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Allergy Risk Assessment for Newly Expressed Proteins (NEPs) in Genetically Modified (GM) Plants

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Abstract

Based on experience and scientific advancements over the past two decades, a revised approach for the assessment of the allergenic potential of newly expressed proteins (NEPs) in genetically modified (GM) plants is warranted. NEPs are most often not native to the crop genome, and thus regulatory reviews of the safety of GM plants include an assessment of the allergenic potential of NEPs. International standards for the assessment of allergenicity first developed in the mid-1990s required a series of characterization studies to be conducted that are, to some extent, still applicable today to the risk assessment of GM plants, with most modern versions represented in the Codex Alimentarius. This standardized guidance on allergenicity assessments, including the required characterization studies, presented two primary challenges. First, there was (and still is) no defined and accepted model (animal or *in vitro*) for directly testing allergy potential. Second, bioinformatic analyses were prescribed using thresholds for hazard identification that were neither universal for all allergens nor tested prior to the implementation of requirements into guidance documents. Herein, risk assessment principles are applied to structure the assessment of the allergenic potential of NEPs. This allergy risk assessment is built on a foundation of: 1) identifying hazard by assessing similarity to known allergens, and 2) assessing exposure when a hazard is identified. Supplementary studies such as IgE binding may need to be performed in special cases. These recommended revisions to current approaches to the assessment of allergy potential are designed to ensure a realistic, case-by-case approach, and consider updated molecular biology, genomics, and bioinformatic techniques that were unavailable when earlier allergy risk assessment approaches were established.

Keywords: genetically modified, allergenicity assessment, food and feed, hazard, exposure, risk, core studies, supplementary studies

Abbreviations: GM, genetically modified; GRAS, generally recognized as safe; HOSU, history of safe use; NEP, newly expressed protein; SGF, simulated gastric fluid; WOE, weight-of-evidence

1. Introduction

There is no single measure or combined set of measures that are universally common to allergens. For example, a high level of stability (e.g., peanut Ara h 1 protein) in the presence of pepsin enzyme (simulated gastric digestion), is common to many proteins, only some of which are allergens, and is not a characteristic of all known allergens. Therefore, by itself, stability across the many structural groups of allergens is too inconsistent to be a predictive indicator of allergenic potential. As no validated animal testing system is available and there is no single criterion that sufficiently predicts allergenic potential, the hazard characterization of potential allergens must use a weight-of-evidence (WOE) approach, combining several biophysical characterizations of the newly expressed protein (NEP) [4]. Fundamental to this hazard assessment approach is the degree of structural similarity to known allergens. A denoted high level of similarity to a known allergen is a hazard threshold that, in most situations, either triggers further studies to more clearly define sensitization/allergy risk or is the basis to discontinue commercial development of the NEP. As described by Nordlee et al. [23], the discovery of similarity between the NEP and the Brazil nut storage protein allergen did not happen until after transformation of the intended soybean crop. In this

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case, appropriate sequence screening tools were not available to allow for early hazard identification; there were no comprehensive protein allergen databases at the time and access to comprehensive sequence databases was limited. Advances in bioinformatics and molecular characterization techniques, along with a better understanding of protein allergens in general, offers the opportunity to revise the approach to allergy characterization to more effectively and accurately inform allergy risk assessment.

2. Scientific Guidance Documents for Allergy Assessment

Experiences early in the history of genetically modified (GM) plant development encouraged the adoption of scientifically reviewed guidelines to assess the allergy safety of proteins introduced into GM plants [21]. The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) revised guidance for food allergy assessments [33], included a flow diagram of characterizations that concluded with the introduced protein being considered as either having, "*no clear risk of being allergenic*" or having, "*evidence for a clear potential allergy risk*". The Codex Alimentarius [3] further refined food allergy assessments with updates in 2009 by providing an integrated, case-by-case "tiered" approach that uses a combination of criteria to assess where, on a continuum of allergenicity potential, the introduced protein fits. Codex uses a WOE approach, recognizing that no single criterion is sufficiently predictive of allergenic potential. The first tier in the Codex process characterizes the source organism of the introduced protein and determines whether that organism is a known source of allergenic proteins. This tier employs bioinformatics to assess whether significant sequence homology and/or structural similarity exists between the introduced protein and known allergens. Other criteria assessed following the Codex process characterize whether the protein is susceptible to degradation by pepsin (many, but not all known allergens are pepsin resistant and many non-allergens are also pepsin resistant), and, if appropriate based on information from the first tier, conducting specific serum screening studies. In the Codex process, if the first tier determines that there is no substantial similarity with known allergens, then risk is characterized as low or negligible. If there is similarity to a known allergen, then an appropriate human serum IgE binding study should be conducted to address potential shared epitope binding between the introduced protein and the known allergen. The Codex process is considered tiered, since it recognizes that none of the individual characterization assessments can be fully predictive of allergenic risk and that potential risk can be more clearly defined by serum-based screening. The guidance does not prescriptively indicate specific protocols but does include relatively specific endpoints about cross-reactivity and shared sequence identity. For example, sequence similarity methods are defined no more specifically than "bioinformatics", but do include a reference for more than 35 percent identity match over 80 or more amino acids when comparing a NEP to allergens.

The guidelines for allergy assessment were built to address two aspects of food allergies, since clinical response occurs in a two-step process: 1) the initial exposure to the protein that sensitizes the individual, and 2) elicitation of a clinically manifested response upon re-exposure to the same or similar protein [22]. Given that proteins are grouped into families that share homology (similarity in structure and function), assessing the elicitation potential of an introduced protein is a key element included in the updated guidance language. Characterization of several distinct physiochemical properties of the introduced protein form the basis for this "elicitation response" assessment. As an example, bioinformatics that assess the sequence similarity between an allergen and a NEP is essentially assessing potential cross-reactivity, or the likelihood that elicitation in sensitive patients to the NEP can occur.

Guidance documents also address the potential risk that the introduced protein may newly sensitize individuals consuming the protein in a food derived from a GM plant that has not previously included the NEP or a related homolog (*de novo* sensitization potential). This second type of risk assessment uses the same physicochemical properties as the elicitation response assessment, but differentially applies them to the WOE conclusion. In other words, without a defined test or criteria that can predict likelihood of allergy, characterization parameters such as sequence similarity are used as a surrogate to assess the likelihood of an allergy risk in an undefined segment of the population.

More recently, proteins introduced into GM plants have been referred to as "novel proteins" in some guidance documents, since the protein is "new" to that plant. However, it should be recognized that, to date, all introduced proteins in GM plants share structural and/or sequence homology with known dietary proteins and are therefore not '"novel" in terms of available data on human dietary exposure to the protein or its homologs [13].

In summary, when guidance was first proposed by Metcalfe et al. [21], later adopted by the World Health Organization in the late 1990s, and expanded in 2001 [33], there were methodological limitations and assumptions made because of limited knowledge about allergens, compared with today. Further along, in 2003 Codex built upon the previous FAO/WHO guidance but stepped away from a decision-tree approach towards a tiered and cumulative WOE approach.

All these earlier guidance documents were effectively based on identifying allergens using approaches that were not empirically tested to distinguish allergens from non-allergens, especially in the case of the bioinformatics thresholds. Since those guidance documents were published, better tools have become available that more accurately and precisely identify allergens (Figure 1). A brief breakdown of the state-of-the-science in 1995 versus the current status of each type of assessment is the following:

i. *1995:* Genome sequencing and databases. As there was no prior knowledge of whole organism genomes, any protein from an organism causing allergy was assumed hazardous. *Currently:* Source organisms and specific genes can be sequenced, identified, and characterized. Allergen databases enable identification of NEPs belonging to an allergen group and allow identification of an al-

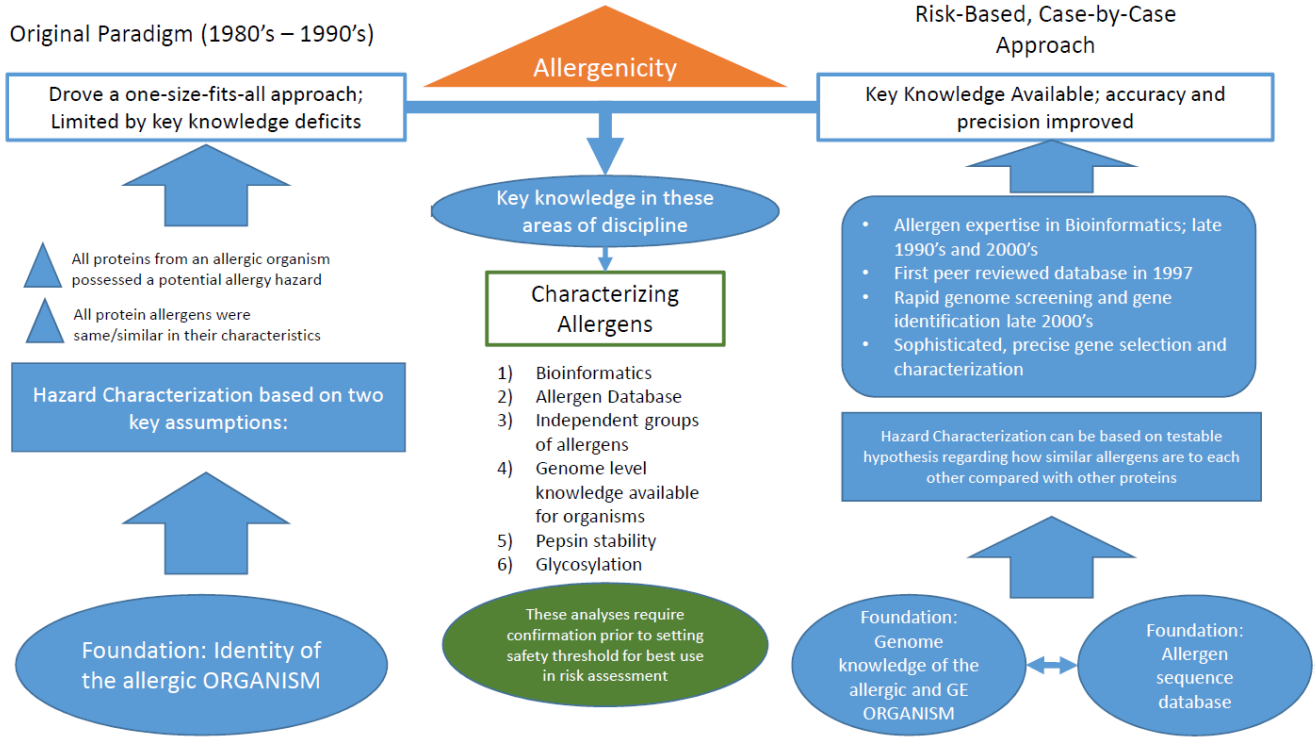

Figure 1: The evaluation of allergenicity of newly expressed proteins in genetically modified plants according to the original paradigm developed twenty-five years ago, and modern approaches using bioinformatics and hypothesis testing

lergen defined by serology (elicitation) risk with known exposure and response by sensitive patients.

ii. *1995:* Gastric fluid simulation (pepsin enzyme stability) was used to assess stability to digestion and exposure of the lower gut immune system. *Currently:* Pepsin is now understood to not be a predictive indicator of a hazard as not all allergens are stable and not all non-allergens are unstable to digestion.

iii. *1995:* Glycosylation of NEPs used as an indicator of hazard. *Currently:* Recognized that glycosylation may support IgE binding, but that it is not a causative factor in the initiation or elicitation of clinical allergy.

iv. *1995:* Serology testing with human serum and IgE-binding readouts were used to identify cross-reactivity. Serology provided the only available method to identify allergens. However, it was only implemented if there was some other hazard identifier for the NEP being similar to an allergen, not as a "test" for allergy potential. *Currently:* Serology testing is still recognized as a valuable method to study patient response to an allergen. However, it does not provide utility in routine screening NEPs for allergenicity without a prior "trigger" being identified, which is typically a concern raised by relevant bioinformatic similarity.

v. *1995:* Abundance of a NEP in the plant was used as a hazard identifier, but without specific quantified guidance thresholds. This approach was based solely on knowing that some allergens, such as plant storage proteins, represent a high percentage of total consumed protein. *Currently:* Abundance has no predictive capacity for allergenic potential (see Exposure section); threshold levels for clinically relevant exposure levels have not been identified for most allergens.

In view of methodological developments and a modern understanding of allergenicity, a revised approach for assessing the allergenic potential of NEPs is warranted. This revised approach supports an overall risk assessment to ensure that foods derived from GM plants are safe for consumption. It assigns various WOE characteristics into respective risk assessment categories of "hazard" and "exposure", and is based on well-recognized risk paradigms that, in the absence of hazard, there is no risk, and therefore no need to assess exposure. Similarly, in cases where there is no exposure there is no need to assess hazard. From a technical standpoint, there is a much better understanding in the last 15-20 years regarding molecular characterization, the bioinformatic assessments of NEPs, and recognition of qualified allergen database(s). A key concept to the revisions discussed herein is to place the biophysical assessments of NEPs into their respective risk-use domains.

A suggested approach for allergenicity evaluation, separating assessment of hazard from assessment of exposure is the following:

- **Allergen-Specific Hazard Assessment (Core Studies)**:

These should be performed for all NEPs.

- **Exposure Characterization (Supplementary Studies)**: If a hazard is identified, exposure characterization should be performed. In the absence of hazard, there would be no risk, and therefore, no need to characterize exposure.
- **Hazard Characterization: Allergenic Potential and IgE Binding (Supplementary Studies)**: These may need to be performed on a case-by-case basis.

3. Allergen-Specific Hazard Assessment (Core Studies)

A critical question to address in the allergy hazard assessment for NEPs is whether the protein is similar to a known allergen. This addresses the potential for cross-reactivity between the NEP and a known allergen (i.e., elicitation), and the potential for a novel NEP to present a hazard as a *de novo* allergen (i.e., sensitization followed by elicitation). The assessment of the latter focuses on primary or first exposure of a *de novo* protein allergen to a person who has potential for sensitization and the consideration of whether that person, upon subsequent exposure, would develop an elicited, clinically-relevant response.

The paradigm of risk assessment for allergens, hinging on the standard risk equation (Risk = Hazard x Exposure), has caveats to distinguish allergens from other toxicants. Specifically, there is no single test or predictive assessment for whether a protein will act as an allergen. Consequently, hazard identification involves measures of several of the physiochemical properties of the NEP. The approach is based on identifying relevant properties that are considered "common" to allergens. The key characterization parameters and revised approaches to assess allergenic potential of NEPs are detailed below.

3.1. History of Safe Use of the NEP

History of safe use (HOSU) of the NEP is one of the fundamental and initial endpoints in the safety assessment. Demonstration of prior human and/or animal consumption provides familiarity with respect to the probable safety of a given protein. This is similar to the GRAS (Generally Recognized as Safe) concept employed by the U.S Food and Drug Administration (FDA) [28]. GRAS classification indicates that a food ingredient is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, either through scientific procedures or through common use in food. FDA extended the GRAS concept to proteins used in biotechnology plants in 1992. The concept of HOSU was also included in a recent European Food Safety Agency (EFSA) assessment guideline document suggesting no need for any specific toxicity or allergenicity testing in cases where both the plant and proteins expressed in the GM plant have a history of safe consumption by humans and animals. The concept of protein HOSU has also been emphasized in peer reviewed publications and other guidance documents related to safety assessment of GM plants [4, 8]. In order to demonstrate HOSU, evidence of similarity and exposure to the other consumed proteins or species expressing these proteins or similar proteins is needed [13]. Protein similarity can be determined, on a case-by-case basis, by primary sequence alignment and by structural or functional similarity, depending on the class of the protein. Protein phylogenetic analysis also helps in determining protein similarity with well-characterized proteins known to be safe. Similarly, familiarity with the mode of action and the specificity of protein activity also contributes to an evaluation of the HOSU of proteins. Depending on similarities, it may not be necessary in all cases to model exposures, as sufficient history of exposure may be demonstrated semi-quantitatively (e.g., by comparing concentrations of the similar protein in foods and relative consumption levels). While epidemiological and experimental evidence should also be considered when available, an extended history of use in the diet with no reported adverse outcomes can suffice for a safety assessment. It is important to note that absence of a clear HOSU for a protein does not represent a hazard but only indicates that further analysis of other lines of evidence may be needed in the assessment of protein safety.

3.2. Familiarity of the Source Organism

Familiarity with the source organism of the protein can also play a vital role in the WOE approach for determining safety of the NEP. The absence of any biosafety risk associated with the source organism provides strong evidence about the safety of the NEP. If the source of the protein has a HOSU then any protein from the source is also likely to be safe [6]. Animal toxicology and nutrition studies, as well as human exposure, with the source organism of the NEP can also support a safety assessment of the NEP. Use of an organism with a HOSU can demonstrate the limited potential for the NEP to be a toxin, allergen, or anti-nutrient [6]. On the other hand, if a source organism does have some pathogenicity, toxicity, or allergenicity, established knowledge of that organism can also support a safety assessment of the NEP. Typically, only a few proteins or a small fraction of an organism's genes are responsible for these properties. With modern molecular characterization of source organisms, fully curated allergen sequence databases, vast knowledge of toxic protein sequences and modes of action, and capability in modeling higher levels of structural similarity, the source organism as such does not necessarily describe a hazard for an NEP. In other words, there is usually clear identification of the genes within a genome that produce an allergen, an allergen homologue, a toxin or an anti-nutrient. Other proteins encoded in the genome would be expected to be non-allergenic and non-toxic, and the organism itself does not define a hazard. It is more important to establish what degree a NEP (not already known to science as an allergen) is significantly similar to a known allergen early in the NEP development process; thus, it becomes a foundation in describing whether a NEP is in any way similar to allergens or more similar to other safely consumed proteins.

3.3. Amino Acid Sequence Similarity and Bioinformatics

Traditionally, assessing protein similarity at the amino acid sequence level was considered as "bioinformatics", and hazard identification involved a binary condition considering two

measures of sequence alignment. The amino acid sequence of the NEP was compared with that of known allergens using algorithms to assess sequence similarity with a focus on locally aligned "domains". Those two measures were: 1) shared percent *identity* > 35 percent, and 2) *overlap* length of alignment ≥ 80 amino acids.

This dual criterion and binary bioinformatic approach has since been demonstrated to limit two key understandings of protein allergens [19, 26]. First, an untested set of identity and overlap criteria do not describe a minimum understanding of similarity between two proteins. Allergens (i.e., an allergen database) were not originally modeled bioinformatically whereby only identity and overlap length were determinately known to predict biological relevance. Therefore, identity was used early on in allergen similarity assessments, but now is not enough to uniformly apply as an indicator of relatedness across the many groups of allergens [20]. A much better understanding of applying bioinformatic when comparing a NEP with allergens has come into place since the initial bioinformatics guidance [32] was published.

Although bioinformatics is a wide-ranging discipline combing informatics and biology, it is a special-case application when using local alignment algorithms or other methods to determine similarity among two or more sequences for the purposes of allergy risk assessments. The best use of bioinformatics for protein safety assessments in this context is the combination of a thorough understanding of existing allergens with a coordinated review of allergens and their placement into a qualified database that has more statistical power to detect structural relationship [5].

The use of bioinformatic tools should be such that the results are both accurate and precise without reliance on arbitrary endpoints (as discussed earlier). This can be enabled by application of structural biochemistry to support structural classifications of all proteins so that individual structural classes of protein allergens are recognized. The structural analyses of allergens then become "case-specific", because it is the combination of the clinical phenomenon of some proteins being allergen sensitizers/elicitors along with their unique biochemistry that allows placing them into an allergen database. It remains to be defined whether there is common structural biochemistry across all allergens; in the meantime, a case-by-case analysis when addressing the similarity of a NEP with known allergens is required.

To date, there is no evidence that a single sequence alignment feature such as percent identity (or percent identity and sequence length) is both conservative (from a safety standpoint) and accurate in describing allergy potential and/or allergy cross-reactivity. In fact, inspection of the Comprehensive Protein Allergen Resource (COMPARE) database reveals some allergen families are composed of highly similar sequences from a highly diverse group of hosts, while other allergen families consist of highly diverse sequences from closely related hosts.

Recent test cases and exploration of allergen similarity using common algorithmic approaches highlight the extent to which accurate measures of similarity, which extend past the use of percent identity and alignment length, can be applied. To enhance the accuracy and reliability of bioinformatics assessments for allergenic potential of NEPs, a step-wise approach is recommended:

Step 1: Sequence level consideration – Does a protein share relevant similarity with any proteins in a qualified allergen database?

Bioinformatic algorithms have been designed to highlight and measure the probability that two sequences share a substantial portion of their structure and otherwise share a common evolutionary origin. The conventional local sequence alignment-based algorithms Fast All (FASTA) and Basic Local Alignment Search Tool (BLAST) produce several output measures that demonstrate shared identity, domain-specific overlaps, and similarity with the summary statistic, expectation value (E-value) typically being the culmination.

The recommended endpoint measure is E-value, the most reliable and sensitive indicator of likely sequence homology. E-value depends on the database size and simply reports the number of times a similarity score is expected by chance, or the number of expected false positives (non-homologs) per search. Generally, an alignment of two sequences with E-value < 0.01 are homologous, but to be certainly homologous for two sequences, E-value needs to be less than 10^{-6} [24]. E-value determinations for specific cases (i.e., specific NEP comparison to allergen databases) should be evaluated depending on the following:

a) Is the E-value biased by amino acid composition, i.e., is the normal distribution of the 20 amino acids expected for a typical protein, or is there reduced distribution? This is critical to understanding alignment scoring due to a bias in "significance" if reduced distribution (i.e., low complexity) is present. This can be assessed by shuffling the sequence of the NEP and repeating the search. If shuffled, and if these sequences yield E-values of ~1.0 or greater, the corresponding alignments demonstrate that alignment results are reliable (i.e., indicate false positivity) because the random shuffling abolished the unique sequence structure only present in the intact, native, and original sequence.

b) Do alignments with significant similarity identify two or more different families of allergen proteins? In such cases, sequence masking should be employed to remove so-called "low complexity" sequences from the search. If masking eliminates apparent significantly similar alignments, the significance of low complexity sequence alignment should be assessed.

Step 2: Structural relatedness – Does the NEP belong to a structurally defined group of allergens?

Allergens can be grouped taxonomically to some degree, and more often structurally, to help determine similarity (when they are well characterized proteins) with the goal of assessing

the potential for cross-reactivity. Regardless of whether a protein possesses relatively low or high allergenic potential, identifying relationships among groups of allergens can be informative.

Step 3: Further structural considerations – Does a protein that is similar (either significantly or borderline) in sequence level consideration share direct measures of similarity using modeling of three-dimensional structures?

Dimensional modeling offers a more sophisticated measure of similarity between a NEP and an allergen, but it would only be performed if necessary, based on the results of sequence level analyses. Because dimensional modeling would be expected to extend beyond the limits of the linear sequence similarity typically performed during sequence level consideration, an additional assessment with modeling may offer clarity. Specifically, modelling may reveal dramatically different 3D structures despite the observed primary sequence similarity.

Specific metrics from modeling would be addressed on a case-by-case basis with the knowledge of any specific allergens and their associated epitopes, and other clinically relevant sequence mapping being a key to understanding similarity with the NEP (i.e., IgE binding epitopes).

4. Exposure Characterization (Supplementary Studies)

If a hazard is identified, exposure should be characterized to obtain an understanding of risk. Digestion, processing, and abundance are studies that address exposure to the NEP.

4.1. Digestion

Stability of proteins in simulated gastric fluid (SGF) was first suggested as a distinguishing feature of food allergens in 1996 [1]. While subsequent studies largely found this correlation to be weak or non-existent [2, 10, 17], this initial study and the intuitive appeal of reduced exposure in the intestine, where sensitization and elicitation were believed to predominantly occur, resulted in the wide adoption of this criterion as part of the WOE approach supporting the allergenic risk assessment of NEPs [33]. The SGF method [29] was adopted as a surrogate for human digestion in the stomach because this is the method for which results were initially reported to correlate with the allergenic status of proteins [1], and because this method has been used commonly to assess the digestion of pharmaceuticals [11].

As follow-up studies began to show that the SGF assay was a poor predictor of the allergenic status of proteins, modifications and expansion of digestion studies to include more physiological gastric conditions and simulated intestinal fluid were explored, without any notable improvement in the contribution to the WOE for assessing the allergenic risk of novel food proteins [16]. This is not surprising, as a robust body of literature exists in the animal science arena showing that a better correlation between *in vitro* and *in vivo* digestion often occurs when non-physiological *in vitro* digestion conditions are employed [9]. As layers of complexity are added through the inclusion of "physiological conditions", it becomes increasingly difficult to draw meaningful conclusions due to the introduction of additional variables. It is important to note that SGF and simulated intestinal fluid are not designed to mimic the highly complex and variable conditions of human digestion, but rather to allow relative rates of digestion among substances (in this case proteins) to be compared [16].

While SGF is often considered to measure pepsin susceptibility, this is typically not the case. Rather, it is the combination of acid denaturation and pepsin digestion. Pepsin is a relatively promiscuous enzyme and will digest most proteins very rapidly when the proteins are linearized by denaturation [14]. It is the folding of proteins, resulting in the shielding of these pepsin cleavage sites, that slows digestion. The acidic conditions in the stomach and those specified for SGF (pH 1.2) denature many proteins, and results of the SGF assay are largely dependent on the kinetics of this denaturation [14]. Furthermore, pepsin describes a family of highly similar enzymes with different amino acid sequences. Multiple pepsins exist in humans, and the porcine pepsin used for SGF assays is even known to cleave proteins into different peptides compared with human pepsin [30]. Thus, the SGF assay is a surrogate for human gastric conditions but is only designed to give relative information among proteins. It is not designed to mimic physiological conditions that are highly complex and variable. Surrogate digestion assays such as SGF have been developed recognizing this reality. Clearly, increased digestion of a protein reduces exposure in the intestines. For sensitized individuals, reduced exposure to the offending allergen reduces allergenic symptoms [27]. With the poor correlation between digestion results and the allergenic status of proteins, these recent findings do not support the use of digestion results in the WOE for assessing sensitization risk for novel food proteins [15, 31] .

The characteristics of hazard and the triggering level of elicitation need to be established for a risk evaluation [7]. Under the risk-based approach recommended, SGF stability provides value only when there is a known hazard, as digestion characteristics would contribute to exposure considerations in the risk = hazard x exposure equation.

4.2. Processing

Processing has typically referred to the assessment of how apparent the stability of a NEP may be when the grain in which it is contained is processed using processes that would be typical for turning grains into food and feed fractions. The premise is that the application of heat during processing can alter protein structure, which is key to both enzymatic/biological function and IgE-binding site access (i.e., allergenic potential). From a safety perspective, the goal is presumably to understand whether some form of unique "stability" is apparent that is not otherwise identified by the SGF pepsin assay. Discussed below is the clarification of the limited hazard characterization utility in performing this type of study on NEPs.

To address the limited use of a "processing stability" assessment, Privalle et al. [25] reviewed this type of characterization of NEPs. The basis of the premise stated above is that several

allergens maintain functional intactness and allergenicity after exposure to various heat conditions during food processing like cooking [25]. As a result, heat stability, also known as thermal stability, is required in Tier I of the Codex WOE approach [4]. Typically, *in vitro* heat stability is measured by two methods, enzymatic/biological function assays and non-human immunodetection assays. These methods were reviewed extensively [25]. Measuring functional intactness after food processing or heat treatment(s) could contribute to an exposure assessment. In the non-human immunodetection assays, animal IgG serum is used to show loss of the immune-binding to the introduced protein after varying levels of heat treatment. The IgG serum is produced by exposing an animal to the protein of interest and serves as a "surrogate" for human IgE serum, which is unavailable due to lack of human exposure to the NEP. However, the animal IgG serum is not a suitable surrogate because its production relies on the animal's immune systems, and there is no way to predict or ensure the human IgE and animal IgG binding sites are the same. As a result, it was concluded that the non-human immunodetection assay measures immunogenicity, not allergenicity, and cannot contribute in any meaningful way to assessments of allergenicity [25]. In the function assays, enzymatic/biological activity of the introduced protein is measured before and after heating; a loss of function suggests a low risk of allergenic potential. However, functional stability is not consistently correlated with allergenic potential because heat conditions have been shown to increase, reduce, or unalter allergenicity in foods such as roasted peanuts, hazelnut, and soybean meal, respectively [25]. Measuring functional intactness after food processing or heat treatment(s) could contribute to an exposure assessment but does not otherwise identify allergy hazard for NEPs if the NEP is not otherwise determined to possess allergen similarity or is clearly an allergen. There is no presumptive endpoint for processing stability that would contribute to allergy risk assessment for the currently approved NEPs that lack allergen similarity and are known to be safe. Exposure assessments may be an expectation for some regulatory agencies but provide no quantitative value for risk assessment in the absence of hazard.

4.3. Abundance

Abundance has been recognized as additional information that can support an allergy safety assessment [3, 4]. The premise is that generally, protein allergens tend to be abundant on a per weight basis and that this is part of the characterization that separates allergens from non-allergens. Several allergens, like Ara h 1 in peanut and glycinin in soybean, are abundant proteins and represent at least one percent of the total protein from the source organism [12, 18]. Due to the high concentration, abundant proteins are more likely to endure digestion in humans and animals and crop processing (although stability is a factor) and may increase the risk of allergenic response in sensitized people. In this regard, abundance has been treated as an associative factor in considering exposure; i.e., more of the protein equates to more exposure and a higher probability of allergy risk. For this reason, abundance of the introduced protein could be considered as a contributing factor to the exposure assessment for known allergens. However, abundance is not understood in a way that endpoints can be applied to proteins that are not otherwise allergens or cross-reactive proteins. And, there are many allergens expressed at low concentrations because they are not seed storage proteins or other proteins expressed at high levels relative to others in plant and animal tissues. Therefore, low abundance suggests a low probability of allergy relevant exposure, but independent of an identified hazard, greater or lower abundance cannot be a contributing factor in describing allergy risk for a NEP.

5. Hazard Characterization: Allergenic Potential and IgE Binding (Supplementary Studies)

Traditionally (Codex), the need to perform human serum IgE binding to a purified NEP was considered as part of a tiered approach. In this approach, the expectation was that either the source organism of the NEP being an allergen-containing source or a bioinformatic similarity trigger, was a requirement. With the advent of more sophisticated bioinformatic techniques and using the proposed refined approach described herein, the necessity of adding IgE binding data to an allergy risk assessment would be considered a "special case". The WOE across all the characterization metrics for the NEP and the details of the bioinformatic analyses will be unique for each NEP. To support rapid and routine safety screening of introduced proteins, bioinformatics is the primary way in which introduced proteins are screened. When determined to be necessary, IgE binding data could help confirm elicitation potential, but the expectation is that this would only be performed in rare cases, if at all. Therefore, it is recommended that IgE binding as an allergy assessment strategy be delegated as "case-by-case".

6. Conclusion

The assessment of a NEP for allergic potential is based on a characterization of its overall biophysical similarity with known allergens. In turn, advancements in allergen discovery and characterization support an increasingly robust characterization of NEPs. A key advancement is an established database of known allergens as well as transparent and well-documented processes for maintaining this as an accessible resource for safety assessments. In addition, molecular characterization of genomes has redefined the safety focus to be on individual genes rather than whole organisms for those that are the source of a NEP and those of allergen-containing organisms. While identifying a source organism for a NEP as a source of allergy requires additional investigation, a greater knowledge of the genes and proteins within organisms allows those genes related to allergy to be separated from those that are not. As such, the vast majority of safe genes and proteins within the source organism are not falsely implicated as contributing to allergenic risk. Overall, addressing NEP health concerns by assessing whether it is an allergen or similar enough to cross-react with a known allergen is the hallmark of the allergenicity assessment. Predicting whether a NEP can "become" an allergen remains the

most difficult health concern to address, as there is no single test for this potential. Yet, improvements in allergen databases (e.g., COMPARE) [5], genome-level gene identification, and the recognition that NEPs are rarely "novel" and are expected to be proteins that can be fully characterized, alleviate most of this concern. In the modern era, allergenicity assessment relies on the newest allergy and molecular science to maintain a low level of risk for the consumer.

7. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

8. Disclaimer

Portions of this policy commentary were used to inform policy commentaries, *Core and supplementary studies to assess the safety of genetically modified (GM) plants used for food and feed* and *Toxicological assessment of newly expressed proteins (NEPs) in genetically modified (GM) plants*. These portions were written by the same authors and the commentaries are published in this journal issue.

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10. Article Information

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