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Toxicological Assessment of Newly Expressed Proteins (NEPs) in Genetically Modified (GM) Plants

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Abstract

This paper details the weight of evidence (WOE) and stepwise approaches used to assess the food and feed safety of newly expressed proteins (NEPs) in genetically modified (GM) plants, based on previously reported principles. The WOE approach is critical, as in a vast majority of cases no single assay or biochemical characteristic can identify a protein as a hazard. A stepwise approach is recommended to evaluate the safety of NEPs taking the totality of information into account. Potential triggers for the need for supplementary toxicology studies are discussed, and an alternative *in vitro* method for the acute toxicology study is proposed.

Keywords: genetically modified, toxicological 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; MOA, mode of action; MOE, margin of exposure; NEP, newly expressed protein; NOAEL, no observable adverse effect level; WOE, weight-of-evidence

1. Introduction

Proteins are a natural part of human and animal diets, and when subjected to rapid degradation by digestive enzymes and acidic conditions in the gastrointestinal tract, are catabolized into individual amino acids and small peptides that can be absorbed by the body. There are many biological barriers in mammals and livestock that restrict the oral bioavailability of intact proteins after dietary consumption [24, 26] and there are many factors, including size, charge (e.g., many proteins are charged, which restricts permeation), and lipophilicity (logP, diffusion across lipid membranes) that affect their absorption. In general, systemic absorption of any orally consumed substance is inversely proportional to the size of the molecule, with smaller molecules more readily absorbed in comparison to larger ones [12]. Thus, even for proteins with the unusual property of resistance to degradation by digestive enzymes (for example, lectin proteins), systemic uptake is limited by their large molecular weight. Unsuccessful attempts to use orally administered proteins for therapeutic purposes exemplify the effectiveness of these natural barriers [13, 15, 31, 36].

Consumption of proteins as a general class of macronutrients is not normally associated with adverse effects. While some proteins have shown toxicity via parenteral routes (non-oral exposure to venoms), very few are known to exhibit evidence of adverse effects following oral exposure. Most of the proteins that are toxic via oral exposure are lectins and tend to exhibit effects at the intestinal epithelium, although in some cases, such as with ricin, systemic effects can also occur [6].

A stepwise assessment approach is recommended to evaluate the hazard of newly expressed proteins (NEPs) taking the totality of information into account [7]:

- **NEP Hazard Identification (Core Studies)**: Key hazard identification studies are required to assess the safety of all NEPs.
- **NEP supplementary toxicology studies (Supplementary studies)**: If the above studies are unable to conclude on the absence of hazard of the NEP with reasonable cer-

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tainty, then additional supplementary studies need to be conducted (Supplementary studies).

- **Exposure assessment (Supplementary studies)**

2. NEP Hazard Identification (Core Studies)

Evidence from initial hazard identification can be built by considering the following elements: (a) history of safe use (HOSU, consumption) of the protein of interest; (b) HOSU of the source organism; (c) protein mode of action (MOA); functional specificity; and (d) bioinformatics for sequence comparison (e.g., primary amino acid sequence homology and overall structural similarity [30] to proteins with a known HOSU and evaluation for similarity to known toxins or other biologically active proteins that produce adverse effects in humans and animals. If a hazard has been identified, exposure can be determined by performing studies (e.g., dietary exposure assessments) as necessary, depending on the NEP.

2.1. History of Safe Use of the NEP

History of safe use (HOSU) is one of the initial analyses in the safety assessment of NEPs in genetically modified (GM) plants. Demonstration of prior human and/or animal consumption of the NEP or closely related proteins, structurally and/or functionally, provides familiarity with respect to probable safety of the NEP.

The concept of HOSU is similar to the GRAS (generally recognized as safe) concept employed by the U.S. Food and Drug Administration (FDA) [41]. 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 (genetically modified) plants in 1992. The concept of HOSU was also included in a recent European Food Safety Authority (EFSA) guideline [9], 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 genetically modified plants [3, 7].

It is important to note that absence of HOSU does not automatically indicate that the protein presents a hazard; it only indicates that further analysis of other lines of evidence is required. In order to demonstrate HOSU, evidence of structural and/or functional similarity and exposure to other endogenous proteins found in foods or other species expressing these proteins or similar proteins is necessary [16]. Protein similarity can be determined by either primary amino acid sequence alignment or structural/functional similarity, depending on the class of the protein. Protein phylogenetic analysis also helps determine protein similarity (with well characterized proteins) in the absence of higher primary sequence identity. Regarding exposure to similar proteins or species expressing these proteins, the appropriate methods for establishing this similarity need to be determined on a case-by-case basis.

2.2. HOSU of the Source Organism

HOSU of the source organism of the protein plays a supportive role in the weight-of-evidence (WOE) approach for determining the safety of the NEP. The HOSU of the source organism as a food ingredient, supplement, pharmaceutical, source of pest resistance (e.g., *Bacillus thuringiensis, B.t.*), or through environmental exposure can provide additional evidence about safety of the NEP. Use of a safe source organism can be used to demonstrate the limited potential for the NEP to be a toxin or anti-nutrient (or allergen) that could be relevant to humans or animals [4]. On the other hand, knowledge of the source organism does not, in and of itself, directly answer the question of whether the NEP presents a likely hazard. Safe proteins can be sourced from "unsafe" organisms because it is very likely that only a small number of an organism's genes are responsible for causing pathogenicity, toxicity or allergenicity.

Tools to characterize the hazard of NEPs derived from organisms known to cause any pathogenicity or toxicity (or allergenicity) include comparison of the amino acid sequence with fully curated protein toxin databases, and mathematical modeling of higher levels of structural similarity (if primary sequence information shows similarity between the protein and a putative toxin and there is information available on conformational epitopes or other key structural features).

Where there is clear identification of those genes in the source organism that produce a toxin or an anti-nutrient, other proteins would be presumed to be non-toxic unless empirical evidence indicates otherwise. We can use this information to demonstrate that the gene encoding the NEP does not have the potential for toxicity, thereby providing supportive evidence in a WOE approach that the protein is not hazardous.

2.3. Mode of Action/Functional Specificity

Knowledge of MOA and functional specificity of the NEP are important elements in the WOE for hazard identification, and may be helpful in determining the NEP's potential for causing toxicity to humans or animals. If the MOA and functional specificity of a NEP are well understood and are shown to have low relevance to humans, it lowers the concern about the safety of the NEP. For example, enzymes generally do not have a toxic MOA, and knowing that a NEP has an enzymatic MOA, for example herbicide metabolism in plants, suggests that the NEP is unlikely to present a dietary hazard. Alternatively, a pesticidal (insect resistant) MOA triggers further investigation into putative hazards and potential risks that can be further understood considering a more detailed mechanism of action. In the case of B.t. insect resistance proteins, the proteins bind to a receptor not present in mammals, which reduces concerns about the protein's potential for human harm.

2.4. Bioinformatics for Toxin Screening

Bioinformatic screens are an excellent tool for placing a protein within the context of related proteins, based on recog-

nizing localized similarity, common domains, and larger protein families or protein super-families. Consequently, bioinformatics plays an important role in the hazard assessment of toxins. This *in silico* screen is typically applied early in the hazard assessment phase and can be useful in providing the preliminary protein and protein family context, which will help determine the need and scientific rationale to conduct any supplementary toxicology (hazard characterization) studies. Bioinformatics results should be regarded as guiding rather than predictive. They allow for a more holistic understanding of a protein or protein family but are not a predictive tool for hazard identification.

The analyses most apt to provide this contextual information are traditional primary sequence alignment algorithms such as Fast All (FASTA) or Basic Local Alignment Search Tool (BLAST), which return localized protein alignments. These alignments can then be reviewed to establish the contextual information, which will serve as the driving reason behind determining the necessity for supplementary studies. Ultimately, as the understanding of domain architecture and function continues to develop, the observed linear alignments - when analyzed in tandem with domain information - will play the greatest role in reconciling protein function and identifying a potential for toxic hazard. For example, use of a domain-based approach has recently been used to help put sequence homology data into context for protein safety evaluation [11, 30]. This analysis demonstrated that simply having a domain or region with homology to a toxin does not necessarily signal potential toxicity.

Bioinformatics will only serve as an identifier of proteins with a "hazard potential" based on some level of similarity. This defined potential, as established by the contextual information gathered by the bioinformatics assessment, will then guide the decision as to whether supplementary toxicology studies are necessary or warranted to enable the classification of a protein as hazardous.

While bioinformatics is an excellent tool for rapid screening and protein identification during the discovery or product development phases, any hazard characterization based upon bioinformatic results must ultimately be examined in conjunction with other hazard and exposure assessment data when generating a risk hypothesis (e.g., HOSU, heat lability, digestibility, MOA, functional specificity, etc.). If a risk is hypothesized, it can be further validated through supplementary toxicology studies. Although bioinformatic analysis may be of limited value for directly demonstrating protein safety, it is an important component of the WOE for hazard identification of the NEP.

3. NEP Supplementary Toxicology Studies (Supplementary Studies)

The weight of the scientific evidence derived from hazard identification can be used to evaluate the necessity for further evaluation, i.e., if the WOE following hazard identification is not sufficient to determine absence of hazard. The toxicological evaluation of all NEPs as a default assessment is not hypothesis driven and is not supported by the WOE established from the history of protein hazard assessments conducted with NEPs in GM plants. Defaulting to *in vivo* toxicology studies, as is often required for regulatory approvals, does not reflect ethical use of animals in scientific research and testing as outlined by the 3R's of responsible animal use (Replacement, Reduction and Refinement) that have been increasingly incorporated into regulatory *in vivo* studies [39]. Such a default approach is, therefore, not science based and is inconsistent with the tiered approach outlined for the safety assessment of NEPs [7]. The initial protein hazard identification should be conducted to build a WOE that can serve as a guide to determine the necessity for supplementary protein hazard characterization.

3.1. Acute Oral Toxicology Study

Evidence to date for NEPs in GM plants indicates that, when no hazard is identified based on the WOE, no evidence of adverse effects is observed in acute oral toxicology studies [2, 7, 23, 28, 38, 44]. Nevertheless, acute toxicology studies are still required by some regulatory authorities regardless of the nature of the protein [29]. These studies have been conducted largely due to the observation that, while most proteins do not present a hazard, most protein toxins elicit their toxicity through acute mechanisms of action [37]. A notable exception to this is the lectins, a group of proteins characterized as anti-nutrients that can cause injury through cell agglutination from binding cell surface carbohydrate moieties.

It is well recognized that the vast majority of dietary proteins are degraded into individual amino acids and small peptides, and absorbed by the intestine for nutritive purposes. This degradation results in a loss of biological activity. Furthermore, most dietary proteins are too large to be absorbed intact, which further minimizes their potential for systemic effects [10, 35]. Lectins have been demonstrated to be highly resistant to proteolytic degradation, and their ability to cause adverse effects is dependent on this property [42].

Given these factors, it is perhaps not surprising that the small number of proteins known to be hazardous when ingested, including ricin and the kidney bean lectin phytohaemagglutinin E (PHA-E), often exert effects on the intestinal epithelium [22, 25, 33, 43, 45]. Lectins can also act systemically [42]. The common features of 'protein toxins' is they typically are cytotoxic, act acutely, and cause damage to an epithelial surface (i.e., non-systemically).

A margin of exposure (MOE) calculation compares the estimated daily exposure that might occur in a given set of circumstances, such as for a specific country/region or sub-population to the No Observable Adverse Effect Level (NOAEL) determined in experimental animals. In the case of NEPs, the NOAEL typically comes from the acute oral toxicity study where the limit dose of 2,000 mg/kg bw is often utilized based on OECD guidelines [40, 32] for testing at high levels when there is no reason to suspect toxicity at lower dose levels.

The MOE is the magnitude by which the NOAEL of the critical toxic effects exceeds the estimated daily exposure, in this case through oral consumption, and is calculated as follows:

MOE = 2,000 mg/kg ÷ estimated consumption (acute consumption values x NEP concentration)

Another method of calculating the MOE is to set dose levels based on multiples of the maximum theoretical human exposure. There may be cases where the test substance solubility is limited or the production of the test substance in large quantities is extremely challenging or virtually impossible, and therefore, using an MOE approach based on exposure estimates, rather than defaulting to testing at a limit dose, would be appropriate. In these cases, one would consider the population and country/region of interest (or highest consumers globally if considering worldwide consumption), and the NEP concentration in a relevant plant commodity or by-product to calculate the MOE.

3.2. Potential Future Approaches to Supplementary Toxicology Studies: In Vitro Evaluations

Conducting an acute toxicology study with a NEP requires the production and isolation of multiple grams of protein from plant or microbial sources. This can be technically difficult for some proteins and virtually impossible for others [1]. Proteins in the latter category include integral membrane proteins and some transcription factors [5, 18, 34]. Proteins such as these have been referred to as intractable proteins, to indicate that it may not be possible to isolate them in quantities required to conduct acute toxicology studies [1].

In view of these protein production challenges, as well as animal welfare consideration, it would be desirable, in the future, to be able to employ *in vitro* methods as a substitute for *in vivo* toxicology studies, as described previously [1]. A feature of toxic proteins is their impact on the intestinal epithelium and/or cytotoxic mechanisms of action. In the unlikely event that a NEP was to be hazardous, it is likely that it would cause damage to the intestinal epithelium. On this basis, intestinal epithelial cell line monolayers from rodents and humans have been investigated to evaluate the effects of known hazardous proteins, including ricin [22] and PHA-E [19]. A number of recently published experiments demonstrate the utility of immortalized [20, 21] and primary [8] human epithelial cell culture models for differentiating proteins with associated hazards from those considered to be innocuous, in both the presence and absence of simulated gastric and intestinal digestive enzymes [6, 27].

4. Exposure Assessment (Supplementary Studies)

As mentioned above, when a hazard is identified by the WOE approach, it is necessary to determine exposure to the NEP. Various factors such as stability of the NEP under different conditions and resistance to digestion influence exposure. Evaluation of these considerations will impact the overall safety assessment. Under conditions where there is no exposure to the NEP, such as in highly-processed foods like oil or sugar, a safety assessment may not be necessary, since there is no apparent risk (Risk = Hazard x Exposure).

4.1. Stability (Heat/pH/Processing)

Demonstration of a lack of biological activity or function following exposure to heat, pH extremes, or processing conditions common in milling, cooking or other processing methods will contribute to the safety assessment of the NEP. This is because these conditions reduce exposure to the functional protein, thereby reducing the hazard potential [17].

4.2. Resistance to Digestion

Proteins, in general, are a natural and necessary part of human and animal diets, and are subjected to rapid degradation by digestive enzymes in the gastrointestinal tract into individual amino acids and small peptides that can be absorbed by the body to support nutritional needs. Large proteins are not known to be absorbed by the intestinal epithelium. As part of the WOE approach, a protein's ability to resist degradation *in vitro*, in the presence of digestive enzymes (pepsin and pancreatin) is tested, and aids in the understanding about the potential digestive fate of a NEP in food. This, in turn, provides information about any potential for systemic absorption of intact active proteins, since proteins that are rapidly and thoroughly degraded by digestive enzymes present no opportunity to be absorbed intact. If the NEP is rapidly degraded in pepsin and pancreatin, it can be inferred that it has limited or no biological activity and is less likely to impart toxic effects upon consumption, and thus less of a concern for safety to humans and animals. However, if proteins are resistant to degradation by digestive enzymes, it does not necessarily indicate that the protein presents a potential hazard, as stability does not, in and of itself, answer the question about whether the NEP is a likely hazard.

5. Conclusion

Toxicological assessment of NEPs in GM plants is performed to inform the overall safety assessment process. In conjunction with allergenicity assessment, the results of toxicity evaluation enable risk characterization and the evaluation of safety of GM plants for food and feed use. A stepwise approach is proposed here to evaluate toxicity that uses WOE gathered from different attributes of the NEP. Key hazard identification studies should first be performed for all NEPs (core studies) and, if a hazard is identified, further toxicity studies and exposure characterization should be done (supplementary studies). An excellent example of the application of this proposed stepwise approach to the safety assessment of a NEP is described in Habig et al., wherein the WOE for safety of the intractable protein VNT1 was successfully concluded using only those approaches described as "core" studies [14]. Acute oral toxicology studies are not informative in the absence of hazard attained from the WOE assessment, and *in vitro* toxicology studies are proposed for intractable proteins. *In vitro* studies are also beneficial for animal welfare. Exposure considerations such as stability and resistance to digestion contribute to the WOE for overall safety assessment and should be done when a hazard is identified. When there is no hazard identified, there would be no risk, and therefore, further hazard and exposure characterization is unnecessary.

6. Declaration of Conflicting Interest

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

7. 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 *Allergy risk assessment for 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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