Appendix VIII

Expert Opinions on the Safety of Soy Leghemoglobin and *Pichia pastoris*

TABLE OF CONTENTS

UPDATED EXPERT COMMENTS ON POTENTIAL ALLERGENICITY
OF SOYBEAN LEGHEMOGLOBIN

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Impossible Foods has met with representatives from the Food & Drug Administration regarding its GRAS Notification (GRN540) for soy leghemoglobin. FDA representatives have shared several critical comments with Impossible Foods with respect to GRN540. Previously, I had submitted my expert opinion on the potential allergenicity of soy leghemoglobin (specifically, soy leghemoglobin preparation (LegH Prep), with soy leghemoglobin as its principal ingredient). Now, I wish to expand upon that previous opinion to address certain key concerns raised by FDA representatives. The concerns raised at various times by FDA regarding GRN540 and the potential allergenicity of soy leghemoglobin are listed below together with my responses based upon my scientific knowledge and expertise.

- FDA concern that Impossible Foods should perform a full allergenicity evaluation on soy leghemoglobin and develop a GRAS dossier patterned after GRN117

In one meeting between FDA and Impossible Foods, 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 soy 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 also 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 soy, even though there is no scientific evidence to suggest that this is the case.

- FDA concern that Impossible Foods should conduct clinical studies on soy-allergic individuals to determine if soy leghemoglobin is a soy allergen

Soy leghemoglobin is very unlikely to pose any risk to soy-allergic consumers. First, soy leghemoglobin is derived from the roots of the soybean plant and not the edible seeds. The known soy allergens are found in soybean seeds. Soy leghemoglobin bears no structural similarity to any of the known soy allergens. But beyond that, Impossible Foods is planning to identify soy leghemoglobin in its ingredient label as "leghemoglobin (soy)" and advise that products containing soy leghemoglobin should be labeled as "Contains Soy". Thus soy-allergic consumers will be alerted that they should avoid consumption of products containing soy leghemoglobin.

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, it is the wisest course for Impossible Foods to reveal that the soy leghemoglobin ingredient is derived from soy. And in fact, Impossible Foods is recommending that the common or usual name for this ingredient should be "leghemoglobin (soy)".

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 et al., 2010*). 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.

- FDA concern that Impossible Foods should evaluate the sensitizing potential of soy leghemoglobin as a novel protein

Impossible Foods has provided evidence of the potential sensitizing capacity of soy leghemoglobin within GRN540. Specifically, they provided evidence of the susceptibility of soy leghemoglobin to pepsin digestion. Soy leghemoglobin was rapidly hydrolyzed by pepsin, a characteristic that makes it less likely to retain any sensitizing capacity as the digested remnants enter the small intestine. 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, this approach remains the only well-accepted procedure.

- FDA concern that Impossible Foods should evaluate the capacity of soy leghemoglobin to cross-react with other known allergens especially legume allergens

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 (allergens from all sources, not just food). This approach is known to provide evidence of cross-reactive potential with known allergens from all sources especially when conservative bioinformatics criteria are used in the assessment as was done in this particular example. Specifically, this assessment did not reveal any sequence homologies between soy leghemoglobin and any known allergens from legume sources.

Cross-reactions within the legume botanical family are not especially common in the U.S. This fact is fortunate because more than 300 edible legume species exist in the human diet. Peanuts are, by far, the most potent and prevalent cause of allergies within the legume family. Soybeans are also considered as commonly allergenic but soybean allergy is considerably less prevalent and

typically less severe. Clinical cross-reactivity among various foods from the legume family is rare (*Bernhisel-Broadbent and Sampson, 1989*). However individuals allergic to a single legume often display positive skin prick tests to other legumes that they can safely ingest (*Bernhisel-Broadbent et al., 1989*). Over the years, many clinical investigators have errantly evaluated potential cross-reactivity among legumes only via the presence of cross-reactive IgE in patient sera or skin test cross-reactive to legume extracts (*Beslar, 2000*). As shown very conclusively (*Bernhisel-Broadbent and Sampson, 1989*), oral challenges are necessary to truly document cross-reactivity among legumes. In that pioneering study, only two of 69 patients (3%) sensitized to legumes (peanut, soybean, pea, green bean, lima bean) were reactive on oral challenge to two legumes (*Bernhisel-Broadbent and Sampson, 1989*). In both cases, these patients were primarily allergic to peanuts with histories of severe reactions and had mild reactions to soybeans. In contrast, 49 of the 69 subjects had positive skin tests or serum IgE tests to two or more legumes.

Similarly, among peanut-allergic individuals, oral challenges revealed the peanut allergy was the sole legume allergy in 94% of 142 subjects while only 8 of the 142 (5.6%) subjects reacted to other legumes on challenge: 4 to pea, 2 to soybean, and 2 to lentil (*Moneret-Vautrin et al., 1998*). Among 187 food-allergic children diagnosed by oral challenge, only 2 children (1.1%) were allergic to more than one legume (peanut-soy in one case; peanut-pea in the other) (*Bock and Atkins, 1990*). In the largest study reported to date in 793 persistent peanut-allergic subjects, 9.5% were considered allergic to other legumes by oral challenge including 48 to soy, 19 to pea, 7 to lentil, 4 to chickpea and 3 to green bean (*Neuman-Sunshine et al., 2012*)

Differing results were obtained in several other clinical studies. Peeters et al. (2009) evaluated 39 peanut-sensitized patients and found that 30/39 individuals were reactive on challenge to peanut while 12/30 subjects (40%) were also allergic to soybean, 6/30 subjects (20%) were also allergic to pea, and 8/30 subjects (26.7%) were also allergic to lupine. Similar results were found among soybean-allergic subjects where 21 of 35 individuals (60%) were also allergic to peanut (*Klemans et al., 2013*). These results might be ascribed to the selection of patients who were cross-reactive because especially in the study of Peeters et al. (2009), the focus of the study was lupine cross-reactivity.

Ibanez et al. (2000) studied a total of 66 legume-allergic subjects but did challenges to more than one legume on only 39 of these subjects. Of those 39 subjects, 21 (54%) reacted to two or more legumes. Of 15 patients challenged with lentil and pea, 11 (73%) reacted to both, 15 of 27 (56%) to lentil and chickpea, 9 of 16 (56%) to chickpea and pea, 8 of 15 (53%) to lentil, chickpea and pea, 3 of 5 (60%) to lentil and peanut and 2 of 5 (40%) to peanut and pea and 0 of 7 to peanut and chickpea.

These studies are the key references to legume cross-reactions that involve oral challenges to confirm that clinically significant cross-reactivity is actually occurring. Several of the studies suggest that cross-reactivity among various species of legumes is rather infrequent, while other studies suggest that certain cross-reactions among legumes are more common. In particular, cross-reactions among lentil, chickpea, and pea seem more common than cross-reactions with peanuts or soybeans.

In my opinion, based upon the prevalence and severity of peanut allergy, potential cross-reactions between soy leghemoglobin and peanut allergens should be the key area of potential concern. However, in that regard, the various peanut allergens are very well identified and characterized. No significant sequence homology exists between soy leghemoglobin and any of these peanut allergens. Clinically significant cross-reactions between peanuts and soybeans occur infrequently even though some homology does exist between the vicilin and legumin allergens in peanuts and

soybeans. The vicilins and legumins are seed storage proteins so some sequence homology might be expected. But, the similarities do not appear to lead to allergenic cross-reactivity in most patients with allergy to either peanut or soybean. Leghemoglobin is found in the root of the soybean plant and bears no structural resemblance or sequence homology to these seed storage proteins.

In my opinion, conducting clinical studies to determine if soy leghemoglobin elicits allergic reactions in peanut-allergic individuals is unwarranted because the results are quite predictable based upon bioinformatics comparisons. And, conducting clinical studies with soy leghemoglobin in individuals with allergies to other legumes is also unnecessary given that the legume allergens are found in the seeds while leghemoglobin is localized in the roots and because the existing evidence suggests that allergic cross-reactivity among legumes is limited to a few species that are not prevalent allergenic foods in the first place.

Conclusion

In my opinion, Impossible Foods has addressed all of the potential allergenicity issues associated with soybean leghemoglobin in a thorough fashion. The labeling of soy leghemoglobin as "leghemoglobin (soy)" will alert soy-allergic consumers to avoid this product. GRN540 addresses all of the potential allergenicity concerns. The available data in GRN540 document that soy leghemoglobin is unlikely to become a novel allergen and demonstrate that soy leghemoglobin is unlikely to cross-react with known allergens from various sources including other foods and legumes. Thus, in my expert opinion, additional testing as proposed by FDA is unnecessary.

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Summary of the Allergenicity and Toxicity Assessment of Soy Leghemoglobin Preparation for Food Use

My laboratory performed a weight of evidence assessment of the potential allergenicity and toxicity of leghemoglobin from soybean (*Glycine max*), expressed in *Pichia pastoris*, for food safety. The evaluation followed the principles of the CODEX Alimentarius Guidelines for risk assessment of foods derived from modern biotechnology (CAC/GL 44-2003). The assessment focused on the soy leghemoglobin protein, with the full 145 amino acid (AA) sequence listed in the NCBI protein database as GI:126241 (Accession #P02236.2). Additional bioinformatics was performed with sequences of the proteins from *P. pastoris* that are present in Soy Leghemoglobin Preparation.

Bioinformatics (sequence comparisons) were made using the AA sequence of the query protein (leghemoglobin) on AllergenOnline.org, version 16, with evaluation of Full-length (looking for sequence matches >50%), Sliding 80-mer (matches with >35% identity over 80) and 8 AA identity comparisons. The highest scoring overall alignments were ~ <26% identity to hemoglobins from a fly larvae (*Chironomus thummi*), which suggests overall an evolutionary relationship. However, it is highly unlikely there is any possibility of allergic cross-reactivity. The 80-mer match and 8 AA identity matches were negative. The sequence was also tested against NCBI Protein using BLASTP with keyword limits (allergen, allergy, toxin and toxic) as well as without keyword limit. There were no "statistically significant" alignments using "allergen". BLASTP alignments with "allergy" were very small and not likely to be important (29% identity over 93 AA with an E score of 7e-5 to *Lephospira yanagawae* and 43% identity over 31 AA with an E score of 0.002 to *Burkholderia multivorans*). Those matches are unlikely to represent cross-reactive matches and do not require additional testing.

Bioinformatics searches for "toxin" and "toxic" were also negative. Using the keyword "toxin" the highest scoring matches were to *Bordetella bronchiseptica* nitric oxide dioxygenase and *Bordetella pertusuis* with 35% identity over 31% coverage. There were no specific matches for toxic. Thus there is no important match to a toxin and no indication for toxicity testing.

Public information from peer-reviewed literature in PubMed was evaluated for evidence of allergy and toxicity associated with soybean protein known as "leghemoglobin". No relevant publications were identified.

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The bioinformatics analysis described above was also performed on 17 proteins of the recombinant host organism, *Pichia sp.* (*Komagataella sp.*). These proteins were identified by Impossible Foods and the Genome Center at the University of California, Davis as residual proteins constituting at least 1% of the total protein fraction of Soy Leghemoglobin Preparation. The 17 proteins were identified by LC-MS/MS and matched to the following proteins:

- Alpha aminoadipate reductase (1400 AA) had no significant alignments to allergens or toxins.
- Cobalamin-independent methionine synthase (768 AA) aligned to a pollen allergen, Sal k 3 of Russian thistle with 49% identity, and 80mer alignment and an 8 AA identity match. Yet Sal k 3 aligns with proteins of many edible foods that do not have shared allergy. There were no clear matches to toxic proteins.
- Aconitase (780 AA) showed no significant identity matches to any allergens. There were a number of statistically significant alignments to proteins in NCBI, but not to toxic proteins, only to enzymes that produce toxic metabolites. There were higher scoring matches to proteins without a label of "toxin".
- Transketolase (679 AA) did not have a significant match to any allergens. There were high scoring matches to proteins using BLASTP with "toxin" or "toxic" as key words, yet the proteins were only from toxic bacteria (e.g. *Bacillus cereus*), but without direct evidence of protein toxicity. There were no direct links for toxicity.
- Glycerol kinase (621 AA) did not have any matches to allergenic proteins. There were low scoring alignments to proteins in NCBI with the keywords "toxic" or "toxin". The aligned sequences were from *Bacillus thuringiensis*, an organism known to be toxic to a number of insects, but there is no direct link to toxic proteins.
- Catalase A (510 AA) had one statistically significant match to an allergen (E score 2.6 e-58), but only 37% identity over 475 AA. This indicates the proteins are apparently evolutionarily related, but not likely to have cross-reactivity as no 80 AA segment was higher than 35% identity and no 8 AA matches were found. The common enzyme was identified as highly similar to proteins from a number of organisms with high identity to toxic protein sources (*Bacillus sp., enterococcus sp., Streptomyces sp., Clostridium sp.*), but there is no direct link to toxic proteins.
- Glucose 6 phosphate dehydrogenase (G6PD, 504 AA) had an alignment of >35% identity (37%) for an 80 AA match to the German cockroach Bla g 3 allergen, but there are no reports of allergic cross-reactivity between fungi and cockroach. The protein did align with a number of G6PD proteins from "toxic" or "toxin" sources, homologues from organisms known to cause toxicity in, but not from the G6PD proteins.
- Hypothetical protein PAS (525 AA) had alignments to three "allergenic" proteins from two molds (*Davidiella sp.*, and *Aspergillus sp.*), and a storage mite (*Lepidoglyphus destructor*) with >35% identity. The proteins are not likely allergens. There were significant alignments with proteins from "toxic" sources (*Bacillus sp.*), but there is no evidence of direct protein toxicity.

- Mitochondrial aldehyde dehydrogenase (501 AA) had two high scoring matches to homologous sequences of two fungi (*Davidiella sp.*, and *Alternaria sp.*), but without direct evidence that these are cross-reactive allergens. There were also high scoring matches to a few homologous proteins associated with "toxin" in NCBI, but they were due to the source organism, *Bacillus thuringiensis,* and not due to direct toxicity.
- Delta-aminolevulinate dehydratase (341 AA) had only low scoring matches to proteins in AllergenOnline. There were high scoring matches to two proteins in *Candida albicans* with keyword matches to "allergen", but those are without proof of allergy. There were modest scoring matches to "toxins" in NCBI, but the proteins are not clear toxins.
- Mitochondria alcohol dehydrogenase (350AA) had a high identity match (76%) with the homologous protein of *Candida albicans,* Can f 1.0101. Yet there is high identity for the Can f 1.0101 protein and homologous proteins from many sources and no evidence of cross-reactivity. The protein also showed modest (36-42%) identity to proteins from bacterial sources of the same type that were identified with "toxin" as keyword limits. The bacteria are toxins, but there is no direct evidence of toxicity to the proteins.
- Malate dehydrogenase (342 AA) had a similar alignment with 51% identity to the malate dehydrogenase protein of *Malassezia furfur* (Mal f 4.0101) as a contact allergen. There is no evidence of cross-reactivity to homologous proteins of other sources. A 36% identity alignment was found with a short 80 AA segment of convicilin of *Pisim sativum* (pea), but again with no evidence of cross-reactivity. High scoring (50%) identity matches were noted for proteins identified in NCBI with "toxins" as a key word term, to proteins of the rat (*Rattus norveicus*) and bacteria (*Vibrio cholera* and *Escherichia coli*), but without direct evidence of protein toxicity.
- Putative protein unknown function (328 AA) had no significant allergen matches. Modest scoring matches were identified in NCBI to proteins listed in various bacteria using the keyword "toxins", but with no direct evidence of toxicity to the protein.
- Triosephosphate isomerase (248 AA) showed high scoring alignments (up to 53% identity) to triosephosphate-isomerase proteins from wheat, house dust mite and shrimp. The same proteins showed alignments of up to 63% identity over 80 AA. These proteins are minor airway allergens and not expected to represent food allergens. The protein aligned with 40-50% identity to homologous proteins from bacterial species listed in NCBI under "toxins" as keywords. No direct evidence of toxicity was found.
- Hypothetical protein cyclophilin (161 AA) matched cyclophilin proteins of diverse fungi, house dust mites and plant sources that have been identified as minor airway allergens. The identities were also found in the 80 AA searches. It is unlikely that these would represent risks of food allergy as cyclophilins are highly conserved across very diverse species. Homologous proteins were identified in NCBI using "toxins" as a keyword, but not direct evidence of protein toxicity was found.
- Cytosolic superoxide dismutase (154 AA) had identities of 53-57% to superoxide dismutase proteins of olive pollen, but the relevance of allergy is weak. There

were also modest to high scoring identities (up to 70%) with similar proteins of various bacteria with identities to "toxins", but without direct evidence of toxicity to the proteins.

- Mitochondria ATPase inhibitor (84 AA) had no significant matches to allergens or toxins.

Literature searches for associations of allergy with *P. pastoris* or *Komagataella sp.* and allergy and toxicity were found, but there were no clear associations with the proteins identified as proteins of interest. Thus, it appears the risks of allergy and toxicity for soy leghemoglobin and for the proteins from *Pichia pastoris* within Soy Leghemoglobin Preparation are not significant.

Finally we tested the stability of the Soy Leghemoglobin Preparation in a model simulated gastric digestion study that includes fixed concentration of protein to pepsin (enzyme) activity and evaluation of digestion resistance at times up to one hour at pH 2.0 and 37 °C. The assay conditions that were used have been published (Ofori-Anti et al., 2008) and used to evaluate proteins in genetically modified crops and novel ingredients. There is a positive correlation between the stability of abundant dietary proteins in this assay and food allergy. In addition, proteins that are rapidly digested by pepsin are unlikely to act as toxins in the digestive tract. Soy Leghemoglobin Preparation was rapidly digested in pepsin at pH 2.0 at both ratio of 1 µg in 10 units (as per standard protocol) and 1 µg in 1 unit pepsin activity (as an experimental protocol). Soy Leghemoglobin Preparation was rapidly digested in this assay to less than 10% residual protein in less than two minutes. No stable fragments were detected either, indicating low potential risk of allergy or toxicity.

My conclusion from this "weight of evidence" approach to dietary protein safety is that the Soy Leghemoglobin Preparation is very unlikely to present a risk of dietary allergy or toxicity to consumers.

Regards,

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