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VIA EMAIL: Lauren.Brookmire@FDA.HHS.GOV

Lauren Brookmire, M.S.
Consumer Safety Officer
Division of Biotechnology & GRAS Notice Review -H FS-255
Office of Food Additive Safety
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: Response to FDA Questions - GRAS Notice 540
Soybean leghemoglobin- Impossible Foods, Inc.

Dear Ms. Brookmire:

Attached is the submission of responses to questions raised by the agency. The agency question is listed in bold type and then the response. In addition to the electronic copy, we can send a hard copy if that is helpful.

Please contact us with any questions.

Sincerely,

Gary L. Yingling

ATTACHMENT

I. General comments

FDA Comment:
The information provided as the basis of the GRAS determination should contain a level of specificity necessary when discussing the ingredient (soybean leghemoglobin protein). The notification should adequately address (i) the safety of soy leghemoglobin for human consumption and (ii) general recognition of its safety for the intended use and level.

Although proteins are a part of the human food supply, not all proteins are safe. Information addressing the safe use of modified soy protein does not adequately address safe use of soybean leghemoglobin protein from the roots of the soybean plant in food.

Impossible Foods Response:

Impossible Foods understands the FDA's concerns regarding the consumption of the root nodule protein. However, as will be elucidated in the responses below to the specific FDA issues raised in regard to GRAS Notification 540 (GRN 540), Impossible Foods does not believe that consumption of this protein presents any issues of safety to the consumer. Though the protein is isolated from the root nodule, it is substantially similar to proteins consumed daily by the global population, in the form of meat and other vegetables. Impossible Foods did not rely on the use of modified soy protein as the sole basis of its determination regarding the safety of the soy leghemoglobin protein. The following responses to the FDA's questions provide additional detail and specificity, and support the use of soy leghemoglobin in meat analogue products, as detailed in GRN 540.

II. Issues regarding the clarity of statements in the GRAS notice

FDA Comment:
1. Please confirm whether the sequence of leghemoglobin that is subject of this GRAS notice has the Geneinfo Identifier (GI) 126241. The database has multiple Glycine max leghemoglobin sequences that are not identical.

Impossible Foods Response:

The sequence of soy leghemoglobin that is the subject of this GRAS notice has the GeneInfo Identifier (GI) 126241.

FDA Comment
2. Page 6 refers to the production strain as Pichia pastoris Bg10, which Page 7 refers to it as MXY022. Please clarify the designation of the production strain.

Impossible Foods Response:

The reference to *Pichia pastoris* Bg10 on page 6 of the notification was a typographical error. MXY022 is the production strain, as correctly identified on page 7. *Pichia pastoris* Bg10 is the parent to the production strain.

FDA Comment:

3. Please provide information about the minimum temperature of denaturation for soy leghemoglobin.

Impossible Foods Response:

The minimum temperature of denaturation for soy leghemoglobin, determined by Impossible Foods using dynamic light scattering, is 64 degrees Celsius. Dynamic light scattering measures the mean effective diameter of a protein as a function of temperature. Increased protein diameter indicates denaturation and aggregation. The minimum temperature of denaturation for soy leghemoglobin is similar to bovine myoglobin, which Impossible Foods determined to be 70 degrees Celcius using dynamic light scattering.

III. Issues regarding the scientific reasoning and availability of public information

FDA Comment:

1. The dietary exposure discussion in GRN 540 includes history of safe use of soy proteins from the soybean plant in general and does not discuss soy leghemoglobin from the roots of the soybean plant, which is the ingredient described in the GRAS notice. The discussion is not relevant in the context of the GRAS notice because soybean root is not a commonly consumed human food. Please provide relevant information, as there is no history or knowledge of human dietary exposure to soy leghemoglobin from roots.

Impossible Foods Response:

Despite an exhaustive literature search, Impossible Foods was unable to document a history of widespread consumption of soy root nodules. However, it is important to note that when developing a safety profile of the soybean leghomoglobin product, Impossible Foods did not base its assessment of the safety of soy leghemoglobin on such a history. Rather, the argument for the safety of leghemoglobin was developed based on its structural and functional equivalence to other widely consumed globin proteins including animal myoglobins and hemoglobins, as well as plant non-symbiotic hemoglobins.

Globins are a large protein superfamily found in all domains of life (Vinogradov et al. 2007). The subfamily of globins that includes the legume symbiotic hemoglobins (leghemoglobins, including the soy leghemoglobin referenced in this notification) also includes the plant non-symbiotic hemoglobins, and animal myoglobins and hemoglobins. The proteins share a common evolutionary origin (Vinogradov et al. 2007) and, based on structural studies and homology modeling, share a common three-dimensional structure involving eight alpha helixes wrapped around a heme B molecule (Ellis et al. 1997). A direct comparison of the structures of leghemoglobin and vertebrate myoglobin shows their high degree of structural similarity (see Annex 1).

The members of this protein family are all involved in selective transport, storage or buffering of oxygen levels in cells and tissues (Vinogradov and Moens 2008). The shared and well-characterized physiology of these proteins strongly supports the inference that the shared

three-dimensional structure of these globin proteins evolved to bind oxygen. Leghemoglobins, which are found exclusively in the nitrogen-fixing root nodules of legumes, play an analogous role, storing oxygen and buffering its concentration into the optimal range for nitrogen fixation (Garrocho-Villegas et al. 2007; Hargrove et al. 1997).

The heme B moiety plays a central role in oxygen binding, and the structure of the globin protein serves to isolate the highly reactive heme from other molecules by creating a small binding pocket inaccessible to most other molecules (Ellis et al. 1997). Thus these heme B-containing globin proteins remain largely inert so long as the three dimensional structure is maintained. When globin proteins are heated, as in cooking, the protein unfolds and the heme B molecule is released. We have shown that heme B, released when myoglobin is heated to cooking temperature, plays a major role in catalyzing the production of the characteristic flavors and aromas of cooked meat. Crucially, however, this catalysis is a function solely of the heme B molecule, and is independent of the specific protein in which it was bound prior to cooking.

The abundant consumption of heme B is widespread in humans and other animals, as heme proteins, like myoglobins and hemoglobins that are abundant in animal tissues consumed as meat, and are also present in the leaves and other routinely consumed parts of plants. Thus, there is overwhelming evidence that heme B-containing proteins, which are functionally equivalent to soy leghemoglobin presented in this notification, have been safely consumed throughout human history.

There is no evidence that any of the globin subfamily that contains the plant hemoglobins and animal myoglobin and hemoglobin have any biochemical activities other than the binding of oxygen (O_2) or the structurally similar nitrous oxide (NO) and carbon monoxide (CO). The three-dimensional structure of leghemoglobin contains no additional active sites to distinguish it from widely consumed proteins like myoglobin, nor is there any biochemical or physiological evidence that this protein has any enzymatic activity or other function outside of controlled binding to oxygen. In addition, our own extensive sequence analysis of the soy leghemoglobin protein has not revealed any structural domains or other features that would suggest any activity other than oxygen binding.

Thus there is no evidence to suggest that soy leghemoglobin in food will behave any differently from the myriad other functionally equivalent and widely consumed globin proteins in the human diet.

FDA Comment:
2. Provide a short description, with reference to the use of Pichia pastoris in the food industry to purify various expressed proteins for human consumption or other systemic use.

Impossible Foods Response:

The use of *Pichia pastoris* as a food production organism has been reviewed by the FDA in the past under GRAS Notification 204. This notification, which received a "No Questions" response from the Agency, describes a recombinant Phospholipase C produced using *Pichia pastoris*. This enzyme is used as an additive to improve extraction of edible oils, such as soy and canola, from their seed sources. *Pichia pastoris* is also the host used for production of nitrate

reductase (The Nitrate Elimination Co. Lake Linden, MI), an enzyme used for treatment of potable water.

Furthermore, *Pichia pastoris* is used as a host to produce recombinant phytase, an animal feed additive that is commonly used to increase the nutritive value of plant material. In fact, *Pichia pastoris* was first developed by the Phillips Petroleum Company in the 1970s as a high protein animal feed, based on its ability to generate high biomass in the presence of methanol. Dried *Pichia pastoris* yeast is approved by the FDA, to constitute up to 10% of total broiler feed (21 CFR 573.750).

As detailed in sections 2.3 (A) and 4.1 of the GRAS notification, *Pichia pastoris* has been proven to be a safe production microorganism for several years, particularly in the development of drug products. Several FDA approved, systemically administered biologic and drug products, as well as those approved by other global regulatory bodies, are currently produced using *P. pastoris* for human use.

Notable examples of proteins used in humans for therapeutic purposes are listed below, in Table 1:[1]

Product	Company/ Country	Use
Kalbitor® (recombinant kallikrein inhibitor)	Dyax (Cambridge, MA)	Treatment of hereditary angioedema
Insugen (recombinant human insulin)	Biocon (India)	Diabetes therapy
Shanvac-B (recombinant Hepatitis B surface antigen)	Shantha/Sanofi (India)	Vaccine for Hepatitis B
Medway (recombinant human serum albumin)	Mitsubishi Tanabe Pharma (Japan)	Blood volume expansion
Nanobody® ALX-0061 (recombinant anti-IL6 receptor antibody)	Ablynx (Belgium)	Rheumatoid arthritis treatment
Heparin-binding EGF like growth factor	Trillium (Canada)	Treatment of Interstitial cystitis/ Bladder Pain syndrome

FDA Comment:
3. The manufacturing specification for total protein content is set at 91% and that for leghemoglobin is set at 73%. Please provide explanation regarding the purity of the ingredient described in the GRAS notice, to account for other proteins that might co-purify with the soy leghemoglobin from P. pastoris. The response to this issue is directly related to the preceding issue of the history of safe use of P. pastoris in the food industry for purifying proteins meant for human consumption.

[1] Table adapted from: http://www.pichia.com/science-center/commercialized-products/

Impossible Foods Response:

The protein of interest, soy leghemoglobin (to be commercially known as RUBIA), will be extracted from *Pichia* cells and purified away from other cellular proteins, with a resultant purity of approximately 73% leghemoglobin. The non-target proteins which may co-purify are expected to be safe for consumption based on history of safe consumption of the whole yeast in animals.

Co-purifying Pichia proteins in RUBIA were identified by Alphalyse (Palo Alto, CA) using liquid chromatography mass spectrometry (LC-MS/MS). We have attached a representative list of co-purifying proteins from batch PP-PGM2-14-127. LC-MS/MS can detect proteins at concentrations as low as 1 fmol and therefore is highly sensitive to even trace amounts of co-purifying Pichia host proteins. Each of the identified proteins represents <1% of the total protein fraction in RUBIA. A representative list of the proteins identified is provided below in Table 2 below, and the full list is presented in Annex 2.

Table 2. Protein Identification Results

Peroxiredoxin
60S ribosomal proteins
Catalase A, breaks down hydrogen peroxide in the peroxisomal matrix formed by acyl-CoA oxidase
6-phosphogluconolactonase, catalyzes the second step of the pentose phosphate pathway
Protein of unknown function that associates with ribosomes
NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate
Translation initiation factor eIF-5A, promotes formation of the first peptide bond
Mitochondrial alcohol dehydrogenase isozyme III
Triose phosphate isomerase, abundant glycolytic enzyme
Translational elongation factor EF-1 alpha
Mitochondrial ribosome recycling factor
Mitochondrial malate dehydrogenase, catalyzes interconversion of malate and oxaloacetate
Non-essential intracellular esterase that can function as an S-formylglutathione hydrolase
40S ribosomal proteins
Transketolase, similar to Tkl2p
Non-ATPase base subunit of the 19S regulatory particle (RP) of the 26S proteasome
Conserved protein of the mitochondrial matrix, performs a scaffolding function during assembly
Nitrogen catabolite repression transcriptional regulator
Ribulose-phosphate 3- epimerase
Unnamed protein products
Mitochondrial intermembrane space cysteine motif protein
Peptidylprolyl-cis/transisomerase
Hypothetical proteins
Phosphatidylinositol 3,5-bisphosphate-binding protein
Non-essential protein of unknown function required for transcriptional induction
Thiol-specific peroxiredoxin, reduces hydroperoxides to protect against oxidative damage

The protein samples were reduced and alkylated with iodoacetamide, i.e. carbamidomethylated, and subsequently digested with trypsin, cleaves after lysine and arginine

residues. The resulting peptides were concentrated by Spec Vac lyophilization, and redissolved for injection on a Dionex nano-LC system and MS/MS analysis on a Bruker Maxis Impact QTOF instrument. The MS/MS spectra were used for Mascot database searching. The data were searched against in-house protein databases downloaded from UniProt and NCBI containing more than 38 million known non-redundant protein sequences. The Mascot software finds matching proteins in the database by their peptide masses and peptide fragment masses. The protein identification is based on a probability-scoring algorithm (www.matrixscience.com) and the significant best matching protein is shown in the result report. Homologous proteins with a lower score are not included in the report. If a matched protein from the source organism is not present in the database, then a significant matching homologous protein from another organism is reported. If several proteins are identified with a significant score then several protein identifications are reported for the sample. It is considered a positive identification when at least 2 peptides have an Ions score above 35 or if a protein under 20kDa has 1 peptide with an Ions score above 50.

FDA Comment:
4. There are published reports of allergic responses in humans to myoglobin (although rare). Please incorporate those reports in the context of your discussion of safety and the general recognition of safety of oxygen-binding globin proteins.

Impossible Foods Response:

Impossible Foods is aware of only a single case of meat allergy linked to bovine myoglobin (Fuentes et al., 2004), although this implication of bovine myoglobin in this case has been disputed (Fiocchi et al., 2005). The reactions observed in this patient were specific to bovine myoglobin, and not porcine myoglobin, suggesting that this is not a general allergy to oxygen-binding globin proteins, but rather a specific response to a bovine-derived protein. Given the widespread consumption of meats containing oxygen-binding globulins at concentrations comparable to those proposed for use soybean leghemoglobin in this notification, the low incidence of meat allergies in general (and the cause of those few reactions is predominantly due to bovine serum albumin sensitivities), and only a single reported case of myoglobin allergy, Impossible Foods believes that this argues that these proteins as a class have low allergenicity.

Among the hundreds of thousands of proteins to which we are exposed in our daily diet, only a very small fraction induces clinically significant allergies. Nevertheless, Impossible Foods recognizes that with any novel protein introduced to the diet, there is a risk of allergenicity. As discussed in our original application, Impossible Foods enlisted Dr. Richard E. Goodman at the Food Allergy Resource and Research Program (FARRP) of the University of Nebraska to assess the potential allergenicity of soy leghemoglobin as well as other hemoglobin proteins derived from a variety of plants and bacterial sources, consistent with the Codex recommendations. Dr. Goodman's assessment (detailed in Annex 3 and Annex 4 of the original notification) found the soy leghemoglobin had no similarity to known allergens or toxins, and that the protein was readily digestible by pepsin. Thus he concluded that the soy leghemoglobin protein that is the subject of this GRAS notification raises no health or safety concern.

FDA Comment:
5. On Page 2, the notifier states that one of the uses of soy leghemoglobin produced in P. pastoris is nutrition. Explain how the use of this ingredient in foods affects dietary protein profile of the proposed foods at the proposed use level.

Impossible Foods Response:

Impossible Foods did not intend to imply that leghemoglobin will affect the dietary protein profile of the proposed foods at the proposed use levels, and apologizes for the confusion. To clarify, Impossible Foods intended to convey that leghemoglobin has a nutritive value as a source of iron, analogous to the role of myoglobin as an iron source in meat. Once cooked and digested, both leghemoglobin and myoglobin release identical heme B molecules into the digestive system. Studies using cell models of iron bioavailability have shown that the bioavailability of iron in soy leghemoglobin is equivalent to that of bovine myoglobin when in a food-like substrate (Reddy 2006).

IV. Issues requiring data/experimentation (or reference of publically available data)

FDA Comment:
1. The dietary exposure assessment is based on 1% market share of beef, pork and poultry consumption. Please recalculate dietary exposure to capture 100% market share to provide a conservative estimate of the consumption of the ingredient from the proposed uses.

Impossible Foods Response:

The most conservative estimate of leghemoglobin intake assumes a consumer would substitute all meat and poultry products from the diet with RUBIA (the commercial name of the soy leghemologbin product) containing meat analogue products. Typically, RUBIA is used at a rate to deliver leghemoglobin at the same concentration (or less) as the myoglobin found in traditional meat and poultry products. As seen in Table 1, the result of such a total switch from a meat-based diet to the meat analogue diet would result in a daily consumption of RUBIA of approximately 773 mg. As marketed, RUBIA contains 73% leghemoglobin. Therefore, the estimated daily intake of leghemoglobin would be approximately 564 mg/person/day. A typical meat containing diet would contain approximately 564 mg of myoglobin/person/day.

Table 3. Estimate of Myoglobin Consumption in the Diet – Meat vs RUBIA-Containing Meat Analogue

Food Category to be Replaced	Mean Consumption[2] (gr/day)	Myoglobin Concentration (mg/gram)[3]	Estimated Typical Daily Myoglobin Intake (mg/person/day)	RUBIA Anticipated Typical Use Rate (%)	RUBIA Estimated Typical Daily Intake (mg/person/day)
Beef	59	8	473	1.10	649
Pork	29	2	58	0.27	78
Poultry	65	0.5	33	0.07	46
TOTAL			564		773

As stated in the GRAS notification, even the base case of 1% of the traditional meat and poultry market represents 5 times the current meat and poultry analogue market. Given the current market of meat-analogue products and known consumption data compared to meat, Impossible Foods does not believe that 100% replacement of meat by RUBIA-containing analogue products is a plausible scenario.

FDA Comment:
2. Please provide reference supporting the methods used for the digestibility experiment and evidence that the method has been used widely in performing such studies. If such references cannot be provided, then please provide justification for the design of the experiment, with emphasis on the enzyme:substrate ratio and how the choice of such a ratio compares with methods commonly done in the study of protein safety in food. This is needed in order to show whether the stability of the protein could have been artificially altered in the digestibility experiment by the use of too much enzyme.

Impossible Foods Response:

The use of *in vitro* pepsin digestibility as part of a weight of evidence approach to assess protein allergenicity has been advocated by several prominent organizations: the 1996 ISLI-IFBC decision tree, the 1996 FAO/WHO consultation on biotechnology and food safety, the 2000 FAO/WHO consultation on food derived from biotechnology, the 2001 FAO/ WHO consultation on allergenicity assessment of GM foods, the 2002 Codex ad hoc task force on safety assessment of biotechnology, and the 2003 Codex Alimentarius Commission guidelines to assess the allergenicity of genetically modified crops (Metcalfe, Astwood, Townsend, Sampson, Taylor, & Fuchs, 1996) (FAO/WHO, 1996) (FAO/WHO, 2000) (FAO/WHO, 2002)

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[2] Retail Food Commodity Intakes: Mean Amounts of Retail Commodities per Individual, 2007-08. U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD and US Department of Agriculture, Economic Research Service, Washington, D.C.
http://www.ncaur.usda.gov/SP2UserFiles/Place/12355000/pdf/ficrcd/FICRCD_Intake_Tables_2007_08.pdf

[3] http://meat.tamu.edu/ansc-307-honors/meat-color/

(FAO/WHO., 2001) (Codex Alimentarius, 2003). These organizations recommend a weight of evidence approach including both pepsin resistance measurements and a comprehensive sequence homology search to assess protein allergenicity. The pepsin digest and sequence analyses included in GRN 540 were performed in accordance with the guidelines referenced above.

Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas, 2004). The pepsin digest protocol in GRN00540 is identical to the robust procedure used in Thomas *et al.*, 2004. Several peer-reviewed studies have shown that *in vitro* pepsin digestibility is an important risk factor for food allergy (Astwood, 1996) (del Val, 1999). Bannon *et al.* (2002) reviewed a broad range of published pepsin digestion studies and found a strong positive predictive value of the digestion protocol when comparing the stability of allergenic and non-allergenic dietary proteins (Bannon, 2003).

Pepsin digestibility measurements to assess the allergenic potential of new proteins are widely used in the food industry by companies such as Monsanto (Fuchs, Ream, Hammond, Naylor, Leimgruber, & Berberich, 1993) (Reed, et al., 1996) (Harrison, et al., 1996) (Hileman, 2006); Bayer (Noteborn, et al., 1995); The Research Institute for Food Science at Kyoto University, Japan (Hashimoto, et al., 1999) (Momma, et al., 1999); Snow Brand Milk products and ENVIRON international corporation (Goodman RE, 2007); and Quincy bioscience LLC (Moran DL, 2014).

FDA Comment:
3. Provide batch analytical data that meet set specifications for 3 to 5 consecutive manufacturing lots of the dry powder formulation of this ingredient.

Impossible Foods Response:

Additional research conducted after the filing of the GRAS notification revealed that the liquid formulation of the soy leghemoglobin product RUBIA is the most effective form of the product for the intended use. In the liquid form, RUBIA maintains full functionality for its proposed use, incorporates into the analog meat products effectively, and is stable as a frozen liquid. Therefore, we are only using liquid formulations and not dry formulations. The batch analyses for the liquid formulations are shown in Annex 5 of the original notification.

FDA Comment:
4. Provide a stability profile of the ingredient when used in a meat or poultry analogue.

Impossible Foods Response:

Stability of leghemoglobin was assessed in a full meat analogue product. RUBIA was added to the analogue at final concentration of 5% w/w RUBIA solution per grams prototype meat analogue product. Four replicate samples were shrink-wrapped in plastic (SW) or vacuum-sealed in plastic pouches (VP). Replicates (SW and VP) were stored under standard refrigeration conditions (4°C) or freezer conditions (-20°C). Leghemoglobin stability was assessed over 10 days by extracting and quantifying total protein in each sample and by densitometry following denaturing gel electrophoresis. The results are provided in Table 4 below.

Table 4. Stability of leghemoglobin in meat analog

	Percentage leghemoglobin[1]			
Storage time (days)	4°C SW	4°C VP	-20°C SW	-20°C VP
0	26.9 ± 2.9			
1	25.8 ± 5.5	25.6± 6.5	25.8± 6.0	26.8 2.3
2	28.3 ± 3.5	29.2 ± 3.2	25.5± 0.4	24.9 ± 7.2
4	30.1 ± 4.8	26.6 ± 1.7	27.8± 1.7	32.4 ± 4.4
6	32.5 ± 5.0	22.0 ± 1.4	24.1± 1.6	26.3 ± 1.6
10	21.8 ± 1.8	20.9 ± 3.6	29.1 ± 2.3	24.9 ± 0.3

[1] Ratio of leghemoglobin protein to total SDS-extracted protein in meat analog as measured by gel densitometry. Percentage = [(intensity of leghemoglobin band)/(intensity of all bands in lane)]

The data doesn't show a statistically significant trend and no degradation products of leghemoglobin were observed at any time point, indicating that the leghemoglobin is stable in a full meat analogue. The 4°C samples have reached their expected endpoint for this study. While not used as a basis for the current stability study, it is important to note that in previous research batches of meat analogue products made with RUBIA there was minimal degradation of the leghemoglobin in meat analogues over months of frozen storage, as observed qualitatively. In the ensuing months, as products are prepared for launch, Impossible Foods has planned additional longer-term, detailed quantitative shelf life studies to ensure the final products achieve an acceptable shelf life.

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Annex 1 from original notification

Structural comparison of plant hemoglobins and animal myoglobins

The globin structural superfamily is a large, well studied family of globular proteins, present in all domains of life: archae, bacteria, and eukaryotes (PFAM PF00042). All members of the globin structural superfamily are thought to share a common ancestor (Punta et al. 2012). The globin structural fold is comprised of eight alpha helical segments and a heme co-factor, which coordinates binding and/or transfer of oxygen. Structural comparisons of animal myoglobin, plant leghemoglobin, and plant non-symbiotic hemoglobin monomers are shown in Figure 1A-H. The crystal structure for cow myoglobin does not exist, so we have included myoglobin structures from tuna, pig, and horse in this analysis. Based on their similarity to eachother (Figure 1F-H), we expect that they are highly similar to cow myoglobin. The crystal structures were superimposed over all backbone atoms using the Super algorithm in PyMOL (Delano, 2007) (Figure 1I-L) and the corresponding root mean square deviations (RMSDs) are shown in Table 1 . Comparison of proteins folds (Figure 1) and RMSD values (Table 1) illustrates that animal myoglobins, plant non-symbiotic hemoglobins, and plant leghemoglobins all adopt the same globin fold and are structurally very similar. Furthermore, animal myoglobins, plant non-symbiotic hemoglobins, and plant leghemoglobins all bind the identical heme prosthetic group, heme B (Figure 1M).

Leghemoglobins, non-symbiotic hemoglobins, and myoglobins each contain the identical heme b co-factor (Figure 1M). Soybean leghemoglobin does not contain peptide sequences that are associated with allergenicity (ANNEX 3) and is completely digested by pepsin leaving only the heme cofactor (ANNEX 4). Therefore, the health effects of ingesting soybean leghemoglobin should be equivalent to non-symbiotic plant hemoglobins and mammalian myoglobins, which are readily consumed in the diet.

References:

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Figure 1. Structural comparison of plant hemoglobins and animal myoglobins showing that proteins adopt the same globin fold. Individual plant leghemoglobins (A-B), plant non-symbiotic hemoglobins (C-E), and animal myoglobins (F-H), are shown in ribbon representation colored in gray, heme porphyrin ring is shown in red stick representation, and iron in blue CPK representation. Superposition of individual proteins shows that the 3D structure of soybean leghemoglobin is highly similar leghemoglobins, non-symbiotic hemoglobins, and myoglobins from different species (I-L).

Figure 1. Plant hemoglobins and animal myoglobins adopt the same structural fold.

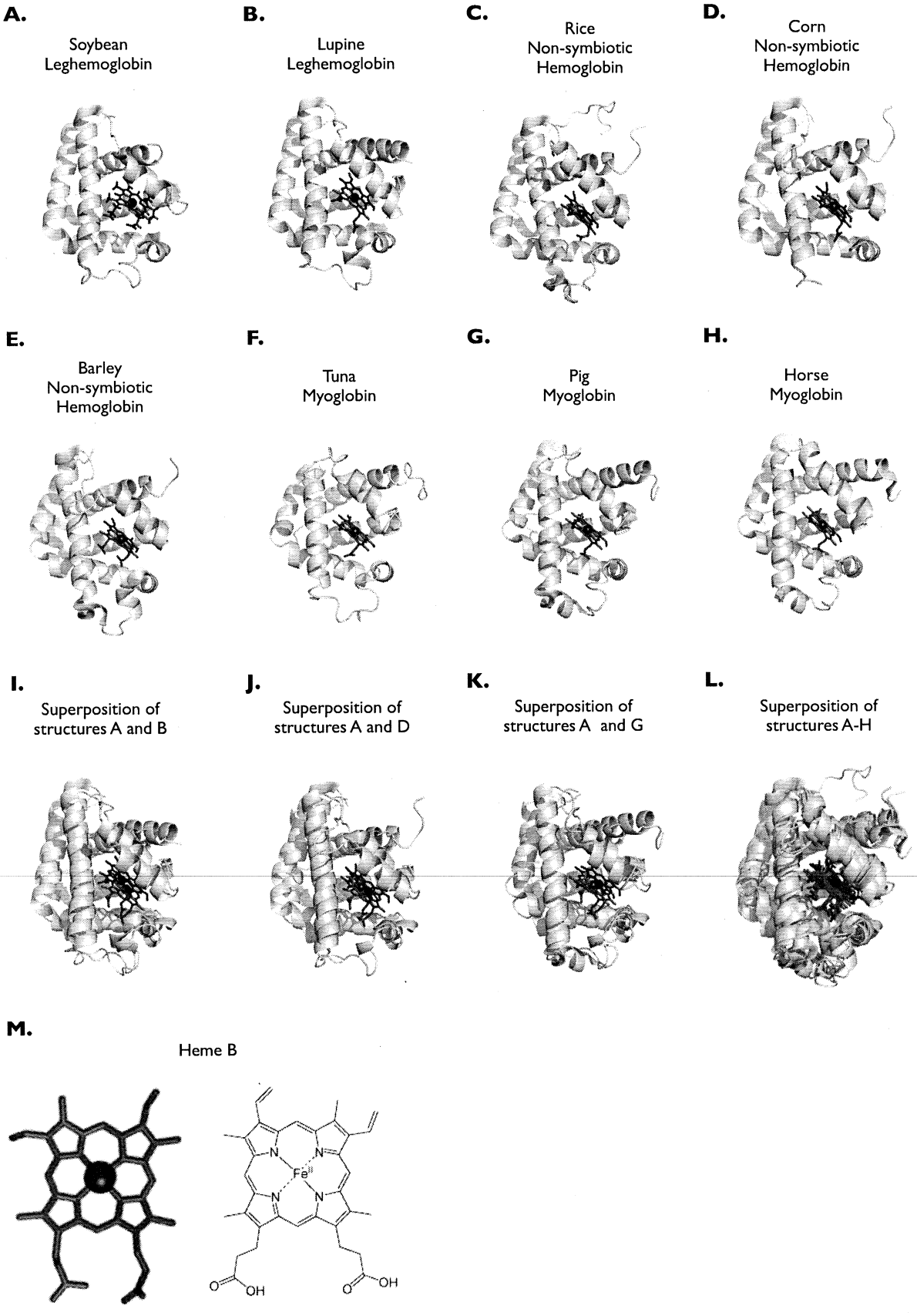

Table 1. Structural comparison between plant hemoglobins and animal myoglobins. Root-mean-square-deviation (RMSD) between all backbone atoms of superimposed X-ray crystallography protein structures (respective PDB codes are shown in parenthesis).

Species		RMSD (Å)
Soybean leghemoglobin (1BIN)	Horse myoglobin (1YMB)	4.5
Soybean leghemoglobin (1BIN)	Pig myoglobin (1PMB)	4.4
Soybean leghemoglobin (1BIN)	Tuna myoglobin (1MYT)	3.6
Soybean leghemoglobin (1BIN)	Barley non-symbiotic hemoglobin (2OIF)	2.5
Soybean leghemoglobin (1BIN)	Corn non-symbiotic hemoglobin (2R50)	1.0
Soybean leghemoglobin (1BIN)	Rice non-symbiotic hemoglobin (1D8U)	1.0
Soybean leghemoglobin (1BIN)	Lupine leghemoglobin (2GDM)	0.8
Soybean leghemoglobin (1BIN)	Soybean leghemoglobin (1FSL)	0.5

Annex 2
RUBIA Protein Identification

Overview

LC-MS/MS

Sample name	Protein found in database	Entry name	Calculated MW	Score	Seq. cov.	Note
PP-PGM2-14-127	RecName: Full=Leghemoglobin C2	gi|126241	15515	1795	95%	
PP-PGM2-14-127	peroxiredoxin [Komagataella pastoris GS115]	gi|254567145	18441	882	61%	
PP-PGM2-14-127	60S ribosomal protein L6 [Komagataella pastoris GS115]	gi|254572856	18595	874	59%	
PP-PGM2-14-127	Catalase A, breaks down hydrogen peroxide in the peroxisomal matrix formed by acyl-CoA oxidase (Pox1 [Komagataella pastoris GS115]	gi|254569930	58114	822	28%	
PP-PGM2-14-127	60S ribosomal protein L18 [Komagataella pastoris CBS 7435]	gi|328350867	41460	734	26%	
PP-PGM2-14-127	6-phosphogluconolactonase, catalyzes the second step of the pentose phosphate pathway [Komagataella pastoris GS115]	gi|254572525	28268	711	61%	
PP-PGM2-14-127	Protein of unknown function that associates with ribosomes [Komagataella pastoris GS115]	gi|254573452	11581	596	44%	
PP-PGM2-14-127	NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate [Komagataella pastoris GS115]	gi|254572123	40399	557	27%	
PP-PGM2-14-127	Translation initiation factor eIF-5A, promotes formation of the first peptide bond [Komagataella pastoris GS115]	gi|254572359	17122	537	55%	
PP-PGM2-14-127	Mitochondrial alcohol dehydrogenase isozyme III [Komagataella pastoris	gi|254568544	37318	537	29%	

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	GS115]				
PP-PGM2-14-127	Triose phosphate isomerase, abundant glycolytic enzyme [Komagataella pastoris GS115]	gi|254572163	27149	432	39%
PP-PGM2-14-127	Translational elongation factor EF-1 alpha [Komagataella pastoris GS115]	gi|254567507	50499	393	27%
PP-PGM2-14-127	Mitochondrial ribosome recycling factor [Komagataella pastoris GS115]	gi|254573108	27998	390	19%
PP-PGM2-14-127	Mitochondrial malate dehydrogenase, catalyzes interconversion of malate and oxaloacetate [Komagataella pastoris GS115]	gi|254568036	34886	385	34%
PP-PGM2-14-127	Non-essential intracellular esterase that can function as an S-formylglutathione hydrolase [Komagataella pastoris GS115]	gi|254571981	33374	369	34%
PP-PGM2-14-127	40S ribosomal protein S15 [Komagataella pastoris GS115]	gi|254567029	16408	360	37%
PP-PGM2-14-127	Transketolase, similar to Tkl2p [Komagataella pastoris GS115]	gi|254571911	79050	351	11%
PP-PGM2-14-127	Non-ATPase base subunit of the 19S regulatory particle (RP) of the 26S proteasome [Komagataella pastoris GS115]	gi|254572439	29657	330	27%
PP-PGM2-14-127	60S ribosomal protein L22 [Komagataella pastoris GS115]	gi|254572676	15051	248	32%
PP-PGM2-14-127	Conserved protein of the mitochondrial matrix, performs a scaffolding function during assembly of ir [Komagataella pastoris GS115]	gi|254567840	19616	238	22%
PP-PGM2-14-127	Nitrogen catabolite repression transcriptional regulator [Komagataella pastoris GS115]	gi|254568582	26834	232	35%
PP-PGM2-14-127	40S ribosomal protein S23	gi|254573324	16042	218	19%

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	[Komagataella pastoris GS115]				
PP-PGM2-14-127	ribulose-phosphate 3-epimerase [Komagataella pastoris CBS 7435]	gi|328353327	25619	207	40%
PP-PGM2-14-127	ribosomal 60S subunit protein L43A [Saccharomyces cerevisiae S288c]	gi|6325300	10369	206	20%
PP-PGM2-14-127	60S ribosomal protein L32 [Komagataella pastoris GS115]	gi|254572377	15281	202	17%
PP-PGM2-14-127	unnamed protein product [Kuraishia capsulata CBS 1993]	gi|584393455	12484	202	35%
PP-PGM2-14-127	Mitochondrial intermembrane space cysteine motif protein [Komagataella pastoris GS115]	gi|254571859	15922	193	13%
PP-PGM2-14-127	Peptidylprolyl-cis/trans-isomerase (PPIase) [Komagataella pastoris GS115]	gi|254569388	19081	191	34%
PP-PGM2-14-127	60S ribosomal protein L20 [Komagataella pastoris GS115]	gi|254570305	21461	190	21%
PP-PGM2-14-127	60S ribosomal protein L2 [Komagataella pastoris GS115]	gi|254565519	27193	189	24%
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254567421	11829	181	34%
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254567441	46634	168	9%
PP-PGM2-14-127	6-phosphogluconate dehydrogenase (decarboxylating) [Komagataella pastoris GS115]	gi|254570771	54232	161	9%
PP-PGM2-14-127	L-ornithine transaminase (OTAse) [Komagataella pastoris GS115]	gi|254571057	47514	142	10%
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254566011	16829	142	22%
PP-PGM2-14-127	hypothetical protein	gi|254570064	19545	137	44%

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	[Komagataella pastoris GS115]				
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254571957	22329	134	22%
PP-PGM2-14-127	40S ribosomal protein S29 [Komagataella pastoris GS115]	gi|254567055	6893	112	32%
PP-PGM2-14-127	60S ribosomal protein L24 [Komagataella pastoris GS115]	gi|254567714	17893	110	13%
PP-PGM2-14-127	Phosphatidylinositol 3,5-bisphosphate-binding protein [Komagataella pastoris GS115]	gi|254566399	42731	107	7%
PP-PGM2-14-127	Non-essential protein of unknown function required for transcriptional induction [Komagataella pastoris GS115]	gi|254573908	52457	95	8%
PP-PGM2-14-127	Putative protein of unknown function [Komagataella pastoris GS115]	gi|254571045	19805	88	14%
PP-PGM2-14-127	Thiol-specific peroxiredoxin, reduces hydroperoxides to protect against oxidative damage [Komagataella pastoris GS115]	gi|254568606	19255	85	25%
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254569418	18257	81	15%
PP-PGM2-14-127	40S ribosomal protein S20 [Komagataella pastoris GS115]	gi|254569654	13478	75	10%
PP-PGM2-14-127	60S ribosomal protein L36 [Komagataella pastoris GS115]	gi|254567471	11475	68	13%
PP-PGM2-14-127	hypothetical protein [Komagataella pastoris GS115]	gi|254566355	17991	67	22%

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Memorandum of Telephone Conversation

Date: August 3, 2015

Between:	Gary Yingling	Morgan, Lewis & Bockius LLP
	Jessica Vaughn	Morgan, Lewis & Bockius LLP
	Nick Halla	Impossible Foods Staff
	Patrick Brown	Impossible Foods Staff
	John (Last Name?)	Impossible Foods Staff
	Chris Davis	Impossible Foods Staff
	Rachel Fraser	Impossible Foods Staff
	Myra Pasek	Impossible Foods Staff
and		
	Lauren Brookmire	HFS-255
	Supratim Choudhuri	HFS-255
	Robert Merker	HFS-255
	Jannavi Srinivasan	HFS-255

Subject: Discussion with Impossible Foods regarding their submission on *Pichia pastoris*-expressed soy leghemoglobin (GRN 000540)

This telephone conference was held to discuss some issues regarding the GRAS notice for the use of *Pichia pastoris*-expressed soy leghemoglobin (SLH) as an ingredient in foods. FDA described the need for the notifier to provide strong scientific evidence when establishing safety. FDA stated that the current arguments at hand, individually and collectively, were not enough to establish the safety of SLH for consumption.

FDA stated that the notifier's basis for the safety of soy leghemoglobin was developed based on its structural and functional equivalence to other widely consumed globin proteins. The primary basis of safety relies on the 3-D structure of the substance, which is not enough evidence when providing a basis of safety. FDA stated that sequence identity could be provided as additional defense information. The notifier then discussed the weight of evidence approach used as part of the safety determination. FDA stated that additional information needed to be included for a robust, evidence-based weight-of-evidence basis of safety.

FDA also made note of the list of proteins (about 20-25% of the final product) co-purified with the SLH. FDA stated that the safety argument should include additional information on these proteins as opposed to solely covering SLH.

FDA suggested that the notifier consult the GRAS notice on Ice Structuring Protein (ISP, GRN 0117). The ISP is a protein from fish source (another common allergenic source) and expressed in yeast (*Saccharomyces cerevisiae*). FDA recommended that the notifier consider as one option developing its safety testing paradigm and protocol for SLH based on how the safety was addressed for ISP.

FDA also noted that although SLH is expressed in *P.* pastoris, its source is soybean, which is one of the most common allergenic foods. Therefore, the substance will be subject to satisfying FALCPA requirements and stipulations.

At the close of the meeting, administrative items were discussed, including the process for withdrawing a GRAS notice and the process for resubmitting a GRAS notice. FDA stated that by withdrawing the notice without prejudice, the notifier can address the deficiencies of the current basis for safety and come back once addressed. FDA personnel are more than willing to review this information prior to the notifier resubmitting the notice. The notifier stated that they would review their options and let FDA know of the notifier's decision.

Lauren Brookmire

GRN 0540 – Soybean leghemoglobin protein derived from *Pichia pastoris*

FDA's Evaluation of the Notifier's Response to FDA's Questions
Information prepared for Telephone Conversation on August 3, 2015.

***Notifier's view of the safety of soy leghemoglobin*:** In the GRAS notice and in the response to FDA's questions subsequent to the review of the notice, the notifier has made an attempt to establish the safety and general recognition of safety of *Pichia pastoris*-expressed soy leghemoglobin (SLH). The notifier's argument hinges on the following assumptions.

1. SLH is safe to consume because its modeled 3D structure is similar to that of hemoglobin and myoglobin– proteins that humans are normally exposed to through oral route.

2. SLH is safe to consume because it belongs to the globin family of proteins that is so widespread in all domains of life.

3. SLH is safe to consume because its function is same as that of hemoglobin and myoglobin, that is, it binds oxygen and other small gas gaseous molecules like CO, NO.

4. SLH is safe to consume because bioinformatic analysis using Allergenonline does not show the presence of 8-mer epitopes or >35% similarity to any allergenic proteins.

***FDA's view of the notifier's analyses of safety of SLH*:** FDA believes that the arguments presented, individually and collectively, do not establish the safety of SLH for consumption, nor do they point to a general recognition of safety, as explained below.

1. Conformational similarity or functional similarity among proteins is not an indication of the safety of proteins for consumption. It is intuitive that similar functions will dictate similar conformation or surface characteristics of proteins. Proteins with such conformational or active site similarity may not even have high sequence (i.e., primary structure) identity. Example, hemoglobin, myoglobin, SLH, hemocyanin.

2. Just belonging to the globin family does not guarantee that the protein will be safe to consume. An example of globin family of proteins that are not so safe for human exposure are the allergenic monomeric and dimeric hemoglobins in Chironomidae, a family of Diptera.

3. Binding oxygen and other similar molecules (CO, NO) is the function of all respiratory proteins. Such function has nothing to do with the safety of the proteins for consumption. An example is the allergenic hemocyanin present in edible shrimps.

4. The bioinformatic analysis using the standard sequence alignment-based approach in Allergenonline does not provide evidence of the lack of sensitization/allergenic potential of

SLH. Analyses using other software, such as SVM module-based software, indicate that SLH could be an allergen.

5. Additionally, the list of proteins (~20-25% of the final product) co-purified with the SLH raises further question on how the safety argument could be made based solely on SLH.

***Going forward—FDA's view*:**

1. The notifier has to establish the safety of consumption of SLH in food by providing direct evidence.

2. FDA recommends that the notifier consults the GRAS notice on Ice Structuring Protein (ISP; GRN 117). The ISP is a protein from fish source (another big allergenic source) and expressed in yeast (*Saccharomyces cerevisiae*). FDA recommends that the notifier develop its safety testing paradigm and protocol for SLH based on how the safety was addressed for ISP.

3. Although SLH is expressed in *P. pastoris*, its source is soybean, which is one of the eight most allergenic foods. Therefore, SLH will be subject to satisfying FALCPA requirements and stipulations.

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November 10, 2015

VIA EMAIL

Lauren Brookmire, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: Withdrawal of GRAS Notification 540

Dear Ms. Brookmire:

On behalf of Impossible Foods, Inc., we submit this letter requesting that FDA withdraw the notification for soybean leghemoglobin from *Pichia pastoris*, filed on September 18, 2014. Impossible Foods, Inc. appreciates the feedback obtained from the Food and Drug Administration, and will submit this application for review in the future, with additional supportive information.

Sincerely,

Gary L. Yingling, RPh, J.D.

cc: Impossible Foods, Inc.

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January 4, 2016

VIA E-MAIL

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Division of Biotechnology and GRAS Notice Review
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Center for Food Safety and Applied Nutrition
Food and Drug Administration
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Re: Request for Meeting to Discuss Filing of GRAS Notification for Soybean Leghemoglobin Protein Derived from *Pichia pastoris*

Dear Dr. Mattia:

This letter is to request a meeting with the Division of Biotechnology and GRAS Notice Review to review Impossible Foods' proposed approach to filing a new GRAS Notice for soybean leghemoglobin. As you may be aware, Impossible Foods filed a GRAS Notice for soybean leghemoglobin protein derived from *Pichia pastoris* and FDA accepted the submission on September 18, 2014, and gave it a GRAS number 540 ("GRN 540").

On August 3, 2015, Impossible Foods engaged in a call with the FDA, during which the Agency discussed some issues that arose during the review of GRN 540. Following that call, Impossible Foods determined that it would be productive to withdraw its review request for GRN 540, which it did on November 10, 2015.

After a careful review of the GRN 540, consideration of the questions and comments provided by FDA, and discussions with leading scientific experts, Impossible Foods believes that it has a plan that will result in a GRAS Notice that will address all of the questions raised by the FDA. However, before commencing testing in support of filing a new GRAS Notice, Impossible Foods wishes to have a meeting with the Agency to review its proposed plan.

The request for a meeting is in part based on Impossible Foods' desire to ensure that it is adequately addressing all of the FDA's concerns. In the August 3, 2015 phone call, the Agency suggested that Impossible Foods should carefully review the approach taken in GRN 117 for an ice structuring protein expressed in *S. cerevisiae*. Impossible Foods has completed that review, and concluded that GRN 117 did provide useful guidance in preparing its new GRAS Notice, but also raised some issues that require clarification. There are some differences between soy leghemoglobin from *Pichia pastoris* and the ice structuring protein from *S. cerevisiae*, which will result in Impossible Foods adopting a testing plan that, while similar, is not identical to that undertaken by the filer of GRN 117. The following is a brief overview of the approach Impossible Foods plans to take in the new submission:

1. Systemic toxicology testing

   Impossible Foods intends to conduct a 90-day feeding study in rat subjects to assess the systemic toxicology of the soy leghemoglobin. Doses of 125, 250, and 500 mg leghemoglobin/kg/day are proposed, in a sample of 10 animals/sex. The 90-day feeding study will be preceded by a palatability study (5 animals/sex) to determine if the animals can tolerate oral ingestion of the leghemoglobin. Due to the flavor associated with leghemoglobin, the rats may experience palatability issues. If this is the case, oral gavage will be employed. The 14-day palatability study will be used to guide the dose and mechanism of delivery (feeding versus gavage) for the 90-day study.

   Questions to the FDA:

   a) Does the FDA agree that a 90-day oral feeding study as summarized above (or oral gavage if necessary) will support the safety of leghemoglobin?

   b) Does the Agency agree that the three doses, 125, 250, and 500 mg/kg/day are appropriate for the 90-day study?

2. Genotoxicology testing

   To evaluate leghemoglobin genotoxicity, Impossible Foods proposes to conduct an Ames assay and an *in vitro* chromosome aberration assay, consistent with other GRAS notices filed. In the event of unusual results, the testing will be supplemented with additional assays, such as the *in vivo* micronucleus test and/or the mouse lymphoma assay, as appropriate.

   Questions to the FDA:

   Does the FDA agree that the Ames assay and the in vitro chromosome aberration assay, in the absence of unusual results, will support the safety of leghemoglobin?

3. Allergenicity testing

In GRN 540, Impossible Foods implemented a multi-factorial approach to assess the potential allergenicity of soybean leghemoglobin. The work was performed by Dr. Richard E. Goodman at the Food Allergy Resource and Research Program (FARRP) of the University of Nebraska and included the following analyses:

1) a full literature search to identify any published reports regarding health issues associated with hemoglobin proteins of any origin (GRN 540 Annex 3),

2) sequence homology comparisons between leghemoglobin's protein sequence and known allergens (GRN 540 Annex 3), and

3) the sensitivity of leghemoglobin protein to pepsin digestion in a simulated gastric fluid (GRN 540 Annex 4).

Dr. Goodman's expert opinion concluded that soybean leghemoglobin is very unlikely to present a risk of dietary allergy to consumers (GRN 540 Annex 2).

As recommended by the Agency, Impossible Foods reviewed GRN 117, and noted that the allergenicity testing was based on a two-pronged approach: Tests that addressed the general allergenicity of the ice structuring protein (ISP), and the tests that addressed ISP-reactivity with fish-allergic individuals. It was apparent from the notification that the authors of GRN 117 did not intend to label, and wished to avoid labeling, the finished food products that would contain ISP as containing fish.

It is the expert opinion of Dr. Stephen Taylor, co-founder and co-director of the FARRP at the University of Nebraska, that the experiments performed in GRN 117 to demonstrate that ISP does not cross-react with fish-allergic individuals are not applicable to leghemoglobin with respect to soy-allergic individuals (Appendix 1). In GRN 117, the primary motivation for including these tests was to eliminate the word "fish" from their label. Unlike GRN 117, Impossible Foods will assign leghemoglobin a common or usual name and will include "soy" on the label. In addition, Impossible Foods will notify consumers that the product "contains soy" as required by the statute. Because Impossible Foods will identify the potential allergen on its label, unlike the authors of GRN 117, there is no necessity to prove that soy-allergic individuals will not react to soy leghemoglobin.

Furthermore, the size of adult population of soy-allergic individuals is insufficient to acquire enough subjects to perform a statistically significant clinical study. While 0.4% of children are allergic to soy, the large majority of them outgrow it by the age of 10 (Savage HJ et al. 2010). Finally, leghemoglobin is natively expressed in the soybean root of the soy plant; whereas, the allergens m4, m5, and m6 are located in the seeds.

This physical separation indicates that the leghemoglobin is highly unlikely to elicit a reaction in a soy-allergic consumer. It is the opinion of Dr. Stephen Taylor that sequence homology and pepsin digest analyses are the most predictive methods known to date; therefore there are no additional tests that Impossible Foods could perform that would strengthen the evidence against potential allergenicity of soy leghemoglobin, as presented in GRN 540.

Because there will be *Pichia* proteins in the leghemoglobin ingredient and the finished product sold by Impossible Foods, to further support the conclusion that its product does not pose an allergenic risk, Impossible Foods will also perform the following evaluations on the *Pichia* proteins that are present:

1) A full literature search to identify any published reports regarding health or allergenicity issues associated with *Pichia* proteins
2) A sequence homology comparison between the *Pichia* protein sequences present and known allergens
3) A sensitivity analysis of the *Pichia* proteins present to pepsin digestion in a simulated gastric fluid.
4) A literature search to determine if any pepsin digestion-resistant *Pichia* proteins have homologs in *Saccharomyces cerevisiae* that are known allergens.

Questions to the FDA:

Does the FDA agree that the proposed allergenicity testing of the Pichia proteins, in conjunction with the previously reported evaluation of the leghemoglobin allergenicity studies from GRN 540 and including soy on the label, will suffice as support of the safety of leghemoglobin?

4. Soybean leghemoglobin protein specifications

As development of the final product has continued to evolve since the previous submission of GRN 540, Impossible Foods anticipates that its proposed soy leghemoglobin product will have the specifications presented in the table below.

Specifications of Heme Protein

Total Protein (%N x 6.25)	8-12% (w/w)
Leghemoglobin/Total Protein	> 65%

Fat	< 2% (w/w)
Carbohydrates	< 3% (w/w)
Ash	< 4% (w/w)
Solids	10-20% (w/w)
pH	6.5-8.5
Lead	< 0.01 ppm
Arsenic	< 0.01 ppm
Mercury	< 0.005 ppm
Cadmium	< 0.1 ppm
Aerobic Plate Count[1]	$<10^4$ CFU/g
E. Coli 0157:H7[2]	Absent by test
Salmonella spp.[3]	Absent by test
Listeria monocytogenes[4]	Absent by test

[1]AOAC OMA 990.12
[2]AOAC R1 020801
[3]AOAC OMA 2011.03
[4]AOAC OMA 2010.02

Most notably, the leghemoglobin content will be greater than 65% versus 80% proposed in the GRN 540 filing. Impossible Foods does not anticipate that this reduction in leghemoglobin will have any impact on the safety of the product. However, the toxicology tests proposed in this document, based on dosage of the specific leghemoglobin content of the preparation, will be conducted with product meeting the intended specifications of greater than 65% of the final product, to support the safety of the leghemoglobin product. Further, Impossible Foods will recalculate the exposure assessment using the current specifications.

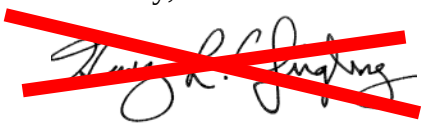
Questions to the FDA:

Does the FDA agree that the specifications are acceptable, and that using product with the new specifications in the toxicology testing will support the safety of the product?

We will provide an update submission that addresses the issues three (3) weeks prior to our scheduled meeting. For the purpose of scheduling the meeting, we will contact Lauren Brookmier who was the contact person on GRN 540.

If you have any questions concerning this request, please have someone contact me.

Sincerely,



Gary L. Yingling

cc: Lauren Brookmier

EXPERT COMMENTS ON POTENTIAL ALLERGENICITY OF SOYBEAN LEGHEMOGLOBIN

Steve L. Taylor, Ph.D.
Taylor Consulting LLC
Lincoln, NE

August 19, 2015

I have been informed that Impossible Foods has met with representatives from the Food & Drug Administration regarding GRN540. In a recent conference, I was informed by Impossible Foods of key comments and requests made by FDA representatives during this meeting. I have served as a consultant to Impossible Foods on the safety/allergenicity assessment of soybean leghemoglobin. I wish to provide my expert input on the ongoing discussions between Impossible Foods and FDA.

Apparently during the meeting, FDA compared GRN540 to GRN117, a notice on ice-structuring protein (ISP) that was advanced several years ago by Unilever. I also served as a consultant to Unilever and a member of the GRAS Panel for ISP. In my view, a major distinction exists between GRN540 and GRN117 that invalidates GRN117 as a model for the type of data that should be submitted by Impossible Foods on soybean leghemoglobin. A key feature of GRN117 was that Unilever did not wish to label ISP as a fish protein. Accordingly, Unilever was obliged to conduct extensive studies to document that ISP was not an allergenic fish protein and that its ingestion would be safe for fish-allergic consumers. The situation with soy leghemoglobin is the exact opposite. Impossible Foods fully intends to label soy leghemoglobin as a soy protein. Products with soy leghemoglobin will be labeled as "Contains Soy" in accordance with FALCPA requirements. Thus, soy-allergic consumers will be advised by these label statements to avoid products containing soy leghemoglobin. In essence, Impossible Foods is conceding that soy leghemoglobin is a possible allergen from soybeans even though there is no scientific evidence to suggest that this is the case.

In my expert opinion, the state of the science on soybean allergens can be summarized in one word – confusing. Many soy proteins have been identified as potential allergens. Expert scientific consensus does not exist with respect to a list of all soy proteins that might be potential soy allergens. Consensus is emerging that Gly m 5 and Gly m 6 are the major soy allergens and these proteins are also the major seed storage proteins of soybean. Because of the confusing nature of the scientific evidence, the possible existence of other soy proteins as minor allergens cannot be excluded. Thus, in my expert opinion, the wisest course for Impossible Foods is to reveal that the soy leghemoglobin ingredient is derived from soy. Thus Impossible Foods is recommending that the common or usual name for this ingredient should be "modified soy protein".

Any FDA request that Impossible Foods should conduct clinical studies on the potential allergenicity of soy leghemoglobin is unreasonable in my opinion. While soybeans are widely considered as a commonly allergenic food, soy allergy appears to occur almost exclusively in young infants and is a transitory condition. The vast majority of soy-allergic infants outgrow their soy allergy by the age of 10 years (*Savage JH, Kaeding AJ, Matsui EC, Wood RA. The natural history of soy allergy. J Allergy Clin Immunol, 2010;125:683-86*). Finding suitable numbers of soy-allergic adults for an oral challenge study would be virtually impossible. My research group (Food Allergy Research & Resource Program) has been attempting to conduct a

soy flour threshold study among adults (the IRB limited us to challenges of individuals age 16 or higher). This study has been ongoing for 11 years and we only have managed to locate 18 subjects on a worldwide basis. In my opinion, it would even be difficult to find a sufficient number of well-characterized soy-allergic subjects to be sources of blood serum to serum IgE-binding studies. Since Impossible Foods is advocating that this ingredient be clearly labeled as derived from soy, the necessity of providing clinical evidence of its potential allergenicity is very questionable in my opinion.

Impossible Foods has provided evidence of the potential allergenicity of soy leghemoglobin within GRN540. They provided evidence of sequence homology comparisons to a database of known allergen sequences (all allergens, not just food). They also provided evidence of the susceptibility of soy leghemoglobin to pepsin digestion. These two approaches are considered as critical in the assessment of the potential allergenicity of novel food proteins derived from genetic engineering. In my expert opinion, these two pieces of evidence are critical components of GRN540. Impossible Foods clearly wishes to market this "modified soy protein" for various uses as described in GRN540. Thus, consumer exposure to soy leghemoglobin could be expected to increase. This increased consumer exposure to soy leghemoglobin carries with it the concern that the increased exposure might result in increased allergic sensitization to soy leghemoglobin. Thus, in my expert opinion, Impossible Foods was prudent and responsible in arranging to have the potential allergenicity of this protein evaluated by these two well-accepted procedures. The results of this allergencity assessment are well described in GRN540. On the basis of the results of these two approaches, soy leghemoglobin does not have the characteristics that are common other allergenic proteins. While I would join other scientific experts in wishing that science could provide additional definitive and discriminatory tests to evaluate the potential allergenicity of novel proteins in the diet, these two approaches remain the only well-accepted procedures. In my expert opinion, no value would be obtained in conducting additional test for the assessment of the potential allergenicity of soy leghemoglobin once consumer exposure to this protein is enhanced.

Finally, I would emphasize that studies on soy-allergic consumers address the concern that soy leghemoglobin might be allergenic and a hazard to existing soy-allergic consumers. As noted, Impossible Foods is going to label soy leghemoglobin in a fashion that existing soy-allergic consumers should avoid it. Impossible Foods has assessed the potential allergenicity of soy leghemoglobin that might result from increased exposure to this protein through these proposed new uses. Impossible Foods has used the two most appropriate allergenicity assessment approaches. No concerns are evident from these results. In my opinion, FDA must view GRN540 from two perspectives: (1) risk to existing soy-allergic consumers and (2) the potential for increased soy allergy as a result of increased consumer exposure to soy leghemoglobin.

In my expert opinion, GRN540 appropriately addresses both perspectives – one through labeling and the other through appropriate documentation of a low risk for increased allergenicity. Further evaluation as encouraged by FDA will not advance decision-making ability on either point. Thus, in my expert opinion, additional testing as proposed by FDA is unnecessary.

Impossible Foods

Soy Leghemoglobin Protein Derived from *Pichia pastoris*

February 3, 2016

Impossible Foods' Mission

Make delicious meat- and dairy-like foods directly from plants

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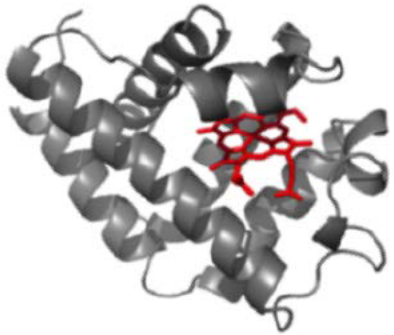
Our Approach

Reverse Engineering

Protein Discovery



Flavor Creation

Material Transformation

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Soy Leghemoglobin Product Usage

- Soy leghemoglobin (b) (4) product under development
  - Amount of the (b) (4) used is (b) (4) based on the soy leghemoglobin concentration
  - The (b) (4) was used to calculate the EDI, which will be used to set appropriate test levels for the (b) (4) studies
- The (b) (4) product will clearly be labeled as "containing soy"

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The Impossible Burger

100% Made from Plants

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Soy Leghemoglobin Toxicology Testing

February 3, 2016

Leghemoglobin Systemic Toxicology Testing

14-Day Dietary Toxicity/Palatability Study in Rats

Non-GLP (OECD 407, FDA Redbook 2000, IV.C.3a, OPPTS 870.3100)

Objective

- Evaluate (b) (4) general toxicity

Experimental Design

- Test article administered in the diet
- (b) (4)
- animals/sex/dose

Analysis

- Clinical observations, food consumption, body weight, hematology, gross necropsy, organ weight

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Leghemoglobin Systemic Toxicology Testing

90-Day Dietary Toxicity Study in Rats

GLP (OECD 408, FDA Redbook 2000, IV.C.4a)

Objective

- Determine leghemoglobin NOAEL for each sex

Experimental Design

- Test article administered in the diet
- (b) (4) n/kg/day
- (b) (4) animals/sex/dose
- Design pending results from the 14-day study

Analysis

- Clinical observations, food consumption, body weight, ophthalmologic evaluation, clinical pathology, histopathology

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Leghemoglobin Genotoxicology Testing

AMES Test

GLP (OECD 471)

Experimental Design

- 4 strains of *Salmonella typhimurium* and 1 strain of *E. coli*.
- 5 test article concentrations
- +/- metabolic activation (S9 mix)

Analysis

- Histidine reverse mutation rate

Chromosome Aberration Test

GLP (OECD 473)

Experimental Design

- Human lymphocytes
- 6 dose groups
- 4 h treatment +/- metabolic activation (S9 mix)
- 3 test article concentrations, 200 metaphases each
- Preparation interval 1-2 cell cycles

Analysis

- Chromosome aberration type and frequency

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Manufacturing Process

February 3, 2016

IMPOSSIBLE™

Soy [redacted] Manufacturing Process

- [redacted] manufacturing process is like many food [redacted] processes
- [redacted] is recovered and concentrated from [redacted]
- The process consists of:
  - [redacted]
  - [redacted]
  - [redacted]
  - [redacted]
  - [redacted]

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Soy (b) (4) Product Specifications

(b) (4)

Lead	< 0.01 ppm
Arsenic	< 0.01 ppm
Mercury	< 0.005 ppm
Cadmium	< 0.1 ppm
Aerobic Plate Count[1]	< 10^4 CFU/g
E. Coli 0157:H7[2]	Absent by test
Salmonella spp.[3]	Absent by test
Listeria monocytogenes[4]	Absent by test

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Soy (b) (4) Allergenicity Assessment

February 3, 2016

Allergenicity Assessment

[redacted]

Multifactorial approach to assess the general allergenicity of leghemoglobin has been completed by Dr. Richard Goodman, U. Nebraska, FARRP

1. Literature search for allergen and/or toxicity reports
   - No publications were found that identified l[redacted] as an allergen
2. Sequence homology comparison to known allergens
   - Sequence comparison [redacted] revealed no homology to known allergens
3. Sensitivity to pepsin digestion in simulated gastric fluid
   - [redacted] digested within [redacted]

Pichia:

The same approach will be used to assess the allergenicity [redacted]

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Allergenicity Assessment (cont'd)

Soy:

Concerning an assessment of leghemoglobin cross-reactivity with soy-allergic individuals:

- (b) (4) [redacted]

Notification of consumers to the presence of soy:

- Impossible Foods label will include soy as an ingredient
- The allergen statement "contains soy" will also be included on the label

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HIGHLY CONFIDENTIAL – DO NOT DISTRIBUTE

MEMORANDUM OF MEETING

Date: February 3, 2016

Time: 1:00 – 2:00 pm

Place: FDA, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, 4300 River Road, College Park, MD 20740

Participants:

Visitors

Gary Yingling	Morgan, Lewis & Bockius LLP
Jessica Vaughn	Morgan, Lewis & Bockius LLP
Stephen Taylor	Taylor Consulting LLC
Don DiMasi	Impossible Foods Inc
Rachel Fraser	Impossible Foods Inc
Myra Pasek	Impossible Foods Inc, General Council

FDA

Lauren Brookmire	HFS-255
Supratim Choudhuri	HFS-255
Terry Deng	HFS-255
Michael DiNovi	HFS-255
Robert Merker	HFS-255
Jannavi Srinivasan	HFS-255

Subject: Discussion with Impossible Foods on the company's potential approach to address FDA suggestions after withdrawal of GRN 000540

Mr. Gary Yingling, Senior Council with Morgan, Lewis & Bockius LLP, requested the meeting on behalf of Impossible Foods Inc. to consult with FDA regarding Impossible Foods' potential approach to addressing the questions raised by FDA during the agency's review of GRN 0540. Impossible Foods recently requested that FDA cease its evaluation of GRN 0540 on November 10, 2015. The subject of GRN 0540 is *Pichia pastoris*-expressed soy leghemoglobin.

At the start of the meeting, the visitors gave a brief presentation that outlined the topic areas and objectives of the present meeting. As part of the presentation, Impossible Foods staff provided an overview of the company and walked through the company's product development process for the soy leghemoglobin. Dr. Steve Taylor provided

information on the allergenicity aspects of the company's safety review. Dr. Taylor stated that no allergen issues have been found. This includes both allergens from food sources as well as non-food sources. He also mentioned that most allergens tend to be stable in the sense that they do not break down in thirty seconds. The notified substance has been found by the company to fully break down within the first thirty seconds in synthetic gastric fluid. Dr. Taylor added that in his professional opinion, oral studies will be quite difficult to perform. It was also noted by Impossible Foods staff that the final product will be labeled as 'contains soy'.

The visitors concluded the presentation and fielded questions and comments from the agency staff. FDA staff asked for clarification on whether the substance currently being discussed differs from the substance previously reviewed under GRN 0540. Impossible Foods staff stated that the current composition of the substance slightly differs from the product detailed in GRN 0540. They stated that their production methods have been evolving during the scaling up process, resulting in changes to the composition of the final soy leghemoglobin product.

FDA then provided feedback on the toxicology aspects of a safety study used to support a conclusion of GRAS status. FDA emphasized that in general, GRAS status requires demonstration of both safety as well as general recognition of that safety. FDA noted that the product being discussed in an ingredient that has not been used in food before. FDA also noted that while a safety review often describes similarities between a new substance and other substances on the market, it is also useful to cover what makes the new substance different and why these differences are not a problem. Other specific areas of research and safety coverage were also suggested by FDA. This included the need for publically available information on the digestibility of the protein.

FDA referred to areas of narrative as well as specific references in GRN 0540 submission and provided feedback on aspects that were lacking and would be valuable for a future submission. An example provided by FDA was the subject of allergenicity potential and cross-reactivity as an area suggested to be covered in the narrative part.

FDA mentioned that it may be useful for the company to talk with Division of Petition Review in the Office of Food Additive Safety regarding the substance being qualified as a color additive. Mr. Yingling noted that color is not the intended use of the substance, but instead an indirect effect.

At the close of the meeting, FDA staff stated that they would be willing to review any additional information prior to the notifier submitting a new notice.

Lauren Brookmire

Morgan Lewis

Gary L. Yingling
Senior Counsel
+1.202.739.5610
gary.yingling@morganlewis.com

August 23, 2016

VIA E-MAIL

Lauren Brookmire, MS
Division of Biotechnolongy and GRAS Notice Review (HFS 255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: Safety Testing of Soy Leghemoglobin-GRN 540

Dear Ms. Brookmire:

As you may recall, Impossible Foods submitted a GRAS notification for its soy leghemoglobin product on September 4, 2014. Following review of the notification, the Food and Drug Administration ("FDA" or "Agency") had several questions for Impossible Foods, and subsequently a meeting was held with the FDA and Impossible Foods on February 3, 2016, to address the issues within the notice.

During the meeting, the issue of safety testing was raised, and Impossible Foods informed the Agency that they were planning to complete a 90-day feeding study in rodents, to support the safety of the product. After consideration of the Agency's feedback during the meeting, Impossible Foods has decided instead to conduct a 28-day study. The company is preparing to conduct the study, and has approached me as regulatory counsel with the proposed study design. Impossible intends to test the doses of 250, 500, and 750 mg/kg bw/day soy leghemoglobin in the 28-day study. The highest dose was selected as it provides a safety factor of 100 times the consumption levels estimated in the 90th percentile estimated daily intake calculations. This dose was not intended to achieve a maximum tolerated dose.

I have advised Impossible Foods that this proposed dosing schedule is appropriate, and consistent with the safety testing that is expected to be included in a GRAS notification. We are now seeking confirmation from the Agency that this dose schedule is acceptable, and would support the safety of the product in a future GRAS notification. Should you have any questions, or require additional information, please do not hesitate to contact me by phone (b) (6) or by email, gary.yingling@morganlewis.com.

Sincerely,

Gary L. Yingling

cc: Robert Merker, HFS 255

Morgan, Lewis & Bockius LLP

1111 Pennsylvania Avenue, NW
Washington, DC 20004
United States

T +1.202.739.3000
F +1.202.739.3001

Brookmire, Lauren

From: Brookmire, Lauren
Sent: Thursday, September 08, 2016 2:23 PM
To: 'Yingling, Gary L.'
Subject: RE: Letter to FDA Re Dosing for 28-Day Study

Dear Mr. Yingling,

Thank you for your patience in my responding. I have discussed your letter with the relevant staff in the Office. It is necessary to emphasize that we cannot provide confirmation that a study – which has not yet been conducted – will support the safety of a product in a GRAS conclusion. We cannot offer such assurances in advance of the conduct of the study. As you are aware, the safety assessment supporting a GRAS conclusion involves multiple types of information, not just a feeding study. A GRAS conclusion is supported by the total 'package' of information, which has multiple dependent factors. Details regarding the substance's chemical composition (which to our understanding is now different from what was submitted in GRN 000540), stability data, digestibility data, and dietary exposure may all be part of the information necessary in a safety assessment as well. The support of one dosing study cannot be assessed independently of the other types of information.

Regarding the levels you mentioned, we feel that the rationale for your selection of the highest level to be tested makes sense. We also feel that the highest level tested should not be considered the maximum tolerable level. In regards to the time frame of the study, we do not provide specific suggestions such as this to a notifier for a GRAS notice.

Thank you, and I hope you find this information useful.

Lauren Brookmire, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: (b) (6)
Email: Lauren.Brookmire@fda.hhs.gov

From: Yingling, Gary L. [mailto:gary.yingling@morganlewis.com]
Sent: Monday, September 05, 2016 11:20 AM
To: Brookmire, Lauren
Subject: RE: Letter to FDA Re Dosing for 28-Day Study

Thanks. They are really anxious to start the study but want to be sure it will generate the data that will be helpful. If you have any questions, give me a call. gary

Gary L. Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW | Washington, DC 20004-2541
Direct: (b) (6) | Main: +1.202.739.3000 | Fax: +1.202.739.3001
gary.yingling@morganlewis.com | www.morganlewis.com

From: Brookmire, Lauren [mailto:Lauren.Brookmire@fda.hhs.gov]
Sent: Friday, September 02, 2016 2:11 PM
To: Yingling, Gary L.
Subject: RE: Letter to FDA Re Dosing for 28-Day Study

Hi Gary,

Thank you for your patience in my responding, as I was out on vacation. I have passed along your letter to the appropriate reviewers/management, and I should be able to get you a response during the middle of next week.

Thank you, and have a nice Labor Day weekend.
Lauren Brookmire

From: Yingling, Gary L. [mailto:gary.yingling@morganlewis.com]
Sent: Tuesday, August 23, 2016 11:27 AM
To: Brookmire, Lauren
Cc: Merker, Robert I; Vaughn, Jessica L.
Subject: Letter to FDA Re Dosing for 28-Day Study

Dear Lauren: Attached is a letter requesting comment on the intent of Impossible Foods to use the doses of 250, 500 and 750 mg/kg bw/day in the 28 day feeding study. A quick response would be most appreciated. gary

Gary L. Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW | Washington, DC 20004-2541
Direct: (b) (6) | Main: +1.202.739.3000 | Fax: +1.202.739.3001
gary.yingling@morganlewis.com | www.morganlewis.com

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July 17, 2017

Kim Richman
Richman Law Group
81 Prospect Street
Brooklyn, NY 11201

Re: FOI Request No. 2017-4553

Dear Ms. Richman:

This is in response to your request of May 17, 2017, requesting information regarding the FDA evaluation of the Impossible Foods Inc. September 24, 2014 GRAS notice submitted to Dr. Antonia Mattia, then Director of the Division of Biotechnology and GRAS Notice Review. The FDA subsequently designated that submission as GRAS Notice No. 540. Your request was forwarded to the Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition.

Enclosed are documents pertinent to your request. Your request is granted in full. Publicly available documents, including the notice and FDA's response letter have not been included. They are freely available on FDA's webstie. After a thorough review of the responsive records, we have determined that portions of the documents are exempt from disclosure under FOIA exemptions (b)(4) and (b)(6) of the FOIA 5 U.S.C. § 552, as amended and delineated below.

The FOIA exemption (b)(4) permits the withholding of "trade secrets" (TS) and "commercial and confidential information" (CCI) that is privileged or confidential. Disclosure of this information would impair the government's ability to obtain necessary information in the future and cause substantial harm to the competitive position of the person from whom the information was obtained. Under the balancing test of this exemption, we are withholding all proprietary information identified as TS and CCI.

The FOIA exemption (b)(6) permits the withholding of information which, if released, would constitute a clearly unwarranted invasion of personal privacy. In this case, it was determined that there is no countervailing public interest qualifying under the standard set forth, under exemption (b)(6), to release the identity of certain third parties.

You may appeal this determination within 90 days from the date of this letter. Your appeal should include copies of your original request and this response, as well as a discussion of the reasons supporting your appeal. The envelope should be plainly marked to indicate that it contains a FOIA appeal and please include the control number. If you decide to appeal this determination, your appeal should be sent to:

Ms. Catherine Teti
Deputy Agency Chief FOIA Officer
U.S. Department of Health and Human Services
Office of the Assistant Secretary for Public Affairs
Room 729H
200 Independence Avenue, S.W.
Washington, DC 20201

Please clearly mark both the envelope and your letter "Freedom of Information Act Appeal."

If you would like to discuss our response before filing an appeal to attempt to resolve your dispute without going through the appeals process, please contact sharon.dodson@fda.hhs.gov or call 240-402-1166. You may also contact the FDA FOIA Public Liaison for assistance at:

Office of the Executive Secretariat
Division of Freedom of Information
U.S. Food & Drug Administration
5630 Fishers Lane, Room 1050
Rockville, MD 20857

If you are unable to resolve your FOIA dispute through our FOIA Public Liaison, the Office of Government Information Services (OGIS), the Federal FOIA Ombudsman's office, offers mediation services to help resolve disputes between FOIA requesters and Federal agencies. The contact information for OGIS is:

Office of Government Information Services
National Archives and Records Administration
8601 Adelphi Road–OGIS
College Park, MD 20740-6001
Telephone: 202-741-5770
Toll-Free: 1-877-684-6448
E-mail: ogis@nara.gov
Fax: 202-741-5769

The following charges for this request to date may be included in a monthly invoice:

Reproduction $ 0.00 Search $46.00 Review $138.00 Other $1.00 (CD) Total $185.00

THE ABOVE TOTAL MAY NOT REFLECT THE FINAL CHARGES FOR THIS REQUEST. **PLEASE DO NOT SEND PAYMENT** UNTIL YOU RECEIVE AN INVOICE FOR THE TOTAL MONTHLY FEE.

Sincerely Yours,

Sharon R. Dodson

for Sheila Wright
FOIA Officer
Center for Food Safety
and Applied Nutrition

Enclosure

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740-3835

October 2, 2014

Mr. Gary Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW
Washington D.C. 20004-2541

Re: GRAS Notice No. GRN 000540

Dear Mr. Yingling:

The Food and Drug Administration (FDA) has received the notice, dated September 4, 2014, that you submitted on behalf of Impossible Foods Inc. in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received this notice on September 6, 2014, filed it on September 18, 2014, and designated it as GRN No. 000540.

The subject of the notice is soybean leghemoglobin protein derived from *Pichia pastoris*. The notice informs FDA of the view of Impossible Foods Inc. that soybean leghemoglobin protein derived from *Pichia pastoris* is GRAS, through scientific procedures, for use as a component of meat and poultry analogue products at levels of no more than 1.4%.

In accordance with proposed 21 CFR 170.36(f), a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)) is available for public review and copying at www.fda.gov/grasnoticeinventory. If you have any questions about the notice, contact me at Lauren.Brookmire@fda.hhs.gov or (b) (6)

Sincerely yours,

Lauren Brookmire
Division of Biotechnology and
GRAS Notice Review
Center for Food Safety
and Applied Nutrition

Hard copy cc: GRN 000540 (1 copy)
Filename: gn0540ak
R/D:LBrookmire:10/2/14
Init:RMerker:10/2/14
F/T:LBrookmire:10/2/14

Questions/Comments for GRN000540 April 8, 2015

I. General comments

The information provided as the basis of the GRAS determination should contain a level of specificity necessary when discussing the ingredient (soybean leghemoglobin protein). The notification should adequately address (i) the safety of soy leghemoglobin for human consumption and (ii) general recognition of its safety for the intended use and level.

Although proteins are a part of the human food supply, not all proteins are safe. Information addressing the safe use of modified soy protein does not adequately address safe use of soybean leghemoglobin protein from the **roots** of the soybean plant in food.

II. Issues regarding the clarity of statements in the GRAS notice

1. Please confirm whether the sequence of leghemoglobin that is subject of this GRAS notice has the Geneinfo Identifier (GI) 126241. The database has multiple *Glycine max* leghemoglobin sequences that are not identical.

2. Page 6 refers to the production strain as *Pichia pastoris* Bg10, which Page 7 refers to it as MXY022. Please clarify the designation of the production strain.

3. Please provide information about the minimum temperature of denaturation for soy leghemoglobin.

III. Issues regarding the scientific reasoning and availability of public information

1. The dietary exposure discussion in GRN 540 includes history of safe use of soy proteins from the soybean plant in general and does not discuss soy leghemoglobin from the roots of the soybean plant, which is the ingredient described in the GRAS notice. The discussion is not relevant in the context of the GRAS notice because soybean root is not a commonly consumed human food. Please provide relevant information, as there is no history or knowledge of human dietary exposure to soy leghemoglobin from roots.

2. Provide a short description, with reference to the use of *Pichia pastoris* in the food industry to purify various expressed proteins for human consumption or other systemic use.

3. The manufacturing specification for total protein content is set at 91% and that for leghemoglobin is set at 73%. Please provide explanation regarding the purity of the ingredient described in the GRAS notice, to account for other proteins that might co-purify with the soy leghemoglobin from *P. pastoris*. The response to this issue is directly related to the preceding issue of the history of safe use of *P. pastoris* in the food industry for purifying proteins meant for human consumption.

4. There are published reports of allergic responses in humans to myoglobin (although rare). Please incorporate those reports in the context of your discussion of safety and the general recognition of safety of oxygen-binding globin proteins.

5. On Page 2, the notifier states that one of the uses of soy leghemoglobin produced in *P. pastoris* is nutrition. Explain how the use of this ingredient in foods affects dietary protein profile of the proposed foods at the proposed use level.

IV. Issues requiring data/experimentation (or reference of publically available data)

1. The dietary exposure assessment is based on 1% market share of beef, pork and poultry consumption. Please recalculate dietary exposure to capture 100% market share to provide a conservative estimate of the consumption of the ingredient from the proposed uses.

2. Please provide reference supporting the methods used for the digestibility experiment and evidence that the method has been used widely in performing such studies. If such references cannot be provided, then please provide justification for the design of the experiment, with emphasis on the enzyme:substrate ratio and how the choice of such a ratio compares with methods commonly done in the study of protein safety in food. This is needed in order to show whether the stability of the protein could have been artificially altered in the digestibility experiment by the use of too much enzyme.

3. Provide batch analytical data that meet set specifications for 3 to 5 consecutive manufacturing lots of the dry powder formulation of this ingredient.

4. Provide a stability profile of the ingredient when used in a meat or poultry analogue.