on safety of the ingredient under its intended conditions of use, it was concluded that further hazard characterization *via* toxicology testing under the ILSI Tier II testing scheme was not required.

Myoglobin is present in all commonly consumed meat sources, such as beef, pork, and poultry, and has an extensive history of safe consumption by the global population. There is a common knowledge of the history of consumption of myoglobin from animal sources. In order to corroborate the history of safe consumption of myoglobin, a comprehensive search of the scientific literature was conducted through March 2021. The literature search was completed using ProQuest and included searches of the following databases for pertinent literature on the safety of bovine myoglobin or myoglobin from *Bos taurus*: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The relevance and specificity of the literature search was increased through the implementation of search terms "bovine myoglobin" or "myoglobin from *Bos taurus*" to reflect the compound of interest in combination with preclinical/clinical endpoints. The search results were retrieved and reviewed in 2 stages (titles and abstracts). The search did not identify any publications, relevant to the safety of bovine myoglobin.

The safety of the bovine myoglobin present in Motif FoodWorks' Myoglobin Preparation is supported by its long history of safe consumption, as well as bioinformatics searches of the protein evaluating its lack of allergenicity and toxigenicity potential. The results of these searches are discussed in Sections 6.3.2 and 6.3.3, respectively, and indicate that Motif FoodWorks' Myoglobin Preparation would not pose an allergenic or toxigenic risk to U.S. consumers.

6.1 Safety of the Production Strain

The safety of the production strain used in the production of Motif FoodWorks' myoglobin was assessed using the same principles for assessing the safety of microbially-derived enzymes for use in food production (Pariza and Foster, 1983; IFBC, 1990; Pariza and Johnson, 2001; Sewalt *et al*., 2016; FAO/WHO, 2020). This approach to the safety evaluation of food enzymes is widely accepted by the scientific community and regulatory agencies and includes an evaluation of the pathogenicity, toxigenicity, and antimicrobial resistance of the production strain, as well as the genetic modification techniques. These points are discussed herein.

P. pastoris is a well characterized non-toxigenic and non-pathogenic microorganism that has a recognized history of safe use in food production. Current *P. pastoris* laboratory strains are from lineages isolated from an oak tree and a chestnut tree and were deposited in a culture collection at the NRRL[5] (www.biogrammatics.com).

Information on the non-pathogenicity and non-toxigenicity properties of *P. pastoris* was discussed in GRN 737 and is incorporated by reference to Section 6.1.3 of the Notice. *P. pastoris* is recognized as a non-toxin producing microorganism and is classified as a biosafety level 1 (BSL1) organism by the ATCC. This species has QPS status in the EU for use in enzyme production (EFSA, 2017), corroborating that *P. pastoris* is a safe and suitable source organism for production of food ingredients and is not capable of producing toxic metabolites when used for food protein production. *P. pastoris* is widely used by the biotechnology industry for the production of recombinant proteins and food enzymes (Cereghino and Cregg, 2000; Cregg *et al*., 2000; Balamurugan *et al*., 2007; Kurtzman, 2009; Reyes *et al*., 2021), with over 300 recombinant proteins produced from this species since the 1980s (Diversa Corporation, 2006; U.S. FDA, 2006). Dried *P. pastoris* is also permitted for the addition to chicken feed as a source of protein under 21 CFR § 573.750 (U.S. FDA, 2020b). This information suggests that *P. pastoris* is non-pathogenic to humans and non-toxigenic and would therefore be a safe and suitable source organism for production of myoglobin (Pariza and Johnson, 2001).

The production strain used in the production of Motif FoodWorks' myoglobin is a genetically modified strain of *P. pastoris*. The production strain was constructed in a similar manner as described in GRN 737 (Impossible Foods, Inc., 2017; U.S. FDA, 2018a) using the principles described by OECD GILSP (OECD, 1992, 1993). Motif FoodWorks' strain of *P. pastoris* (strain t486367) meets the criteria for a safe and suitable source organism described by Pariza and Johnson (2001). The production strain was obtained from a wildtype strain of *P. pastoris* (t303048). A synthetic gene encoding for bovine myoglobin was inserted into the wildtype strain; this strain also contains extra copies of the heme biosynthetic enzymes native to *P. pastoris*. The synthetic gene encoding for bovine myoglobin is the only non-native gene present in the production strain and has been confirmed by bioinformatics to not confer any pathogenic, virulent, or toxigenic factors to the production strain. The genetic stability of the production strain was confirmed after growth on non-selective fermentation media. The production strain does not contain any plasmids or antibiotic resistance genes, as confirmed by phenotyping and whole genome sequencing of the production strain. Overall, it can be concluded that Motif FoodWorks' *P. pastoris* production strain is derived from a strain lineage with a long history of safe use.

Motif FoodWorks' Myoglobin Preparation may contain ≤0.2 mg/L *Pichia* protein. The toxigenicity of *Pichia* proteins was recently discussed by Jin *et al.* (2018) and Reyes *et al.* (2021) following a proteomics assessment of the native proteins expressed by *P. pastoris*. It was concluded that the native *Pichia* proteins do not share structural homology with known toxins and would not cause a toxigenicity concern (Jin *et al*., 2018; Reyes *et al*., 2021). Therefore, on the basis that Motif's production strain was derived from the same host strain lineage as that investigated by Jin *et al*., (2018) and Reyes *et al*., (2021), Motif has concluded that the small concentrations of residual *Pichia* proteins present in the ingredient would not pose a safety concern.

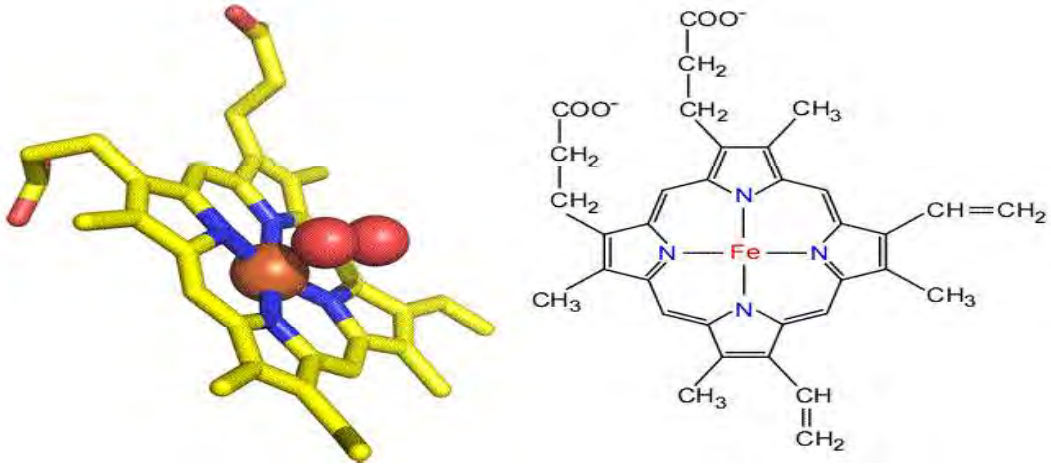
[5] The NRRL collection has been renamed to the Agriculture Research Service Culture Collection and is maintained by the Microbial Genomics and Bioprocessing Research Unit (MGB) of the National Center for Agricultural Utilization Research (NCAUR).

6.2 Safety of Myoglobin

6.2.1 History of Safe Consumption of Myoglobin

Myoglobin is part of a superfamily of heme-containing globular proteins involved in binding iron and/or transportation of oxygen. This globular heme protein is ubiquitous in nature and present in most organisms including bacteria, protozoa, fungi, plants, and animals (Hardison, 1998). Hemoglobin and myoglobin are structurally similar to other heme proteins and contain the identical heme B cofactor. The chemical structure of myoglobin is presented in Figure 6.2.1-1. Consumption of the heme B cofactor is widespread in humans and other animals as heme proteins, such as myoglobins and hemoglobins are abundant in animal tissues where they are consumed as meat. Heme proteins, specifically myoglobins, have been present in the human diet since the beginning of recorded history. Heat treatment of these iron-binding proteins potentiates the meat-like, serum and metallic flavors typically associated with cooked muscle tissue (AMSA, 2015). The Myoglobin Preparation is standardized to contain ≥3% heme protein (see Section 2.3 for further details).

Figure 6.2.1-1 Chemical Structure of Myoglobin



The myoglobin present in Motif FoodWorks' Myoglobin Preparation is 100% identical to bovine myoglobin and shares structural homology to hemoglobin proteins from other commonly consumed animal sources of meat. Considering myoglobin is widely distributed in commonly consumed meats, such as beef and pork, the protein itself has an apparent long history of safe consumption in the human diet. As discussed in Section 3.1, myoglobin is currently consumed at a level of approximately *ca*. 1 g/person per day from red meat, poultry and fish sources in the U.S. diet. In addition, meat extracts and concentrates produced using meats from sources such as bovine, containing myoglobin, are widely consumed in the U.S. population.

Meat extracts, meat protein extracts, and beef protein, which include myoglobin, are considered safe and suitable ingredients for use in the production of meat, poultry, and egg products under FSIS Directive 7120.1 (USDA-FSIS, 2021). The GRAS status of beef protein when used as a binding agent at levels up to 0.89% was filed by the U.S. FDA without objection under GRN 313 (U.S. FDA, 2010).

Therefore, there is a long history of safe consumption of bovine myoglobin in the human diet. To date, there have been no reports of adverse effects following consumption of bovine myoglobin. Similarly, although allergenicity to red meat has been reported in the scientific literature, only 1 case has been associated with myoglobin (see Section 6.2.2 for further details). The amino acid sequence of bovine myoglobin does not contain significant sequence homology to known toxins or allergens, and therefore, would not raise toxigenicity or allergenicity concerns.

6.2.2 Allergenicity of Myoglobin

Food allergies reportedly occur in about 8% of children and less than 2% of adults in the U.S. population, and are most frequently associated with one of the "Big Eight" major allergens [*i.e*., milk, egg, fish (*e.g*., bass, flounder, or cod), Crustacean shellfish (*e.g*., crab, lobster, or shrimp), tree nuts (*e.g*., almonds, pecans, or walnuts), wheat, peanuts, and soybeans] that require allergen labeling under the *Food Allergen Labeling and Consumer Protection Act* of 2004 [(FALCPA) U.S. FDA, 2018b; National Academy of Medicine, 2016]. Although not considered one of the major allergens, red meat allergies have been reported in some individuals, but these cases are rare. The incidence rate of beef allergy was reported to be between 3.28% and 6.52% among children with atopic dermatitis, and about 0.3% in the general population (Fiocchi *et al*., 2000). Most reported cases of allergenic responses to meat, specifically beef, involve sensitization to bovine serum albumin (BSA), and, to a lesser extent, bovine gamma globulin (BGG) (Werfel *et al*., 1997; Fiocchi *et al*., 2000; Vazquez Fuertes *et al*., 2013). BSA and, to a lesser degree, BGG, also are identified as allergenic proteins from cows' milk. BSA and BGG are heat-labile proteins and meat-allergic individuals are typically reactive to undercooked meat. Nevertheless, Fuentes *et al.* (2004) reported a singular case of a 35-year-old woman having allergic episodes after exposure to beef, lamb, and fish and without allergic response to milk. Negative skin prick tests were reported from the subject's assessment while IgE responses differed among proteins presented under differing environmental conditions. Largely degraded proteins were identified in heated meat extracts except a heat-stable, 17 kDa protein, identified as bovine myoglobin, which stayed in solution. While researchers reported significant amino acid sequence homology among myoglobins from different species, the amino acid sequences are not identical. Nevertheless, the evidence indicates that myoglobin was the probable cause of the allergic reaction occurring in this patient. The relevance of bovine myoglobin in this allergenic case report has been disputed (Fiocchi *et al*., 2005) and reviewed in GRN 737 (Impossible Foods, Inc., 2017; U.S. FDA, 2018a). Bioinformatics on myoglobin from different animal species, including cow, pigs, sheep, goats, and chicken, indicates that bovine myoglobin shares structural similarities with myoglobin from goat, sheep, and pig meat, and may cause allergenicity (Chakraborty *et al*., 2014). These findings may explain the reported allergic responses after exposure to beef and lamb in the 35-year-old woman reported by Fuentes *et al.* (2004). Nevertheless, it should be reiterated that beef allergy is rare considering widespread consumption of beef and other meats containing oxygen-binding globin proteins in the global population, and a search of the scientific literature[6] indicates other cases of myoglobin allergy have not been reported since 2004.

[6] Databases searched included: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®.

Over the past decade, meat allergy also has been associated with a carbohydrate, α-Gal (galactose-α-1,3-galactose) linked to meat proteins (Platts-Mills et al., 2020). Affected individuals are typically sensitized to α-galactose from being bitten by one of several species of ticks (ACAAI, 2014). The symptoms of this specific type of meat allergy, known as "α-Gal syndrome," have a delayed onset time of several hours and occur with all species of mammalian meats. According to Kuehn (2018), an unusual set of human physiological associations exist with α-Gal mammalian meat allergy, including subject blood type, past infection, co-existing allergy, and a previous tick bite (often Lone Star Tick). Not all individuals who are sensitized to galactose-α-1,3-galactose have reported allergic reactions to meat. Platts-Mills et al. (2020) reported that of 300 hunters and forest workers in Germany, 58 individuals were positive for α-Gal syndrome, of which, only 5 individuals had allergic reactions to mammalian meat or innards. The causes of the α-Gal allergy are not known but the presence of galactose-α-1,3-galactose in the human body initiates an antigen reaction. Galactose-α-1,3-galactose exists in all mammalian species except humans where it is not naturally present and can be transmitted to humans *via* ticks. The only apparent treatment of α-Gal syndrome is elimination of red meat from the diet. As described in Section 2.2, Motif FoodWorks' myoglobin is not glycosylated, and as a taxonomically distant species *P. pastoris* would not produce endogenous *alpha*-gal epitopes.

A sequence homology search was conducted using the AllergenOnline database version 21 (updated 14 February 2021) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2021) to determine whether the bovine myoglobin[7] shares significant sequence homology to known allergens. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. A sequence homology search was conducted according to the approach outlined by the FAO/WHO (2001) and the WHO/FAO (Codex Alimentarius, 2009). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length bovine myoglobin amino acid sequence. The 80 amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility (FAO/WHO, 2001). Using this search strategy, no matches were identified. A sequence homology search conducted using the exact 8-mer approach did not produce any matches. The results of the sequence homology search indicate that bovine myoglobin present in Motif FoodWorks' Myoglobin Preparation does not pose an allergenic risk to consumers.

Based on the totality of evidence, meat allergy is rare and is not associated with consumption of myoglobin in the human diet. Allergic responses to meat consumption, specifically red meat from bovine sources, have been associated with the heat-labile proteins BSA and BGG; only 1 case of allergic reaction to myoglobin has been reported in the scientific literature in 2004. Likewise, Motif FoodWorks' Myoglobin Preparation does not contain galactose-α-1,3-galactose and would not pose any risk of α-Gal syndrome. Bioinformatics on the amino acid sequence of bovine myoglobin suggest that the protein does not share significant sequence homology with known allergens, and cross-reactivity of the protein to known allergens is unlikely. Therefore, the available evidence suggests that consumption of Myoglobin Preparation would not pose any significant allergenic risk in the U.S. population.

[7] The allergenicity search was performed using the amino acid sequence of bovine myoglobin available under UniProt Accession No. P02192.

In addition to myoglobin, the Myoglobin Preparation may contain residual levels of native proteins from *P. pastoris*. The potential allergenicity of *Pichia* proteins was addressed in publications by Jin *et al.* (2018) and Reyes *et al.* (2021). LC-MS/MS Proteomics was used to identify and semi-quantify residual *Pichia* proteins in a leghemoglobin preparation, the subject of GRN 737, which were then investigated for potential allergenicity using *in silico*-based methods based on recommendations by the Codex Alimentarius (2009). The authors concluded that residual proteins originating from the source organism that may be present do not share significant sequence homology with known allergens, and therefore, do not pose a risk of cross-reactivity (Jin *et al*., 2018; Reyes *et al*., 2021). Conversely, the *Pichia* proteins share significant sequence homology with proteins from common yeasts, such as *Saccharomyces* spp. Based on the available data, the authors concluded that *Pichia* proteins are unlikely to present a risk of allergenicity. As the *Pichia* proteins evaluated by Jin *et al*., (2018) and Reyes *et al*., (2021) were derived from the same host strain lineage as that used by Motif, conclusions that residual *Pichia* proteins are of low toxicity risk can be extended to Motif's Myoglobin Preparation.

6.2.3 Toxigenicity of Myoglobin

The myoglobin present in Motif FoodWorks' Myoglobin Preparation is 100% identical to bovine myoglobin (UniProt Accession No. P02192). The amino acid sequence of the bovine myoglobin was compared against downloaded protein sequences obtained from a curated database of animal venom proteins and toxins maintained in the UniProtKB/Swiss-Prot Tox-Prot database[8] (Jungo *et al*., 2012) using the Basic Local Alignment Search Tool (BLAST) maintained by the National Center for Biotechnology Information. Searches were performed using the default search parameters (E-value threshold = 0.05; BLOSUM62). The search was conducted on March 23, 2021 and the toxin database included 7,271 proteins. One match to a delta-conotoxin from *Conus gloriamaris* was identified with an identity score of 28% and E-value of 0.02. The query cover was 41% and maximum bit-score was 29.3. Currently, there are no formal guidelines established for what would constitute a significant sequence similarity between a query protein and a known protein toxin (Hammond *et al*., 2013). However, Pearson (2013) reported for protein alignments, an E-value or E-score of <0.001 can reliably be used to infer homology, and alternatively, the bit-score may be used to infer homology and is considered to be a more reliable indicator of significant sequence homology. A bit-score of 50 is "*almost always significant*", while a bit-score of 40 is only significant (E-value <0.001) in searches of protein databases with less than 7,000 entries (Pearson, 2013). Furthermore, Pearson (2013) reported that "*homologous sequences that share more than 40% identity are very likely to share functional similarity*" and the E-value or E-score is commonly used to determine the statistical significance of excess similarity. Therefore, based on these criteria, the identified match with delta-conotoxin from *C. gloriamaris* is not considered to be suggestive of significant sequence homology, and bovine myoglobin does not share structural homology or similarity to any known animal venom protein or toxin, and would not harbor any toxic potential on the basis of the *in silico* search.

Myoglobin Preparation may contain ≤0.2 mg/L of proteins from the source organism, *Pichia pastoris*. The production strain is genetically modified to express a gene encoding for bovine myoglobin and does not contain any other exogenous sources of DNA; with the exception of the synthetic DNA encoding for bovine myoglobin, the production strain only contains DNA that is native to *P. pastoris*. The toxigenicity of native *Pichia* protein was discussed in 2 separate publications on a genetically modified strain of *P. pastoris* used as a production strain for soy leghemoglobin (Jin *et al*., 2018; Reyes *et al*., 2021). In these publications, it was concluded that the native *Pichia* proteins do not share sequence homology with any toxins, thus maintaining that *P. pastoris* is a non-toxigenic organism. The toxigenicity of the same *P. pastoris* production strain was addressed in GRN 737 in which the U.S. FDA did not raise any safety concerns with the

[8] Available at: https://www.uniprot.org/program/Toxins.

production organism (Impossible Foods, Inc., 2017; U.S. FDA, 2018a). As previously discussed, *P. pastoris* has a long history of safe use in food production, and to date, no toxic effects of this species have been reported in the scientific literature. Analytical data demonstrated that Myoglobin Preparation is a highly purified ingredient (purity >98%), in which the production strain is removed from the final product. Therefore, Motif FoodWorks' Myoglobin Preparation is not anticipated to pose any toxigenic concern from either the myoglobin itself or arising from the manufacturing process.

6.3 General Recognition of Safety

Motif FoodWorks has concluded that Myoglobin Preparation containing bovine myoglobin is GRAS for use in meat analogue products, as described in Section 1.3, based on scientific procedures. The safety of *P. pastoris* was evaluated using generally recognized safety assessment practices applied to food enzymes under the Pariza Johnson decision tree (Pariza and Johnson, 2001). In this regard Motif FoodWorks has demonstrated that the production strain is from a safe lineage of *P. pastoris* that has been previously demonstrated to be safe for use in food production of similar food ingredients (*e.g*., soybean leghemoglobin).

The safety of myoglobin was evaluated using the 2-tier testing paradigm developed by the ILSI for evaluation of proteins used in the context of agricultural biotechnology (Delaney *et al*., 2008). Bovine myoglobin has a long history of apparent safe consumption from ingestion of beef. Myoglobins are highly conserved among animal species and therefore are also consumed from ingestion of other red meats, poultry, and fish. Although bovine myoglobin from *P. pastoris* does not have a history of consumption, Motif FoodWorks has demonstrated that bovine myoglobin expressed by *P. pastoris* is qualitatively highly identical to bovine myoglobin that is present in red meat. Bovine myoglobin is intended for use in meat analogue products that will substitute 1:1 for meat products on the marketplace and in the absence of qualitatively meaningful differences in the identities of bovine myoglobin from *Pichia* to bovine myoglobin from beef, the history of safe consumption of myoglobin from meat consumption can be extended to Motif FoodWorks' ingredient.

Motif FoodWorks has concluded that use of the company's Myoglobin Preparation in meat alternative products, as described in Section 1.3, are GRAS on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of myoglobin and *P. pastoris*, as discussed herein, and on consensus among a panel of experts qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Professor Emeritus Stephen L. Taylor, Ph.D. (GRAS Panel Chair), (University of Nebraska; Professor Emeritus Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine); and Professor Emeritus Michael W. Pariza, Ph.D., (Food Research Institute University of Wisconsin-Madison).

The GRAS Panel, convened by Motif, independently and critically evaluated all data and information presented herein, and also concluded that Motif's Myoglobin Preparation is GRAS for use as a 1:1 substitution for meat in meat alternative products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel is presented in Appendix A.

6.4 Conclusion

Based on the above data and information presented herein, Motif FoodWorks has concluded that the intended uses of Myoglobin Preparation in meat alternative products intended to substitute for current red meat and poultry sources on a 1:1 basis, as described in Section 1.3, is GRAS based on scientific procedures.

General recognition of Motif FoodWorks' GRAS conclusion is supported by the unanimous consensus rendered by an independent panel of experts, qualified by experience and scientific training, to evaluate the use of Myoglobin Preparation in food, who similarly concluded that the intended use of Myoglobin Preparation as described herein is GRAS.

Motif FoodWorks' Myoglobin Preparation therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. § 170.255 List of Supporting Data and Information

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From: Janet Collins
To: Anderson, Ellen
Cc: Kulkarni, Deepti A.
Subject: [EXTERNAL] Motif GRN1001
Date: Thursday, August 12, 2021 2:18:07 PM
Attachments: image001.png

Dear Ellen:

On behalf of Motif FoodWorks Inc. (Motif), I write regarding the company's GRAS Notification for myoglobin preparation (designated as GRN 1001), which is currently under evaluation by OFAS. It has come to our attention that the Notification included a drafting error. Per our teleconference with you earlier this week, I write to correct the error. As we discussed, the Notification states that myoglobin preparation may contain ≤0.2 mg/L of residual *Pichia pastoris* protein (see pgs. 5,6, 11, 19, 21 and 25). It, instead, should have stated that the myoglobin preparation may contain ≤0.2 mg/L of residual *Pichia pastoris* DNA.

As we also discussed, this clarification does not change the company's conclusion that its myoglobin preparation is generally recognized as safe, within the meaning of section 201(s) of the Federal Food, Drug, and Cosmetic Act (21 USC 321(s)) and FDA's implementing regulations (codified at 21 CFR part 170), for the reasons set forth in the Notification.

Best regards,

Janet E. Collins, Ph.D., R.D.
Vice President Regulatory, Government and Industry Affairs
Motif FoodWorks
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September 2, 2021

Ms. Ellen Anderson
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Sent via email

Dear Ellen - On behalf of Motif FoodWorks, and in response to your letter of August 16, 2021, we are providing the following responses to the Agency's questions on Motif's GRAS Notification (GRN 1001).

1. *FDA. The manufacturing process described in the notice includes a step in which the production strain cells are lysed. Please elaborate on the method used to induce lysis (e.g., whether it is a mechanical process or involves the use of reagents).*

   The production strain cells are lysed using a mechanical process that relies on a bead mill. The cells enter the mill as part of the fermentation broth and are disrupted by bead milling to release the myoglobin protein. Sodium phosphate buffer is added at the end of the lysing process to clear the cellular debris from the bead mill.

2. *FDA. Please confirm that none of the components of the fermentation media are derived from major food allergens.*

   Motif confirms that none of the components in the fermentation media are derived from a "major allergen" as defined in section 201(qq) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321(qq).

3. *FDA. Motif states that all methods of analysis are internationally recognized, and the notice includes citations for the methods used for specifications parameters except for the method used to assay*

myoglobin protein and myoglobin protein purity. Please confirm that all methods are validated for their respective purposes.

Motif confirms that all methods of analysis used in the specification are validated and fit for purpose.

The assay to assess presence of myoglobin protein (HPLC), and presence and purity (UV Vis) was revised for suitability to this fermentation-derived heme protein, validated by Ginkgo Bioworks. According to Nobel (2014[1]), the measurement of a solubilized protein concentration in solution is an important assay in R&D labs. Spectrophotometric protein quantification assays use UV and visible spectroscopy to rapidly determine the concentration of protein, relative to a standard, or using an assigned extinction coefficient, and to establish total protein in a mixture. The UV value of 280nm is used to quantify total protein while the concentration of specific proteins is associated with specific wavelengths. Heme proteins have a targeted specific wavelength- for myoglobin, the wavelength to establish concentration of the protein is at the absorbance wavelength of 420nm.

A copy of the SOP for the myoglobin determinations is attached (Attachment 1).

4. *FDA. We note that the specified limits for lead and cadmium are relatively high in comparison to the reported batch analyses results. Please discuss whether any heavy metals are expected to be present in the final product and how the specification limits compare to the limits of quantification for the method used.*

We do not expect cadmium or lead residues to be present in the Myoglobin Preparation owing to the use of food grade ingredients and processing-aids and treatment of the municipal water by reverse osmosis. We recognize that the specifications provided for all heavy metals are incongruous with levels reported from our third-party analytical laboratories.

Upon reviewing these data, after your question, we recognized that the manufacturing inputs will not be sources of arsenic, cadmium, or mercury and therefore these parameters will be removed

[1] James E. Noble, 2014. Quantification of Protein Concentration Using UV Absorbance and Coomassie Dyes, In Methods in Enzymology. Vol 536, p 17-26. Editor(s): Jon Lorsch, Academic Press.

from the specification. Although lead is similarly not expected to be a contaminant of the myoglobin preparation Motif will retain a lead specification to be consistent with the Agency's expectations of food grade ingredients. Based on the batch results Motif will set a lead specification of ≤ 0.01ppm, using AOAC 2015.01 Mod 2232.

5. *FDA. The manufacturing process described in the notice includes the use of methanol as a carbon source. Please confirm whether any residual methanol is expected to be present in the final product and if so, discuss whether a specified limit has been established.*

In the manufacturing process used to make myoglobin, fermentation continues until the methanol concentration is undetectable (0.00g/L methanol) *via* the YSI 2950 analyzer. HPLC analysis confirms that no methanol remains in the fermentation broth.

6. *FDA. Please confirm whether the three batches of myoglobin preparation presented in the notice are consecutive or non-consecutive production lots.*

Samples used to support GRN 1001 are consecutive production lots. Although they are consecutive lots, they are not continuous production runs. A complete CIP process is used between each of the productions. Each part of the equipment used in the process is taken down, sterilized, and replaced for subsequent fermentation runs.

7. *FDA. Motif states that the myoglobin is formulated with excipients, and the final preparation is stored as a frozen liquid at -20 °C. However, the notice does not include a discussion of the stability of myoglobin preparation. Please provide a narrative of the stability of myoglobin preparation under the recommended storage conditions, including a summary of the results of any stability studies and estimates of shelf life.*

Myoglobin protein present in Motif's Myoglobin Preparation is a stable protein at pH 6.5-8.0 and remains in suspension in a sodium phosphate buffer that includes sodium chloride and sodium ascorbate. Myoglobin Preparation underwent four freeze-thaw cycles with no impact on stability of the protein or on the quality of cooked patties prepared using the previously frozen

Myoglobin Preparation. Freezing and thawing the Preparation had no impact on microbial growth, sensory quality, or color stability when used to formulate and cook soy burger patties.

Motif further assessed stability by studying the impact of heat and temperature changes on Myoglobin Preparation. Myoglobin Preparation remains as a liquid dispersion of myoglobin protein in water up to 60°C with stabilizers added. Myoglobin protein begins to unfold, and a decrease in color (redness) is noted at 40°C although the protein remains suspended. While myoglobin stability is maintained over a range of temperatures as heat is applied (as in cooking), the protein does not degrade but rather destabilizes and denatures at temperatures above 40°C. UV-VIS analysis confirms that the myoglobin protein in the Myoglobin Preparation changes color as the heme iron is reduced from oxymyoglobin to metmyoglobin when heating Myoglobin Preparation higher than 40°C but the myoglobin protein remains intact.

Based on studies assessing raw and cooked burger quality and appearance of burgers prepared using the Myoglobin Preparation, we estimate the shelf life to be 12 months at -20°C, and a refrigerated shelf life (upon thawing) of ≤7 days when stored at ≤8°C.

8. *FDA. The notice includes estimates of dietary exposure to myoglobin protein from the intended uses of the myoglobin preparation. Please provide estimates of dietary exposure to the myoglobin preparation. In addition, please include estimates of dietary exposure for myoglobin preparation and myoglobin protein for upper percentile consumers (e.g., those at the 90th percentile). Motif states that estimates of exposure at the 90th percentile were unreliable due to a limited sample size of consumers of plant-based meat and poultry analogues in NHANES dataset. We note that in guidance to industry (https://www.fda.gov/regulatoryinformation/search-fda-guidance-documents/guidance-industry-estimatingdietary-intake-substances-food) dietary exposure at the 90th percentile can typically be estimated as approximately twice the mean.*

To address this question, analytical data was obtained characterizing the nutritional profile of Motif's Myoglobin Preparation and the estimated intakes of each nutrient from the intended food uses of the Preparation in simulated meat products were calculated using the NHANES Data Derivation [2013-2018 (CDC, 2019)]. The nutrients reported include total protein, iron, yeast, sodium ascorbate, sodium phosphate, sodium chloride, total sodium, and phosphorus.

Dietary intake estimates were conducted for the total population and users-only of simulated meat products at a use level of 1.0, 1.5, and 2.0% of the Preparation in plant-based product formulations.

A second query was conducted to estimate the nutritional impact of the intended food uses on the ***total diet from inclusion of the Myoglobin Preparation*** at 1.0, 1.5, and 2.0% in addition to background dietary intakes of each nutrient.

Our analysis was conducted using statistical modeling software (SAS 9.44) and data provided from the 2013-2018 NHANES survey. The 2013-2018 survey included dietary intake information provided from a population of 22,818 individuals over the age of 2 years, excluding incomplete data and individuals pregnant or lactating women. The population of individuals in the survey reporting consumption of plant-based meats and poultry, as reported in the GRN 1001, was small including 116 participants in the subgroup of a population of 22,818 (NHANES, 2013-2018).

Various plant-based meat analogue products included in the 2013-2018 NHANES food codes were used to represent meat analogue products (soy-based burger, grain-based sausages, vegetarian hot dogs) to determine the impact of the addition of the Myoglobin Preparation to the usual diets of individuals consuming plant-based meat analogues. Dietary intake estimates were established for the total population and for consumers-only, to estimate exposure to various nutrients by the total population and by heavy consumers of plant-based meat analogue foods.

Data presented in Table 1.0 represents dietary intake estimates of the individual components of the Myoglobin Preparation from the intended food uses of the Preparation in meat-analogue products and includes data for myoglobin protein, water, sodium phosphate, sodium chloride, sodium chloride, and yeast (from *Pichia pastoris*). Data is presented for the total population of consumers and for consumers-only. Due to the low number of consumers of meat analogue products dietary intakes for total population was negligible. Due to the small sample size for consumers only, dietary intake estimates for 90th percentile consumers were considered statistically unreliable; however, Motif notes that the 90th percentile intake values were approximately 2x the mean intake values and were therefore considered reasonable approximates of consumption by heavy consumers of meat

analogue products and as recommended by the FDA's guidance to industry for estimating 90th percentile dietary exposures[2].

Table 1. Dietary Intake of Nutrients [Means and (90th Percentiles[3])] Among Total Population, and Consumer's Only from the Intended Food Uses of Various Levels of Myoglobin Preparation

Nutrient	Population	Inclusion Levels (%)					
		1		1.5		2	
		Mean	90th%	Mean	90th%	Mean	90th%
Iron	Total Population	0.01	0.02	0.01	0.02	0.01	0.02
	Consumers-Only	1.06	2.17	1.60	3.21	2.13	4.35
Phosphorus (mg)	Total Population	0.09	0.18	0.13	0.26	0.17	0.340
	Consumers-Only	13.97	29.56	20.95	42.84	27.93	4.35
Protein (g)	Total Population	0.01	0.02	0.01	0.02	0.01	0.02
	Consumers-Only	0.73	1.50	1.10	2.24	1.46	2.98
Sodium (mg)	Total Population	0.24	0.48	0.35	0.70	0.05	0.10
	Consumers-Only	37.85	77.41	56.77	116.11	75.70	154.91
Sodium chloride (g)	Total Population	0.001	0.002	0.001	0.002	0.002	0.004
	Consumers-Only	0.14	0.29	0.21	0.44	0.29	0.58
Sodium phosphate (g)	Total Population	0.001	0.002	0.002	0.004	0.003	0.006
	Consumers-Only	0.202	0.414	0.304	0.621	0.405	0.828
Sodium ascorbate (g)	Total Population	0	0	0	0	0.001	0.002
	Consumers-Only	0.05	0.10	0.07	0.15	0.10	0.19
Yeast (g)	Total Population	0.01	0.02	0.01	0.02	0.02	0.04
	Consumers-Only	1.19	2.43	1.79	3.65	2.38	4.87

Exposure estimates of each nutrient ***resulting from consumption of foods containing the Myoglobin Preparation in addition to background intakes of each nutrient from the total diet*** are presented in **Table 2**. Estimates for nutrient exposures are presented for the survey subset of 22,818 individuals represented as "Total Population users as well as and 'Consumers-only' users (n=116) who reported consumption of plant-based meat and poultry analogues. Due to the small sample size of consumers

[2] *https://www.fda.gov/regulatoryinformation/search-fda-guidance-documents/guidance-industry-estimatingdietary-intake-substances-food*

[3] 90th percentile numbers for "All" are calculated as 2x the mean for the category; "Consumers 90th percentile represent data from the NHANES data.

of meat analogue products, the data provided in **Table 2** provide a good comparison of background intakes for the general population as well as the dietary impact of adding myoglobin to the diet from simulated meat products. As can be seen in the Table, the introduction of Myoglobin Preparation to simulated food products produced trivial changes in the dietary intakes of most nutrients relative to background intakes by the general population. Dietary intakes of iron were increased marginally (*ca.* +10%) in most population groups, an effect that is nutritionally desirable.

Table 2. Nutrients [Means and (90th Percentiles)] Calculated from Intended Food Uses and Background Intakes with Various Levels of Myoglobin Preparation

Nutrient	Population	At Inclusion Levels (%) and 90th Percentile Consumption					
		1		1.5		2	
		Mean	90th%	Mean	90th%	Mean	90th%
Iron (mg)	Total Population	14	24	14	24	14	24
	Consumers-Only	16.8	26.1	17.4	26.7	18.1	27
Phosphorus (mg)	Total Population	1358	2193	1358	2192	1358	2192
	Consumers-Only	1447	2072	1456	2080	1465	2086
Protein (g)	Total Population	79	130	79	130	79	130
	Consumers-Only	82	135	83	136	83	136
Sodium (mg)	Total Population	3400	5646	3401	5646	3403	5647
	Consumers-Only	3612	5428	3636	5472	3660	5538

9. *FDA. Please address the 90th percentile consumption of heme iron from the 90th percentile consumption of myoglobin and explain why such consumption is not expected to pose any iron-mediated safety problem.*

The Food and Nutrition Board (FNB) of the Institute of Medicine (IOM) has established tolerable upper intake levels (UL's) for most nutrients, and this value represents the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. The UL for iron after age eight for boys and 13 for girls is 45 mg/day; all others

at a younger age have a UL of 40 mg/day (IOM, 2001[4]). As demonstrated from the data derived from the 2013-2018 NHANES data set, the 90th percentile of consumers of plant-based meat analogues containing the Myoglobin Preparation at the maximum use level of 2% myoglobin would result in a daily intake of **4.40 mg of iron from the Myoglobin Preparation (Table 1)**. This value is roughly 50% of the Recommended Dietary Allowance (RDA) for iron across all age groups of men and postmenopausal women at 8 mg/day and therefore would not present a risk for overconsumption of iron. When compared to the UL of 45 mg, an intake of 4.40 mg of iron would be <10% of the UL value.

This conclusion is re-enforced by further evaluation of the dietary intakes of iron from all potential food uses in meat substitutes in combination with background dietary intakes of iron from the total diet. For example, the background dietary intake of iron from the total diet among heavy consumers of foods containing iron was 24 mg per person per day at the 90th percentile. When consumption of simulated meat products containing myoglobin was considered in conjunction with background intakes of iron containing foods, the dietary intake of iron increased from 24 mg to 27 mg among heavy consumers of simulated meat products. This dietary increase in iron intake would be considered nutritionally desirable and remains well below the UL value of 45 mg.

There is no reason to conclude that this level of additional iron to the diet would pose any risk of excess consumption of iron by the general population.

10. *FDA. Myoglobin has been historically consumed by human beings and is widely consumed all over the world through the consumption of meat. The notifier has addressed this in the GRAS notice. The rare case reports of myoglobin allergenicity only confirms the fact that any protein could be allergic to some people in the world (i.e., no protein is totally free from the potential of causing an allergic reaction). However, certain allergenicity prediction programs (e.g., AlgPred, and AlgPred 2.0) call myoglobin as a potential allergen. These algorithms are support-vector machine-based methods.*

Motif has obtained findings from an independent scientific report prepared by allergenicity expert Dr. Steve Taylor to provide context for the findings of potential allergenicity of myoglobin when

[4] Institute of Medicine (US) Panel on Micronutrients. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Washington (DC): National Academies Press (US); 2001. 9, Iron. Available from: https://www.ncbi.nlm.nih.gov/books/NBK222309/

evaluated using certain allergenicity prediction programs (*i.e.*, AlgPred, and AlgPred 2.0) incorporated. In brief, Dr. Taylor concluded that these findings are artifacts of questionable decisions to place bovine and equine myoglobins on the allergen sequence databases used by the algorithms as a result of a single case report. That decision was then magnified when three of the most frequently used allergen prediction programs included bovine and equine myoglobins as allergenic proteins in the training sets used for their programs. A copy of Dr. Taylor's evaluation is provided in Attachment 2.

11. *FDA. Please explain why the allergenicity prediction by AlgPred and AlgPred 2.0 is not relevant in predicting the true allergenic potential of myoglobin.*

Motif contracted with Dr. Steven Taylor and Dr. Rick Goodman to address Question #10 and #11. A detailed assessment of the literature related to the question are provided in Attachment 2.

We appreciate the opportunity to address these important questions and provide the answers to each one. Please let me know if you have any specific further questions.

We look forward to your positive response.

Best Regards,

Janet E Collins (electronic)

Janet E. Collins, Ph.D., R.D.
Vice President Regulatory, Government and Industry Affairs

Helping to make plant-based foods
better tasting, more nutritious.

Attachment 1: SOPs for Myoglobin Protein Analysis

Attachment 2: Scientific Report on Allergenicity of Bovine Myoglobin (Dr. Steven Taylor)

Attachment 1: SOPs for Myoglobin Protein Analysis

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Attachment 2: Scientific Report on Allergenicity of Bovine Myoglobin (Dr. Steven Taylor)

Response to FDA Question Posed to Motif FoodWorks, Inc. on 16 August 2021 Regarding GRN 001002
Predicted Allergenicity of Bovine Myoglobin Based on Bioinformatics Assessment

August 23, 2021

Taylor Consulting LLC
Steve L. Taylor, Ph.D.
Richard E. Goodman, Ph.D.
Lincoln, NE, USA

In response to their GRAS Notice, GRN 001001, Motif FoodWorks received a response from FDA on 16 August 2021 posing several questions. Taylor Consulting LLC was requested to respond to Question #10 from the FDA response letter as copied below.

10. Myoglobin has been historically consumed by human beings and is widely consumed all over the world through the consumption of meat. The notifier has addressed this in the GRAS notice. The rare case reports of myoglobin allergenicity only confirms the fact that any protein could be allergic to some people in the world (i.e., no protein is totally free from the potential of causing an allergic reaction). However, certain allergenicity prediction programs (e.g., AlgPred, and AlgPred 2.0) call myoglobin as a potential allergen. These algorithms are support-vector machine based methods.

FDA seems to concede that bovine myoglobin is unlikely to be an allergenic protein based on a long history of safe use through widely consumed beef products and a literature search revealing only one possible case report of an allergic reaction attributable to myoglobin consumption. These two elements were contained in GRN 001001.

However, FDA apparently plugged the sequence of bovine myoglobin into two allergenicity prediction programs (AlgPred and AlgPred 2.0) that indicated that bovine myoglobin was a potential allergen. These allergenicity prediction algorithms are in disagreement with the observations in GRN 001001. Thus, Taylor Consulting LLC has now conducted additional investigation to try to determine the basis for this disagreement.

Several approaches were taken in this investigation:

(1) The sequence of bovine myoglobin was plugged into several allergen databases and allergenicity prediction programs. Variable results were obtained as discussed below leading to questions about several allergen databases and their use in allergenicity prediction programs.
(2) Personal exchanges were conducted with several scientists involved in the generation of allergenicity prediction programs.
(3) The manuscript by Fuentes et al. (2004) describing the single published report of myoglobin as a beef allergen was reviewed in more detail.
(4) Several manuscripts cited in the Allergome allergen database as the basis for the listing of equine (horse) heart myoglobin as an allergen were reviewed.

Bioinformatics Searches:

The sequence of bovine myoglobin used in the bioinformatics searches was Uniprot Accession No. P02192. This is the same sequence that was provided in GRN 001001.

AllergenOnline database: This peer-reviewed database is maintained by the Food Allergy Research & Resource Program at the University of Nebraska-Lincoln (Goodman et al., 2016). For this search, version 21 was used and contains 2233 proteins in 912 protein allergen groups. AllergenOnline was searched for full-length identity matches, matches of >35% identity over sliding 80-amino acid windows, and exact matches for 8 amino acid (8 mer) segments.

For full-length FASTA matches, any matches of 50% identity would certainly be considered as possible cross-reactive targets. In this case, the only matches were of low identity and length, to globin proteins of larval flies, ranging from 24.8% identity down to 22% identity. The E scores were larger than 0.008, not very significant.

No identity matches of >35% over 80 amino acid windows were found for bovine myoglobin when searched against this database. No 8-mer exact matches were identified either.

COMPARE database: This peer-reviewed database is maintained by the Health & Environmental Sciences Institute (HESI) of the International Life Sciences Institute (van Ree et al., 2021). This database emerged from AllergenOnline several years ago and the allergen sequences in the database are similar although no longer identical.

The ful-length FASTA search of this database revealed similar results to the one from AllergenOnline with low identity matches to several globin proteins from larval flies. No identity matches of >35% over 80 amino acid windows were found. No 8-mer exact matches were found.

WHO/IUIS database: This database is maintained by a scientific committee of the International Union of Immunological Scientists (IUIS) under the auspices of the World Health Organization (WHO) (Goodman and Breiteneder, 2019). The main purpose is to establish

consistent short-hand nomenclature for allergenic proteins from all sources. Bioinformatics sequence-based searches are not provided.

This database lists 12 bovine protein allergens from milk, dander, and blood/meat. Myoglobin is not listed as a bovine allergen. The major bovine meat allergen is bovine serum albumin (BSA) as described in GRN 001001.

This database lists 6 equine protein allergens. Myoglobin is not listed as an equine allergen. One allergen is listed in this database for *Equus asinus* (donkey) and it is not myoglobin.

Allergome database: This allergen database is maintained by Prof. Adriano Mari in Italy (Mari et al., 2006). Decisions about inclusion of proteins in this database are not peer-reviewed and criteria for inclusion do not appear to be fixed.

A search of the Allergome database for myoglobin revealed 9 entries including myoglobin from beef, ox/yak, chicken, ostrich, horse, and pork. The evidence supporting the inclusion of the bovine myoglobin is weak consisting of the single case report of Fuentes et al. (2004) already discussed in GRN 001001. A further discussion of the Fuentes et al. (2004) manuscript can be found below but a single case report should not be sufficient to include a protein in an allergen database. For equine (horse) myoglobin, several other publications were also identified in the Allergome file – publications by Gieras et al. (2016) and Gattinger et al. (2019). These publications involved equine myoglobin which shares 93.8% identity with bovine myoglobin. These two publications will be discussed further below but neither implicate bovine or equine myoglobin as allergens. The inclusion of myoglobins from ox/yak, chicken, ostrich, and pork in the database was likely based solely on the degree of sequence homology with bovine and equine myoglobins as the file did not contain any publications describing allergic reactions to these myoglobins.

AllerCatPro database: AllerCatPro was developed as an allergen prediction program from the Bioinformatics Institute in Singapore (Maurer-Stroh et al., 2019). AllerCatPro uses information on the 3-dimensional structure of proteins together with their amino acid sequence to predict potential allergenicity. AllerCatPro takes its list of known allergen sequences (4180 proteins) from AllergenOnline, COMPARE, WHO/IUIS, UniProtKB, and Allergome.

AllerCatPro identified bovine myoglobin as a strong allergen based upon its high (93.8%) sequence identity to equine myoglobin (UniProt accession no. P68082.

R. Goodman contacted S. Maurer-Stroh on 20 August 2021 and determined that equine myoglobin was identified as an allergen because it was in the Allergome database. AllerCatPro did not make any independent determination regarding the evidence for allergenicity of equine myoglobin but simply used the information contained in Allergome.

AllerTOP version 2.0 database: AllerTOP is another allergen prediction program. It was developed by Prof. I. Dimitrov and colleagues at the Medical University of Sofia in Bulgaria (Dimitrov et al, 2014). AllerTOP uses information about a set of allergens as the basis for predictions about the allergenicity of query proteins. The allergen set was assembled from the

Central Science Laboratory (U.K.), AllergenOnline, the SDAP (Structural Database of Allergenic Proteins) from Texas A&M, and Allergome. The non-allergen dataset used in AllerTOP was assembled from Swiss-Prot using proteins from several sources of food including tomato, potato, wheat, rice, and bell pepper excluding any proteins containing the key word, allergen.

AllerTOP identified bovine myoglobin as a probable allergen. Since equine myoglobin from Allergome is a protein in the AllerTOP dataset, the prediction that the highly homologous bovine myoglobin is an allergen is not surprising.

Immune Epitope database (IEDB): IEDB, based in La Jolla, CA, is a free database maintained with funding from the NIAID (National Institute of Allergy & Infectious Disease in the U.S.). IEDB catalogs experimental data on antibody and T cell epitopes and allows prediction of epitopes based on comparisons to those data (Vita et al., 2019).

The epitopes catalogued in IEDB include IgG, IgM, IgA, IgD, and IgE epitopes. R. Goodman contacted Nina Blazeska, the manager of bioinformatics at IEDB, to inquire about the number of allergenic proteins with epitopes catalogued in the IEDB. Together, they searched the IEDB and could determine that IEDB contains epitopes from only two known allergenic proteins. Thus, the use of the IEDB for prediction of allergenic epitopes of proteins seems quite premature although IEDB has become a valuable resource for prediction of immunogenic epitopes (IgG, IgM, IgA, and possibly IgD).

AlgPred and AlgPred 2.0: AlgPred and the most recent version (AlgPred 2.0) were developed from the Intraprastha Institute of Information Technology in New Delhi, India. The basis of AlgPred 2.0 is summarized in a recent publication by Sharma et al. (2021). Algpred is an allergen prediction algorithm based on SVM (support vector machine). The machine learning approach of AlgPred 2.0 relies upon 10,075 known allergens and 10,075 known non-allergens. The list of allergens was taken from various sources include AllergenOnline, COMPARE, the original version of AlgPred, AllerTOP, and Swiss-Prot (Sharma et al., 2021). The developers claim that AlgPred 2.0 has 10,451 experimentally validated IgE epitopes which seems like a very high number based on our knowledge of the allergen literature. The AlgPred 2.0 output is a hybrid with ML Score, MERCI Score, BLAST Score and hybrid score. As the final output, AlgPred 2.0 predicts if the protein will or will not be an allergen.

When bovine myoglobin is assessed by AlgPred 2.0, this protein is identified as an allergen. However, many unknowns remain about the construction of the databases of known allergens and non-allergens and their epitopes that would serve to train AlgPred 2.0 to assess the allergenicity of bovine myoglobin. As noted above, AlgPred 2.0 relies in part on a list of known allergens maintained by AllerTOP. The AllerTOP database in turn relies on several sources of known allergens including Allergome. Since bovine and equine myoglobins are listed as an allergen in the Allergome dataset, that information was carried forward to AllerTOP and AlgPred 2.0.

R. Goodman corresponded with GPS Raghava in India about the basis for AlgPred 2.0. Dr. Raghava claimed that he and his students obtained over 15,000 B cell epitopes from IEDB.

However, as noted above, IEDB has very few known IgE B cell epitopes in its database. Perhas, AlgPred 2.0 has included many of the B cell epitopes from IEDB as "experimentally validated IgE epitopes" and used them in the SVM prediction tool.

Bioinformatics Summary: Bovine myoglobin is not listed as an allergen in multiple databases including AllergenOnline, COMPARE, and WHO/IUIS. The only allergen database that lists bovine myoglobin as an allergen is Allergome. The main evidence for that listing was the single case report by Fuentes et al. (2004) that was discussed in GRN 001001 and will be further discussed below. Also Allergome contains myoglobins from other species including equine myoglobin probably based upon high sequence homology with bovine myoglobin (unclear). Several publications are provided in the Allergome file for equine myoglobin regarding the use of this protein in allergenicity studies. These publications will be discussed further below.

Three allergen prediction programs (AlgPred, AllerCatPro, and AllerTOP) identify bovine myoglobin as an allergen or probable allergen. However, the allergen datasets used in all three of the prediction programs are derived partially from Allergome and thus contain bovine and equine myoglobin as allergens.

Personal Scientific Communications:

As noted above, the identification of bovine myoglobin as an allergen or probable allergen in several of the allergen prediction program occurred because the Allergome allergen database which includes bovine and equine myoglobin were used in the development of these programs. Personal communication with S. Maurer-Stroh, the developer of the AllerCatPro prediction program confirmed that bovine and equine myoglobins were used in the training dataset because the dataset was derived in part from Allergome. Publications from AllerTOP and AlgPred 2.0 indicate that the Allergome database was used in part to create the training sets for these two allergen prediction programs as well. Additional personal communications with the bioinformatics manager of IEDB and with P. G. S. Raghava of AlgPred confirm that the IEDB database contains few IgE epitopes and yet was extensively used in the training set for AlgPred 2.0. Thus, AlgPred appears (not entirely clear) to rely upon the sequences of immunogenic epitopes from proteins that are not validated as IgE epitopes and further relies on an allergen dataset derived from Allergome (via AllerTOP) that contains bovine and equine myoglobins as allergens.

Detailed Critique of Fuentes et al. (2004):

One allergen sequence database (Allergome) lists bovine myoglobin as an allergen while other allergen sequence databases do not. The Allergome file contains only one publication supporting the assertion that bovine myoglobin is an allergen. That publication by Fuentes et al. (2004) is a single case report that does not merit the listing of bovine myoglobin as an allergen. That is especially true when consumer exposure to bovine myoglobin has been quite substantial over many decades. The publication by Fuentes et al. (2004) was previously identified and discussed in GRN 001001.

To date, only this one publication by Fuentes et al. (2004) provides any evidence of allergy to bovine myoglobin. This observation is significant because meat is widely consumed and consumer exposure to bovine myoglobin from meat is high. Beef allergy is rare and studies have indicated that bovine serum albumin is the major beef allergen. Fuentes et al. (2004) described several allergic episodes occurring in a 35 year old nonatopic female after ingestion of several types of meat, primarily tested by prick-to-prick tests and specific IgE measurements. Using immunoblotting, her serum IgE bound to a 17 kDa protein in a semi-purified protein fraction obtained using ethanol fractionation. They also tested binding to 80 other atopic subjects and none had serum IgE that bound to this 17 kDa protein. This protein could be isolated using 70%-90% ethanol fractionation and characterization revealed that it was a heat-resistant protein that did not contain disulfide bonds. N-terminal sequencing was used and identified a segment of 16 amino acid residue that matched myoglobin. The sequence "GLSDGEWQLVLNAWGK" was tested by us in NCBI Protein BLASTP and shows 100% identity match to myoglobins of many species including sheep, goat, bovine, some rodents and 94% identical to human myoglobin as well as pig myoglobin. The methods described by Fuentes et al. (2004) are not commonly used today. The immunoblotting image shows a broad band that may represent glycol-protein or the possibility of multiple proteins (figures 1 through 3). Since no other data are shown to characterize the protein. Thus, in our opinion, it is not possible to be certain that myoglobin from beef was the primary protein binding with serum IgE during immunoblotting used serum from this individual.

We concede that bovine myoglobin may have been responsible for the allergic episodes occurring with this single individual. The evidence is not strong because myoglobin was not carefully isolated away from other bovine proteins. Also, bovine myoglobin was not confirmed to cause mediator release in this individual via skin testing or basophil testing with the purified bovine myoglobin. Additionally, no other cases of suspected allergy to bovine myoglobin have surfaced either before or after this single case.

In our opinion, insufficient evidence (one case report) exists to place bovine myoglobin on allergen sequence databases as was done with Allergome.

Involvement of Equine Myoglobin in Allergenicity Studies:

Equine (horse) heart myoglobin is also listed in the Allergome database perhaps owing to its high sequence homology with bovine myoglobin. Other myoglobins are also listed in the Allergome database including ox/yak, chicken, ostrich, and porcine. No publications are provided in the Allergome file to indicate any reports of allergenicity with ox/yak, chicken, ostrich or porcine myoglobins so these proteins must have been included in Allergome based upon high sequence homology alone. With equine myoglobin, the Allergome file includes several publications on the use of equine heart myoglobin in allergenicity research studies (Gieras et al., 2016; Gattinger et al., 2019).

These two publications come from the laboratory of the imminent allergist/immunologist, Rudolph Valenta in Austria. Valenta and colleagues were interested in exploring the fundamental relationship between the number of IgE-binding epitopes on a protein and the efficacy of mediator release from IgE-armed mast cells of basophils. They conceived the rather

brilliant idea of binding known linear epitopes peptides from known allergens to a non-allergenic scaffold protein. They chose equine heart myoglobin as the non-allergenic scaffold protein on the basis that equine heart myoglobin was non-allergenic. Geiras et al. (2016) specifically state in their manuscript that the basis for selection of equine heart myoglobin for these experiments was its non-allergenicity. Gieras et al. (2016) constructed artificial allergens by grafting IgE epitopes in different numbers and proximities onto the myoglobin scaffold protein. The IgE epitopes were taken from the major allergen of timothy grass pollen (Phl p 1). The number and location of epitopes on the myoglobin were characterized. They used mouse IgE against these known Phl p 1 epitopes and armed rat basophilic leukemia cells with the murine IgE. Then they evaluated mediator release from the basophils by challenging with the recombinant Phl p 1-grafted scaffold protein. They demonstrated that the closer proximity of IgE-binding epitopes causes more mediator release. This elegant study demonstrated that the minimal spatial distance of IgE epitopes is likely around 30 amino acids. They myoglobin scaffold did not show any activity.

A similar approach was taken in Gattinger et al. (2019). Equine heart myoglobin was used as the non-allergic scaffold protein. The myoglobin was modified by grafting CCD (cross-reactive carbohydrate determinants) epitope site onto the protein. The proteins were produced in an insect cell line that has the enzymes to make the complex carbohydrate determinants on the sites. The recombinant proteins were used as inhibitors of IgE binding for individual subjects' sera that had IgE binding to CCD. They demonstrated dose-specific inhibition of binding using the constructs and could remove binding with glycanase that removed the carbohydrate. Again the myoglobin was a non-allergic scaffold protein.

We do not know how these publications can be used to justify the placement of equine heart myoglobin in the Allergome database as an allergen.

Conclusions:

The allergenicity assessment found in GRN 001001 remains sound. Beef allergy is rarely reported especially in relation to its consumption. The major allergen responsible for beef allergy is bovine serum albumin. Only one case report exists of an allergic reaction to ingestion of myoglobin involving a single individual. The role of bovine myoglobin in these single case was not fully confirmed but sufficient evidence was presented to create some suspicion that bovine myoglobin is an exceptionally rare food allergen especially in light of high levels of consumer exposure to this protein in their diets.

Although we would disagree with this action, the single case report of an alleged allergic reaction to bovine myoglobin was sufficient to list that protein and its sequence in the Allergome allergen sequence database. It is noteworthy that other allergen sequence databases do not list bovine myoglobins (or myoglobin from other species). The Allergome database also contains myoglobins from other species probably due to the high level of sequence homology with bovine myoglobin.

At least three allergen prediction programs identify bovine myoglobin as an allergen or probable allergen. But in all three cases, these programs have included allergen sequences from Allergome as part of the set of allergen sequences used to predict the allergenicity of query proteins. When

bovine and equine myoglobins are present in the allergen training sets for these prediction programs, then it is not surprising that these programs will predict that bovine myoglobin is an allergen.

Specifically to answer Question #10, Allergome made the questionable decision to place bovine and equine myoglobins on its allergen sequence database as a result of a single case report. That decision was then magnified when three of the most frequently used allergen prediction programs included bovine and equine myoglobins as allergenic proteins in the training sets used for their programs.

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An ingredient innovation company

18 October 2021

Ms. Ellen Anderson
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Sent via email

Dear Ms. Anderson:

On behalf of Motif FoodWorks (Motif), I write in response to your email of October 1, 2021, in which you raised additional follow-up questions regarding Motif's Myoglobin Preparation GRAS Notification (GRN 1001). Below, please find the specific questions posed in your October 1st email (in italics), immediately followed by Motif responses (in bold).

A. *FDA: In Motif's response to Question 8 in the September 3rd amendment in which you provided estimates of dietary exposure to the components of the myoglobin preparation, we request the following:*
    1. *FDA: For the estimates of iron exposure provided in Table 1, please confirm that the units are in milligrams.*

       Motif: We confirm that units for iron in Table 1 of the Notice are milligrams (mg).

    2. *FDA: The estimates included dietary yeast exposure from the intended uses of myoglobin preparation, however the source of the yeast exposure is not clear. In the description of the manufacturing process provided in the notice, you state that the myoglobin preparation does not contain viable cells of the production strain, which are lysed and removed during the manufacturing process. Please provide a discussion of the presence of yeast (Pichia pastoris) in the preparation that is the source of this exposure.*

       Motif: The presence of 'yeast' in the Myoglobin Preparation is noted in GRN 1001 (page 13, Table 2.3-1) where footnote 'b' states that the presence of protein beyond that contributed by myoglobin protein is residual *Pichia* protein. The residual *Pichia* protein was referred to as 'yeast' in the response to FDA, which in retrospect was confusing as this term was not used in the GRAS Notice. The oversight was further compounded by an error that occurred in our calculation of 'yeast' in the Myoglobin Preparation, resulting from a data input error.

The following is a *Revised* Table 1 that provides data for the Myoglobin Preparation, total protein, myoglobin protein, and *Pichia* protein.

Revised Table 1. Dietary Intake of Substance [Means and (90th Percentiles)] for the Total Population and Consumers Only from 3% *Myoglobin Preparation* at Three Myoglobin Levels of Inclusion

Substance	NHANES Population Categories	Myoglobin Protein Inclusion Level (%)					
		1%		1.5%		2.0%	
		Mean	90th %	Mean	90th %	Mean	90th %
Myoglobin Preparation (g)	Population	10.18	31.90	15.27	47.85	20.36	63.80
	Consumers	21.87	48.78	26.79	73.17	31.70	97.56
Total Protein (g)	Population	0.01	0.02	0.01	0.02	0.01	0.02
	Consumers	0.73	1.50	1.10	2.24	1.46	2.98
Myoglobin protein (g)	Population	0.0098	0.0196	0.0098	0.0196	0.0098	0.0096
	Consumers	0.715	1.47	1.078	2.195	1.431	2.862
Pichia protein (g)	Population	0.0002	0.0004	0.0002	0.0004	0.0002	0.0004
	Consumers	0.015	0.03	0.022	0.045	0.029	0.118

*Myoglobin protein: Calculated as protein (g) x 98% purity= myoglobin protein (g)

**Pichia* protein: Calculated as total protein (g) – total myoglobin protein= *Pichia* protein (g)

3. *FDA: For completeness of the record, please provide estimates of dietary exposure to the notified substance, i.e., whole myoglobin preparation, for the intended uses. Given the high water content of myoglobin preparation, we expect these estimates may be determined on a total dry matter basis.*

   Motif: Responses to Q2 (above), we provide the requested information regarding dietary exposure to Myoglobin Preparation derived from NHANES data (2013-2018) modeled across the entire population and consumers of plant-based meat analogues into which the 3% Myoglobin Preparation is included at 1%, 1.5%, and 2% of the analogue formulation.

B. *FDA: In Motif's response to Question 3 in the September 3rd amendment, you stated that all methods of analysis are validated and fit for purpose and provided a copy of the SOPs for myoglobin protein analysis as Attachment 1. We note that the SOPs are labeled as "proprietary and confidential". Please confirm that this information is indeed propriety and confidential and considered exempt from public disclosure.*

   Motif: Motif confirms that the SOP for myoglobin protein analysis (as Attachment 1) is proprietary and confidential, and exempt from public disclosure pursuant to FOIA Exemption (b)(4), 5 U.S.C. 552(b)(4), and FDA's implementing regulations. The method for myoglobin protein analysis is corroborative of the presence of myoglobin in the preparation and is not significant regarding the basis for a GRAS determination. As such, the SOP has no impact on reaching a conclusion that the myoglobin preparation is generally recognized as safe. Accordingly, pursuant to FOIA Exemption (b)(4), 21 CFR 20.61, and 21 CFR 170.250(d) and (e), this information should not be subject to public disclosure.

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We appreciate the opportunity to provide clarification to questions raised by FDA in response to the Motif earlier correspondence on these issues. Should you have further questions, please contact me directly [jcollins@motiffoodworks.com; (+1-703-868-3280)].

Respectfully submitted,

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Vice President, Regulatory, Government and Industry Affairs