Critical Reviews in Toxicology

http://informahealthcare.com/txc
ISSN: 1040-8444 (print), 1547-6898 (electronic)

Crit Rev Toxicol, 2013; 43(S2): 25–42
 DOI: 10.3109/10408444.2013.842956

informa
healthcare

REVIEW ARTICLE

Toxicological evaluation of proteins introduced into food crops

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Abstract

This manuscript focuses on the toxicological evaluation of proteins introduced into GM crops to impart desired traits. In many cases, introduced proteins can be shown to have a history of safe use. Where modifications have been made to proteins, experience has shown that it is highly unlikely that modification of amino acid sequences can make a non-toxic protein toxic. Moreover, if the modified protein still retains its biological function, and this function is found in related proteins that have a history of safe use (HOSU) in food, and the exposure level is similar to functionally related proteins, then the modified protein could also be considered to be ''as-safe-as'' those that have a HOSU. Within nature, there can be considerable evolutionary changes in the amino acid sequence of proteins within the same family, yet these proteins share the same biological function. In general, food crops such as maize, soy, rice, canola etc. are subjected to a variety of processing conditions to generate different food products. Processing conditions such as cooking, modification of pH conditions, and mechanical shearing can often denature proteins in these crops resulting in a loss of functional activity. These same processing conditions can also markedly lower human dietary exposure to (functionally active) proteins. Safety testing of an introduced protein could be indicated if its biological function was not adequately characterized and/or it was shown to be structurally/functionally related to proteins that are known to be toxic to mammals.

Keywords

Food processing, food safety, genetically modified crops, history of safe use, threshold of toxicological concern

History

Received 15 May 2013
Revised 4 September 2013
Accepted 6 September 2013
Published online 25 October 2013

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Introduction

Crop plants enhanced through the use of biotechnology, also known as genetically modified (GM) crops, are typically modified to express one or more newly encoded proteins

(''introduced proteins'') that impart a trait not previously present in the crop. Agronomic traits such as insect and virus resistance and herbicide tolerance have been the most common traits introduced to date; however, crops with traits for improved nutritional value and environmental stress tolerance are under development and nearing commercialization. The safety of GM crops must be assessed and then approved by regulatory agencies and/or authorities in various countries where the crop will be cultivated or imported. The *Codex Alimentarius* Commission guidelines developed and adopted by the World Trade Organization (WTO) countries describe an integrated approach for the assessment of the safety of proteins expressed in plants derived from recombinant DNA technology (Codex, 2009). These guidelines center on evaluating whether the GM crop is ''as safe as'' conventional crops that have a ''history of safe use'' (HOSU). In general, the food safety assessment is conducted in terms of changes imparted to the host plant, including those resulting from the newly introduced trait (intended effect) and the potential for modification of existing traits (unintended effects).

Proteins are one of the three major nutrient types ingested by humans and animals. Here we focus on the safety assessment of introduced proteins to determine whether they have the potential to exert adverse effects. This paper does not discuss allergenicity, as multiple comprehensive reviews cover this topic (EFSA, 2010a; Goodman et al., 2008; Ladics et al., 2011; Randhawa et al., 2011; Stagg et al., 2013; Thomas et al., 2009). The safety assessment of small RNA molecules (Parrott et al., 2010) and general schemes for evaluating protein safety in GM crops (Chao & Krewski, 2008; Delaney et al., 2008a; Hammond & Cockburn, 2008) have been previously reviewed. Building on these earlier reviews, we now address the following topics: (1) additional considerations for what constitutes a HOSU for proteins; (2) the effects of food processing on the function and integrity of introduced proteins, and thus on dietary exposure; (3) the potential utility of applying the threshold of toxicological concern (TTC) concept for proteins; (4) an assessment of the potential for harmful interactions between introduced proteins in combined-trait agricultural crops; and (5) an assessment of whether genotoxicity assays with introduced proteins are needed to confirm their safety on an *ad hoc* basis.

Summary of current protein safety assessment considerations

In an earlier report, a two-tiered testing system was proposed by the International Life Sciences Institute (ILSI) for the assessment of protein safety (Delaney et al., 2008a). In Tier 1, no *in vivo* toxicology testing is considered necessary if the protein meets all of the following criteria: (1) there is a HOSU of the protein or related proteins in foods; (2) the protein is not structurally or functionally related to proteins considered to be toxic (or allergenic) to humans or animals; (3) the protein has a molecular or biological function that raises no safety concerns; and (4) the protein is readily digested in validated *in vitro* digestive tests. If potential safety issues are raised during the Tier 1 assessment, or if assurance of safety is not possible due to limited available information, then additional safety testing, such as acute or repeat-dose *in vivo* toxicity studies, may be indicated for Tier 2 safety assessment. For example, hypothesis-based toxicology studies may be needed when the protein's biochemical function suggests it may be potentially toxic to non-target organisms.

Safety concerns have been raised about various proteins that have been introduced into GM crops. Some argue that since proteins introduced into GM crops to control insect pests have been modified from those found in nature (e.g. *Bacillus thuringiensis* derived Cry insect control proteins) these changes may cause unknown health consequences in those who consume the crop. Thus, the GM crops should be subjected to chronic toxicity testing in animals as is done for pesticidal chemicals applied to agricultural crops (Seralini et al., 2011). In a different publication, *Bt* Cry insect control proteins have been reported to cause hematotoxicity in mice when *Bt* microbial spore preparations containing various Cry proteins were administered by stomach tube to mice (Mezzomo et al., 2013). Others have reported that administration of Cry proteins to mice by various routes (stomach, rectum, nasal or by intraperitoneal injection) caused immunogenic effects (Moreno-Fierros et al., 2000; Vasquez-Padron et al., 2000). There were similar reports of immunogenic effects in rats fed *Bt* rice containing Cry1Ab protein (Kroghsbo et al., 2008) and chronic feeding of *Bt* maize in mice was reported to have caused alterations in the intestinal and peripheral immune populations (Finamore et al., 2008).

Regarding the food safety implications of modifying proteins, the genes from bacteria encoding for introduced proteins (e.g. *Bacillus thuringiensis* Cry proteins) must be modified slightly to facilitate translation of the genes in plants. Modifications of proteins may also be done to enhance their functional activity, but as will be discussed later, such changes are highly unlikely to make a non-toxic protein toxic. There are also fundamental differences in the biological properties of proteins compared to smaller molecular weight chemicals, e.g. pesticides) which greatly limit the potential of proteins to produce chronic toxic effects when ingested. Hematotoxic effects have not been reported in the many animal studies with Cry proteins and *Bt* crops following repeated dietary exposures as discussed later. Indeed the reported hematologic effects (Mezzomo et al., 2013) may have been an artifact since the study was not properly controlled. The authors used distilled water instead of *Bt* spores lacking the Cry genes as the negative control. The *Bt* spores contain many other bacterial proteins that could have confounded the study results. Doses of Cry proteins reported to cause immunogenic effects in mice were often many thousands of times higher than potential human dietary exposure, and used routes of exposure that were not relevant to dietary exposure (intranasal, intraperitoneal injection, etc.) Immunogenic responses to proteins that are considered foreign to the species tested are normal responses. However, the predictive value of immune findings in mice to humans has not been generally accepted as no animal models are considered validated for predicting immune responses in humans (Codex, 2009; Goodman et al., 2008; Thomas et al., 2009). Some of the results of other studies (Moreno-Fierros et al., 2000; Vasquez-Padron et al., 2000) were not reproduced. The authors of the repeat study attributed the immunogenic effects observed in the original

studies to possible contamination of test articles with endotoxins that can cause immunogenic effects in mice (Adel-Patient et al., 2011). Thus the relevance of the aforementioned study results with Cry proteins to human food safety assessment has been questioned (Hammond & Koch, 2012). It would be helpful to summarize some of the key considerations on protein safety from the prior literature, including the 2008 ILSI publication (Delaney et al., 2008a). Some of these considerations also address the aforementioned safety concerns.

(1) Proteins are fundamentally different from low-molecular-weight chemicals because they are very large macromolecules composed of amino acids. In the gastrointestinal tract, protein macromolecules, unlike low-molecular-weight chemicals, are subject to degradation by digestive enzymes into individual amino acids and small peptides that can be absorbed by the body to support nutritional needs. In general, the propensity for systemic absorption of any orally consumed substance is inversely proportional to the size of the molecule, with smaller molecules more readily absorbed intact than larger ones (Gardner, 1988). As such, the potential for systemic absorption of a protein from the digestive tract as an intact molecule is very much less than that of low-molecular-weight chemicals.

(2) Numerous physical and biological barriers in humans and other animals restrict the movement of intact proteins into cells after dietary consumption (Kier & Petrick, 2008). These barriers reduce the likelihood that an ingested protein can access critical intracellular spaces such as the cytoplasm and nucleus to impact cellular physiology or DNA integrity.

  (2a) As noted in point 1, the normal fate of most dietary proteins is digestion into peptides and amino acids that are subsequently absorbed into the body (Caspary, 1992). For a protein to be absorbed intact into the systemic circulation, it would first need to survive the proteolytic environment of the stomach, which is highly acidic and contains multiple proteases.

  (2b) To affect cell/tissue physiological functions, the protein may need to traverse the plasma membrane of intestinal epithelial cells or intercellular junctions into the systemic circulation, pass from the circulation into another tissue, and survive hydrolytic degradation during these discrete transit steps. Plasma membrane lipid bilayers of the digestive tract epithelium are effectively impermeable to exogenous proteins in the absence of specialized membrane transporters that facilitate uptake of specific proteins, although infants on colostrum readily absorb protein nutrients (Gardner, 1988).

  (2c) These biological barriers generally limit the amount of intact protein that enters the body. The effectiveness of these barriers has been well established by unsuccessful attempts to orally administer proteins for therapeutic benefit (Goldberg & Gomez-Orellana, 2003; Hamman et al., 2005; O'Hagan et al., 1988; Shah et al., 2002).

(3) Proteins found in living organisms have been isolated, characterized, and assigned to different functional classes having related structures and functions. Despite the large number of known proteins, only a small number are known to elicit adverse effects in vertebrates following oral intake (Delaney et al., 2008a).

(4) Toxicology testing of proteins is recommended in cases where the weight of evidence regarding safety following Tier 1 assessment is considered to be either incomplete or inconclusive. Subsequent testing should be hypothesis based to resolve specific safety questions. Because proteins known to be toxic to mammals and other organisms generally work through specific mechanisms to cause adverse acute effects, testing can often be performed using acute toxicity tests (Delaney et al., 2008a; Sjoblad et al., 1992). For example, *Bacillus thuringiensis* (*Bt*)-based Cry insect-control proteins work through acute mechanisms to control target insect pests. To assess the potential hazards of *Bt* Cry insect-control proteins that have an acute mode of action, the US Environmental Protection Agency (US EPA, 2000) requires the use of high (hazard) acute doses (~gram/kg body weight [BW]) where feasible. Due to limited solubility of some Cry proteins in dosing vehicles, it is not always possible achieve gram/kg dosage levels.

(5) Candidate proteins intended for use in new GM crops are chosen to avoid potentially adverse consequences. In the unlikely scenario that an introduced protein has a biochemical function similar to a known anti-nutrient proteins found in plants, such as lectins or protease inhibitors, then an acute toxicity study may be insufficient to assess potential toxicity. These anti-nutrients generally manifest their toxicity within a few weeks of dietary exposure by interfering with protein digestion (protease inhibitors) or by damaging cells lining the gastrointestinal tract (lectins) (Delaney et al., 2008a; Leiner, 1994; Pusztai & Bardocz, 1995). They can be tested for potential toxicity by using the study design adapted from the Organization for Economic Cooperation and Development (OECD) test guideline 407 for 28-day toxicity studies with chemicals (OECD, 1995). The dose administered may be based on a limit dose of 1000 mg/kg as is done for chemicals (Delaney et al., 2008b; Mathesius et al., 2009), although this is not scientifically relevant for proteins in GM crops because the exposure levels are many orders of magnitude lower than the limit dose achievable for chemicals. Therefore, it is preferable to employ dosages that provide at least a 100-to-1000-fold margin of safety (Juberg et al., 2009; Stagg et al., 2012). Additional toxicological studies may be indicated if the 28-day study finds evidence of toxicity. As will be discussed, the European Food Safety Authority (EFSA) requires testing of introduced proteins without a history of safe use (HOSU) in a 28 day study. They do not normally require acute tests on introduced proteins.

(6) There have been no reports of direct carcinogenic, teratogenic, or mutagenic effects associated with ingestion of proteins in general (Pariza & Foster, 1983; Pariza

& Johnson, 2001; US EPA, 2000). Food safety scientists in regulatory agencies have not considered it necessary to conduct chronic testing of proteins introduced to date into GM crops based on the aforementioned considerations. These proteins are not considered to be toxic based on their known biochemical function. The intact proteins and potential peptide degradation products are also not similar to known protein/peptide toxins as determined by bioinformatics searches. Residual levels of introduced proteins are present at very low levels in food products and are capable of being digested so that the potential for systemic absorption of the intact protein would likely be negligible. Chronic testing is not generally needed to identify the potential toxicity of known protein toxins as this is usually manifest after acute or short term dietary exposure based on the known biochemical functions of protein toxins.

(7) Proteins can be denatured and inactivated during normal food processing, which may have an impact on dietary exposure. Where there are low levels of dietary exposure to functionally intact introduced proteins, the TTC concept could be used to assess thresholds of exposure that have been shown to have very large margins of safety (Delaney et al., 2008a), which would preclude the need for toxicity studies.

Additional factors for protein safety assessment

Additional factors which will be discussed include (1) the relevance of HOSU to the safety assessment of proteins, (2) the effects of thermal or other kinds food processing conditions on dietary exposure to introduced proteins, (3) the potential for interactions between introduced proteins in combined-trait crops, and (4) the testing of proteins for genotoxicity.

History of safe use

The European Food Safety Authority (EFSA) has referred to proteins that are introduced into GM crops that do not have a history of safe use (HOSU) as ''novel'' (EFSA, 2008). The word novel is defined as ''new and not resembling something formerly known or used'' (Webster's New Collegiate Dictionary, 1973). According to this definition, referring to a specific protein that lacks direct evidence of being safely consumed (i.e. without a HOSU) as ''novel'' may not be appropriate if the protein is a member of a family of structurally/functionally related proteins with a HOSU in foods. If the protein shares either structural or sequence homology with known proteins with a HOSU, it is not necessarily ''novel''. As discussed in the section on HOSU, there is considerable evolutionary divergence in the amino acid sequence of functionally related proteins across plant and animal species, and therefore amino acid changes may not make a protein ''novel''.

EFSA has recommended that all introduced proteins without a HOSU be tested for safety in 28-day repeat-dose toxicology testing unless there is reliable information to demonstrate their safety (EFSA, 2009a). To date, the completed 28-day mouse studies on proteins introduced into GM crops have shown no evidence of adverse effects (Delaney et al., 2008b; Juberg et al., 2009; Mathesius et al., 2009; Stagg et al., 2012). The proteins evaluated in these 28-day studies had also been assessed by the widely accepted weight-of-evidence approach (e.g. lack of homology to known toxins and allergens; rapid digestion in simulated digestive assays) (Codex, 2009; Delaney et al., 2008a). Therefore, the 28-day study may not contribute any new, useful information where the weight of evidence indicates that there are no meaningful concerns raised during the risk assessment process. Thus the use of these studies should not be a routine requirement.

Relevance of HOSU concept to protein safety assessment

Very few traditional whole foods, including those which contain anti-nutrients and toxins, have been subjected to systematic toxicological assessment. However, because such foods have a long HOSU, the protein components in such foods are generally regarded as safe to eat (Constable et al., 2007). Some foods require special preparation to minimize the potential for associated health risks from consumption of natural protein anti-nutrients, such as trypsin inhibitors and lectins in soy or kidney bean. While the application of the HOSU concept may be somewhat subjective, it remains a useful tool for the hazard assessment of introduced proteins (Delaney et al., 2008a; Hammond & Cockburn, 2008) and the comparative analysis is based on the use of it.

There is general agreement that an introduced protein has a HOSU if it is identical to a protein that is already present in food and known to be safe for consumption at the levels expected in the GM crop. When the sequence of an introduced protein differs from the sequence of an endogenous protein already present in food, then the degree of acceptable similarity for safety assessment purposes needs to be decided on a case-by-case basis. General protein biochemical principles can provide useful guidance. As previously noted, there can be considerable variation in amino acid content within families of proteins without changes in protein structure or function. It is extremely rare that a few amino acid changes can impart to a protein a meaningful new functionality; most typically, such changes result in loss of function (Bloom & Arnold, 2009).

As an example, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is a key enzyme in the shikimate pathway, which is required for aromatic amino acid biosynthesis in plants, and is inhibited by the herbicide glyphosate. The 2mEPSPS protein, which is derived from the maize EPSPS, contains two site-directed mutations (I103T and S107P) (Herouet-Guicheney et al., 2009) that do not alter its metabolic function in the shikimate pathway, but render it insensitive to glyphosate inhibition. Compared to the wild-type enzyme from maize (Table 1), 2mEPSPS is nearly

Table 1. Amino acid sequence similarities between the 2mEPSPS protein and EPSPS from various crops.

	Maize	Rice	Grape	Lettuce	Tomato	Canola
% Sequence identity	99.5	86	79	77	75	75

EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase.

identical (99.5% sequence identity). Importantly, the sequence variation between the EPSPS proteins from maize, tomato, lettuce, grape, and rice is much greater, as these enzymes share 75–99% identity; thus, the HOSU for this family of enzymes provides strong evidence for the safety of 2mEPSPS (Herouet-Guicheney et al., 2009). Moreover, structural comparison of the EPSPS proteins from canola, maize, rice, soy, *Agrobacterium*, and *Escherichia coli* (Figure 1) indicates that sequence variation has little effect on overall protein structure and function. For example, CP4 EPSPS protein, which also imparts tolerance to the herbicide glyphosate, shows approximately 50% sequence identity to plant-derived EPSPS enzymes (Figure 1).

As described later, homologous proteins (i.e. those derived from a common ancestor having similar functions) share related amino acid sequences and three-dimensional structures, as well as common function (Doolittle, 1990). Introduction of a gene into a GM crop for the expression of a protein that is homologous to proteins with a HOSU can ensure a more robust safety assessment (Delaney et al., 2008a; EFSA, 2006; US FDA, 1992). Homologous EPSPS proteins are ubiquitous in plant, yeast, and microbial food sources and show considerable variety in amino acid sequence, but share the same function in aromatic amino acid synthesis. In addition, these enzymes are not present in mammalian cells, which do not synthesize their own aromatic and branched-chain amino acids. No forms of EPSPS have been reported to be toxic or allergenic. EPSPS enzymes as a component of food and feed, including plant materials consumed after processing, have never been associated with any health concerns. Based on the weight of evidence, EPSPS proteins can be deemed safe for consumption, and CP4 EPSPS (see the section on application of the TTC for proteins) and 2mEPSPS (this section) should not be considered as ''novel'' (Herouet-Guicheney et al., 2009; Padgette et al., 1996) and are ''as-safe-as'' to consume as endogenous plant EPSPS enzymes.

Structural/functional relatedness – lessons from protein engineering

Proteins with conserved sequence motifs and/or structural features are often grouped into protein families, which can provide important information about the possible biochemical role of a given protein (Buljan & Bateman, 2009; Ganfornina & Sánchez, 1999; Moore et al., 2008; Thornton et al., 1999). As of November 2011 (Punta et al., 2012), there were nearly 16 million unique protein sequences in public databases that could be grouped into ~13 500 protein families based on sequence and structure. The relatively limited number of homologous protein families highlights the constraints that evolution, protein structure and folding, and biological function place on amino acid sequences. The relatedness and diversity of proteins in food can help guide a HOSU evaluation. For example, if an introduced protein does not have a HOSU because it was derived from a plant species not used for food, then information might be available from homologous proteins that are already consumed in food. The evidence based on a wide variety of studies shows that variation in these proteins have no effects on their safety in vertebrates.

Proteins in GM crops can be modified to enhance their functional properties, but the selection of changes is constrained by the requirement to maintain the structure, stability, and activity of the protein. Modification of enzymes to improve catalytic properties has been an objective for the last 30 years. The safety of enzymes modified by protein engineering has been discussed previously in light of the abundant natural variation of enzymes along with the conservation of general tertiary structural features and catalytic activities within enzyme families (Pariza & Johnson, 2001). Engineered enzymes still retain the three-dimensional structure and functional characteristics of the enzyme family from which they were derived, and may exhibit similar or even less variation in amino acids than what occurs through natural sequence variation (Pariza & Cook, 2010). Most evolutionary changes resulting in amino acid substitutions are conservative and maintain the stability of the protein, as structure is more conserved than sequence (Illergård et al., 2009).

Based on experiences to date with *in vitro* engineering of proteins, modifications in amino acid sequence may have little impact on structure and/or function (Behe et al., 1991; Lattman & Rose, 1993; Rose & Creamer, 1994). Changes in amino acid sequence can be conservative, such as substitution of one amino acid residue for another (e.g. a glutamate for an aspartate), or radically alter the chemical properties of a

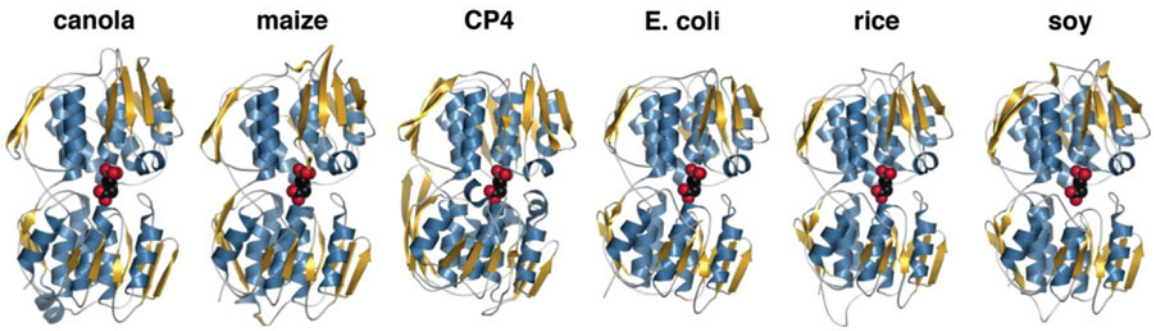

Figure 1. Comparison of three-dimensional structures of EPSPS from plants and bacteria. The X-ray crystal structures of the EPSPS from *E. coli* and *Agrobacterium* CP4 were used to generate homology models of the EPSPS from canola, maize, rice, and soy using SwissModel. The plant enzymes have ~90% sequence identity with each other and are ~50% identical to the bacterial enzymes. Secondary structure corresponding to α-helices and β-strands are colored blue and gold, respectively. The position of glyphosate (from the *E. coli* and CP4 crystal structures) is modeled as a space-filling molecule (red) into each structure to show the active site location.

particular position in terms of size, shape, and/or charge. For example, substitution of a large amino acid, such as tryptophan, for a smaller amino acid, such as cysteine, results in side-chains with different physical and chemical properties. Moreover, a substitution made in the context of a three-dimensional structure may also have major consequences. Amino acid changes will have no impact on function if they are in regions that are not required for function (e.g. flexible loops) in contrast to substitutions in areas essential for protein function (e.g. active sites required for catalysis or ligand-binding sites). Many modifications are deleterious to protein function because of alterations in protein folding that reduce or eliminate functional activity (e.g. enzyme activity). It has been estimated that 50–70% of random modifications in amino acid sequence are neutral, 30–50% are strongly deleterious, and 0.01–0.5% are beneficial to protein function (Bloom & Arnold, 2009). Ultimately, it is much easier to disrupt protein function than to improve it.

Evolutionary changes within protein families used as food processing enzymes have not resulted in the enzymes becoming toxic to humans (Pariza & Cook, 2010). It is highly unlikely that modification of a protein sequence (and structure), regardless of the source organism, will turn a non-toxic protein into a toxic protein. First, the natural drift of sequence changes in a gene or protein occurs gradually on an evolutionary time-scale (Creighton, 1993). Second, the odds that a set of specific mutations will occur to convert a non-toxic protein into a toxin are extremely low. For example, the probability that engineering nine amino acid substitutions into a non-toxic enzyme could make it into a protein toxin was calculated to be only 1 in 2×10^{11} (Pariza & Cook, 2010). Third, any sequence changes would need to be consistent with a biological mechanism of toxicity. Protein toxins generally need to recognize specific molecular targets, such as metabolites or other proteins, in a susceptible organism to have a toxic mode of action (Rappuoli & Montecucco, 1997). For example, some proteins of the cystine-knot family bind to specific ion channels, which can result in toxic effects; however, changes in sequence can alter channel specificity, change the range of species susceptible to the toxin, or render the toxin ineffective because the structure and sequence of the toxin must match the structure and sequence of the targeted channel (Craik et al., 2001).

Bioinformatics and HOSU

If the HOSU concept can be expanded to include those proteins within the same family that have ''similar'' structure and function, then ''similarity'' can be assessed using bioinformatics and animal toxicity testing may not be needed. While no formal guidelines have been established for what constitutes a significant sequence similarity between an introduced protein and familiar proteins normally present in foods, one general recommendation based on evolutionary sequence analysis is that proteins sharing <20% identity over 100 or more amino acids should not be considered homologs (Doolittle, 1990).

As part of the Tier 1 safety assessment described by Delaney et al. (2008a), a bioinformatics evaluation is conducted very early in the development of a potential product. Analysis of sequence and structure databases addresses a fundamental safety question: does the introduced protein share any sequence or structural similarity with proteins known to pose possible toxicological hazards? These databases range from repositories of protein sequences derived from translations of nucleic acid sequences to curated databases that include additional information about proteins that have been validated for expression and/or function (Table 2). Because high sequence and structural similarity often correlates with a conserved biological role (e.g. Mills et al., 2004), computer algorithms to evaluate phylogenetic relationships of proteins are useful in safety evaluations.

Table 2. Bioinformatics resources.

Database	Type	URL
NCBI Entrez Protein*	Sequence repository	www.ncbi.nlm.nih.gov/protein
RefSeq	Sequence repository	www.ncbi.nlm.nih.gov/RefSeq
PIR†	Curated database	pir.georgetown.edu
UniProt-Swiss-Prot	Curated database	www.uniprot.org

*National Center for Biotechnology Information.
†Protein Information Resource.

Sequence comparisons help define superfamilies of proteins that catalyze similar chemical reactions on different molecules or families of homologous (and orthologous) proteins. This type of analysis can provide a useful tool for safety evaluation. For example, cytochrome c oxidases from bacteria, fungi, and animals are related by <30% sequence identity but share a common overall three-dimensional structure (Figure 2). One could conclude, therefore, that if cytochrome c oxidase from a species currently used for food (e.g. tuna) were to be introduced into a food crop, it would pose no safety concerns, as there is a HOSU for tuna consumption. However, if cytochrome c oxidase from an organism with no HOSU, such as the bacterium *Paracoccus*, were introduced into food, should this form of cytochrome c oxidase be considered as ''novel,'' thus requiring it to be tested in a 28-day toxicology study (EFSA, 2009a)? Given the fact that the various cytochrome c oxidases perform the same biochemical function due to conservation of the active site across species, one could rationally conclude that none of the aforementioned forms of this protein are ''novel'', as they share common structural, sequence, and functional relationships and would be safe to consume. As part of the safety assessment, the technology developer would still submit the amino acid sequence of the *Paracoccus* cytochrome c oxidase to bioinformatics analysis to confirm it was not related to known toxins or allergens and perform *in vitro* studies (e.g. digestibility and heat stability assays) to confirm its potential to be readily degraded or denatured during food processing and consumption. Assuming that this assessment of the *Paracoccus* protein raised no safety concerns, then scientifically there should be no need for further toxicity testing, since it is functionally related to protein homologs that have a HOSU.

Dietary exposure: impact of food processing on introduced proteins

Many crops and other foods are subjected to a variety of processing conditions to improve their nutritional quality (e.g. heat and/or pressure denaturation, alkaline

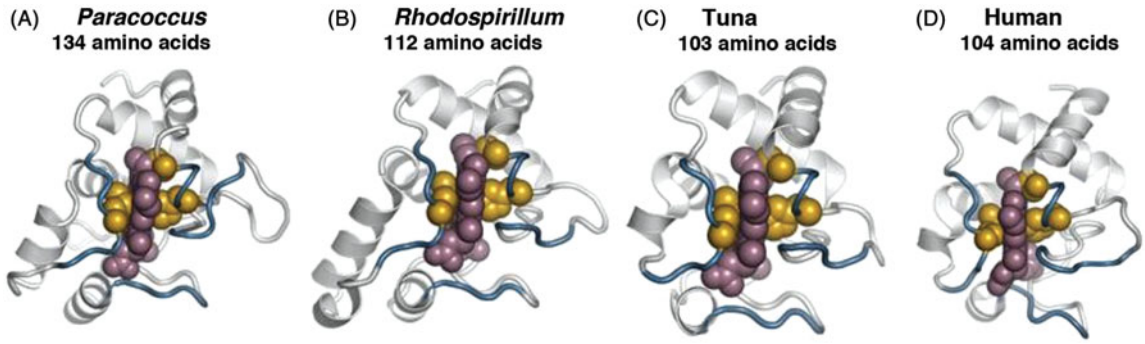

Figure 2. Cytochrome c oxidase structural homology. Comparison of the three-dimensional structures and active sites of cytochrome c oxidases from various species. Overall structure is shown as a ribbon diagram. Invariant residues (gold spheres) that maintain the position of the catalytic heme-group (rose spheres) are highlighted.

nixtamalization of maize) and/or to enhance flavor, texture, taste, and appearance (Rooney & Serna-Saldivar, 2003; van Boekel et al., 2010). Food processing can denature, inactivate, degrade, or even remove proteins from the final product. For instance, digestibility of soy is improved by cooking soy meal to inactivate endogenous anti-nutrients such as trypsin inhibitors (which interfere with digestion of dietary proteins) and lectins (which impair nutrient absorption from the gastrointestinal tract) (Concon, 1988; Rackis, 1974). Cooking and pasteurization also destroy microbes that reduce storage stability, and thermal processing can modify protein content and structure to achieve desired technical or nutritional effects (Thomas et al., 2007; van Boekel et al., 2010). Likewise, extensive chemical and physical processes are used to refine edible oils. These steps involve acid pre-treatment, degumming, neutralization, bleaching and deodorization by heating the oil up to 230 °C. Such processes usually alter the proteins normally present at low levels in these oils. Protein solubility is decreased, which reduces total protein content in the final processed fraction to the point that the introduced protein is not detected in immunological tests (Codex, 2010; de Luis et al., 2009; Grothaus et al., 2006; Margarit et al., 2006; Thomas et al., 2007; Venktesh & Prakash, 1993). A detailed summary of the different processing conditions used to make food products derived from maize and soy can be found in the Supplementary data (Appendix A) of Hammond & Jez (2011). How a food/feed crop containing an introduced protein is processed and/or cooked, how that processing affects the dietary levels of the protein, and how processing alters protein structure and activity all need to be considered for safety assessments.

Influence of structure on protein function

The amino acids in a protein are linked together by covalent peptide bonds into polypeptide chains that can consist of thousands to tens of thousands of amino acids (Branden & Tooze, 1991). The specific sequence of amino acids in a protein dictates the formation of secondary structure (e.g. α-helices, β-pleated sheets) and their arrangement into a stable tertiary or three-dimensional protein structure. The amino acid sequence can also influence the quaternary formation of multi-subunit proteins. Folding of a protein into the correct three-dimensional structure is essential for protein function (Figure 3) (Branden & Tooze, 1991). Proteins that perform either similar or identical biological functions in different organisms typically share related secondary and tertiary structures and, to varying degrees, primary amino acid sequence.

The proper folding of an amino acid sequence into a functional protein involves a combination of physico-chemical forces that include short-range repulsions, electrostatic forces (i.e. charge–charge interactions and dipole moments), van der Waals interactions, hydrogen bonds, and hydrophobic interactions (Branden & Tooze, 1991; Creighton, 1993). Electrostatic, hydrogen bond, and van der Waals interactions in aqueous environments such as the cell are weak compared to interactions of hydrophilic amino acids with the water surrounding a protein; however, proteins also contain regions of hydrophobic amino acids. Generally, this leads to orientation of hydrophilic amino acids on the exterior of a protein and hydrophobic amino acids packed into the interior to exclude water from the protein core. This hydrophobic effect, that is, the exclusion of water from the protein interior, shapes the overall structure of a given protein and allows it to function biologically as either a membrane protein or a soluble protein (Kilara & Sharkasi, 1986; Pace et al., 2011). As described in the following section, the forces that hold a protein in its properly folded form can be severely affected by food processing and cooking.

Alteration of protein structure triggered by changes in the microenvironment

In general, protein structures are only stable under a limited range of physiological conditions and are easily disrupted by any changes in their surrounding microenvironment (e.g. shifts in temperature, variation of pH, or physical disruptions) that overcome the forces keeping them folded (Creighton, 1993; Kristjánnson & Kinsella, 1991). Denaturation of proteins is characterized by a drastic change in structure that invariably results in a loss of biological function, as the denatured polypeptide is more like a random coil than a properly folded protein. As the microenvironment of a protein is gradually altered toward conditions that favor unfolding, the folded structure initially changes very little, if at all, but suddenly, the favored tertiary structure of the protein is sufficiently disrupted to unfold. The abruptness of the protein denaturation transition results because the process is highly cooperative. For example, as pH varies, multiple charged

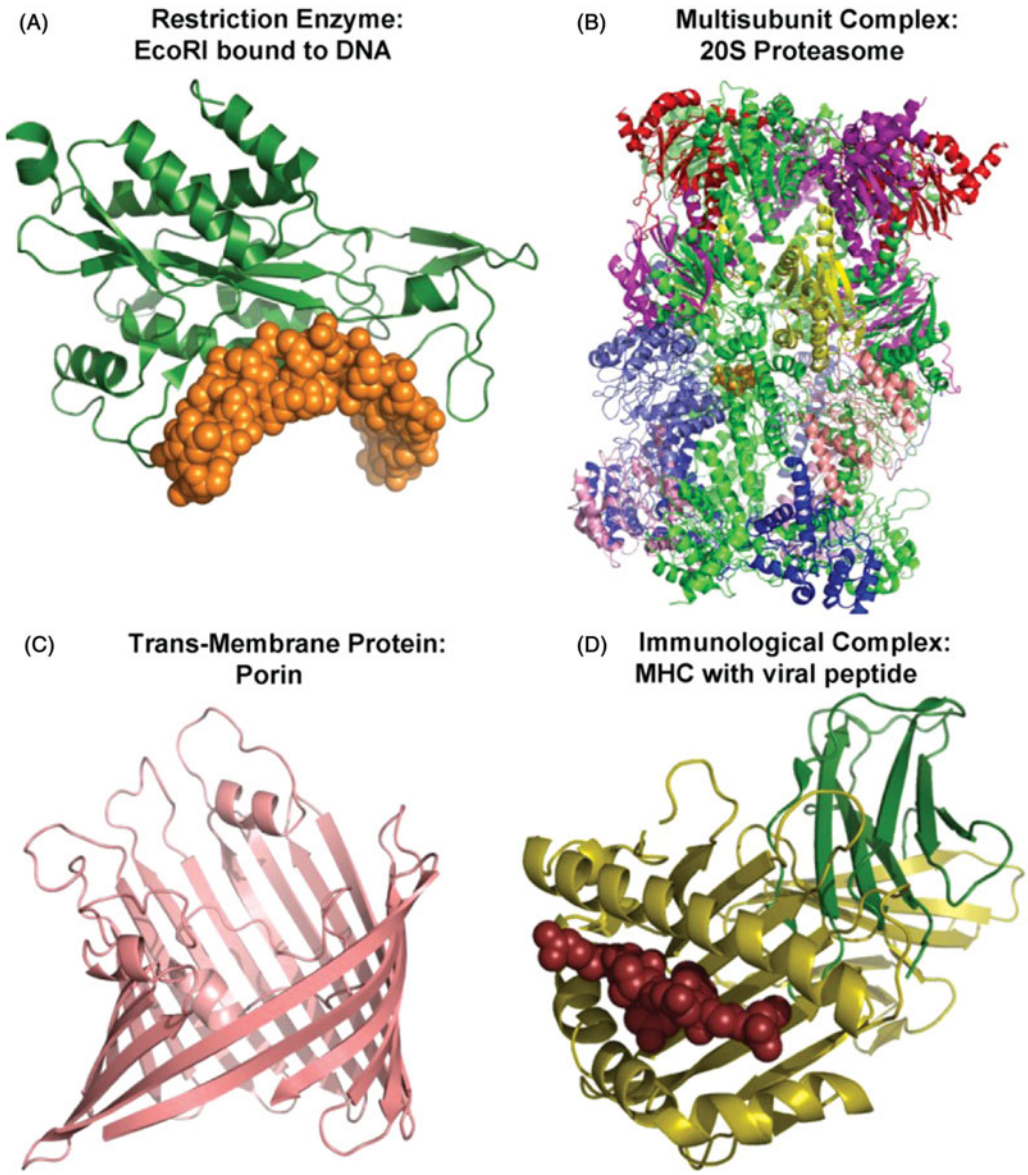

Figure 3. Variety of protein structures. Examples of different types of proteins are shown to highlight variations in secondary, tertiary, and quaternary structures. In panel A, the gold space-filling model represents DNA and the green ribbon diagram the protein. In panel B, each subunit of the proteasome is colored differently. In panel C, the monomeric structure of porin is shown as a ribbon diagram. In panel D, the viral peptide is shown as the brown space-filling model and the major histocompatibility complex (MHC) molecule as a ribbon diagram.

amino acids change ionization state simultaneously. Similarly, increased temperature affects all of the forces that maintain a stable protein structure at the same time. Denaturation can also result from other changes to a protein, such as altered oxidation state or the removal of cofactors and prosthetic groups. Typically, denaturation does not involve changes in the primary structure of a protein, such as degradation or cleavage of the polypeptide chain. Although the conditions that cause denaturation may differ for each protein (Pearce, 1989), proteins that function under normal physiological conditions tend to have similar resistance to unfolding, even though they have different amino acid sequences and three-dimensional structures (Creighton, 1993). It should be noted that peptides and proteins containing covalent disulfide bonds require reduction of these bonds for complete unfolding.

Changes in the environment of food/feed proteins routinely occur during processing, such as cooking. For example, grain/seed from maize and soy are not consumed raw by humans, but are first cooked. During the processing of maize and soy into food fractions, heating, extrusion under high pressure, mechanical shearing, changes in pH, and the use of reducing agents are all employed. These processes will unfold a native protein structure and/or alter the primary structure of a protein by hydrolysis of peptide bonds (Kilara & Sharkasi, 1986; Meade et al., 2005). During typical processing of soy into food fractions, temperatures of 95–100 °C for several minutes are commonly encountered (Kilara & Sharkasi, 1986), and even higher temperatures and longer times are possible depending on the processing method. These elevated temperatures can lead to irreversible denaturation and loss of protein function (de Luis et al., 2009; Thomas et al., 2007). These processing conditions do not generally degrade the nutritional value of the protein as a source of dietary amino acids; in many cases, they actually enhance digestibility and improve the bioavailability of the component amino acids. This is particularly true for some vegetable proteins, which

Table 3. Impact of heating on functional activity of introduced proteins and food processing enzymes.

Protein	*In vitro* heat treatment	Function	Activity after treatment	Reference
CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)	65–75 °C; 30 min	Enzyme*	None detectable	EFSA (2009d)
2mEPSPS	65 °C; 30 min	Enzyme*	None detectable	EFSA (2007b)
Phosphinothricin-N-acetyl transferase (PAT)	55 °C; 10 min	Enzyme†	None detectable	Hérouet et al. (2005)
Glyphosate acetyltransferase (GAT)	56 °C; 15 min	Enzyme‡	None detectable	Delaney et al. (2008b)
Cry1Ab	80 °C; 10 min	Insecticide¶	None detectable	de Luis et al. (2009)
Cry1F	75–90 °C; 30 min	Insecticide	None detectable	EFSA (2005d)
Cry3A	95 °C; 30 min	Insecticide	None detectable	US EPA (2010)
Cry9C	90 °C; 10 min	Insecticide	No loss of activity	de Luis et al. (2009)
Cry34Ab1/Cry35Ab1	60–90 °C; 30 min	Insecticide	None detectable	EFSA (2007a)
Acetolactate synthase	50 °C; 15 min	Enzyme§	None detectable	Mathesius et al. (2009)
β-Glucuronidase	60 °C; 15 min	Enzyme||	50% loss of activity	Gilissen et al. (1998)

*Catalyzes the conversion of phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate.
†Catalyzes the conversion of L-phosphinothricin to N-acetyl L-phosphinothricin.
‡Cleaves the thioester bond of acetyl-CoA.
¶Measured against target lepidopteran pests in an insect bioassay.
§Catalyzes the conversion of pyruvate to acetolactate.
||Catalyzes the hydrolysis of β-D-glucuronides to D-glucuronic acid and the aglycone.

are poorly accessible in uncooked material (van Boekel et al., 2010). Similar denaturation of proteins also occurs during processing of maize, which exposes the grain to high heat during cooking and can also involve the use of harsh conditions such as exposure to alkaline conditions during nixtamalization (a process in which grain is soaked and cooked in an alkaline solution) to produce maize tortillas, chips, and comparable foods (Hammond & Jez, 2011).

Depending on the desired outcome of a processing method, changes in conditions can be used to manipulate the physical properties of a protein. For example, precipitation is often used to separate proteins from lipids and/or sugars. Changes in the physical properties of a protein can reduce its solubility, lead to either aggregation or precipitation, or cause a multimeric protein to dissociate into monomers, thus resulting in loss of function (Meade et al., 2005; Schultz & Liebman, 2002; van Boekel et al., 2010). Cooking proteins can often aid their digestion in the gastrointestinal tract because proteases (e.g. pepsin and trypsin) are able to access proteolytically susceptible cleavage sites of the random-coiled primary and secondary structures of a denatured protein more quickly and efficiently compared with the same protein in its native tertiary conformation (Herman et al., 2006).

Testing for denaturation by heat treatment

Since cooking is one of the most common processing methods applied to foods, assessing the susceptibility to heat treatment of proteins introduced into GM crops can contribute useful information to the overall weight-of-evidence assessment of the introduced protein. The potential for a protein to be denatured by heat treatment can be assessed *in vitro* by heating it in aqueous solution at temperatures ranging from 25 to 100 °C for 15–30 minutes, conditions commonly found in the kitchen and in the processing of maize and soy into human food products (Hammond & Jez, 2011). For example, a protocol for measuring the heat stability of proteins has been proposed by the Indian Department of Biotechnology (DBT, 2008). Following heat treatment, the protein solution is returned to room temperature and the activity of the protein is measured. For enzymes, this involves a functional assay. For insect-control proteins, a bioassay that measures insect survival and growth can be used. It is important to assess functional activity at room temperature to rule out the possibly that the protein might renature when cooled, which has been reported for a limited number of proteins (Albillos et al., 2009).

Enzymologists traditionally use similar *in vitro* methods to determine the optimum temperature for enzyme function, as well as the temperature at which an enzyme denatures and loses functional activity (Kristjánsson & Kinsella, 1991). For proteins lacking functional assays, biophysical methods such as protein fluorescence, circular dichroism spectroscopy, and nuclear magnetic resonance spectroscopy can be used to evaluate the degree of denaturation and/or unfolding of protein structure (Cantor & Schimmel, 1980; Greenfield et al., 2001; Rehm et al., 2002). Activity assays and analytical methods can also be applied to examine the effects of pH, solvents, or other treatments on protein structure and function.

Several proteins introduced into GM crops have been subjected to *in vitro* tests to measure heat stability. A few studies have also assessed the impact of processing on the functional activity of introduced proteins (Tables 3 and 4). Results from these studies indicate that the tested proteins are effectively degraded when exposed to temperatures and processes similar to those used to prepare food fractions from maize and soy. The impact of food processing on the functional activity of introduced proteins may be relevant for other processed crops that are cooked before consumption, such as wheat and rice. It would not apply to food crops that are not processed and are consumed raw, such as certain fruits and vegetables.

- Protein denaturation during processing of crops such as maize and soy into foods also creates technical challenges for the detection of proteins introduced into GM crops. Detection methods for identifying foods derived from GM crops are mandated in many countries for labeling purposes. These methods often employ antibodies that bind to epitopes on the introduced protein (Codex, 2010; Grothaus et al., 2006; ILSI, 2007). Denaturation of

Table 4. Impact of food processing on functional activity of introduced proteins and food processing enzymes.

Protein	Food processing conditions	Function	Results	Reference
Malt α-amylase	Bake 68–83 °C; 4 min	Enzyme	None detectable	Pyler & Gorton (2009)
β-amylase	Bake 57–72 °C; 2 min	Enzyme	None detectable	Pyler & Gorton (2009)
CP4 EPSPS	Toasted soy meal	Enzyme*	None detectable	Padgette et al. (unpublished results)
CP4 EPSPS	Soy protein isolate	Enzyme*	None detectable	Padgette et al. (unpublished results)
CP4 EPSPS	Soy protein concentrate	Enzyme*	None detectable	Padgette et al. (unpublished results)

*Catalyzes the conversion of phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate.

proteins often makes immunological detection in final food products difficult (de Luis et al., 2009; Grothaus et al., 2006; Margarit et al., 2006). Moreover, protein aggregation during food processing can also reduce the effectiveness of protein extraction from the food matrix (Codex, 2010; de Luis et al., 2009; Grothaus et al., 2006; Margarit et al., 2006; Terry et al., 2002; Thomas et al., 2007). Immunological recognition in immunoassays such as ELISAs or lateral-flow test strips can depend on the epitope adopting a defined three-dimensional structure, which is lost after processing. However, for allergenic proteins, linear IgE binding epitopes, which are based on sequence rather than structure, may be intact after denaturation (Thomas et al., 2007). In addition to immunoassays based on antigen–antibody interactions and PCR-based methods to detect transcripts encoding an introduced protein, mass spectrometry has often been successfully applied to detect specific proteins even in several complex food matrices (Heick et al., 2011).

It is possible to develop antibodies to epitopes on a denatured protein as it exists in the processed food. These antibodies can be used for detection purposes if the test is validated and fit for the purpose (Grothaus et al., 2006). This has been applied to detect certain denatured introduced proteins in processed soy meal and food and feed ingredients derived from maize grain. However, because of the technical challenges inherent in the detection of denatured introduced proteins in processed food, protein-based immunologic detection methods are mostly applied to testing crops that are minimally processed for food or feed (Bogani et al., 2008; de Luis et al., 2009). For example, the Cry1Ab protein, which was introduced into maize to protect against caterpillars that bore into crop tissues, can be readily detected in grain and plant tissues, but it has not been detected in processed food products (de Luis et al., 2009; Margarit et al., 2006). Therefore, monitoring for GM products in processed food and feed relies more routinely on DNA-based detection methods (Alderborn et al., 2010). When a trace level of Cry9C DNA was detected in taco chips (CDC, 2001), Cry9C protein levels were also tested. Cry9C (as either the intact protein or fragments that retained binding epitope) was detected in maize bread, muffins, and polenta at levels that were 13%, 5%, and 3%, respectively, of that found in whole grain (de Luis et al., 2009). In highly processed foods such as maize tortillas, puffs, or flakes, <0.2% or in some cases, no protein was detected, even though this protein is more heat stable than Cry1Ab protein (de Luis et al., 2009).

Overall, these scientific considerations support the conclusion that risk assessors should consider the impact of food processing on the levels of the introduced protein, otherwise they most likely will over-estimate potential dietary exposure. As stated by EFSA,

> . . . food products are often processed into ingredients and/or incorporated in formulated processed food products, where the new protein and/or the novel secondary gene product attrition will occur. This may result in significant reduction in the theoretical maximum daily intake (TMDI) of the novel gene product, resulting in over-estimated exposure levels and even larger margins of safety for man. (EFSA, 2008)

If a protein is rapidly denatured by *in vitro* heat stability assays and does not renature and regain activity, and if there is evidence that it can be rapidly degraded by proteases, then this should be sufficient evidence to conclude that a 28-day repeat-dose animal toxicology study would provide no additional useful information for risk assessment. Once again, this must be judged on a case-by-case basis, as there are certain food crops that are either not processed or minimally cooked.

Application of threshold of toxicological concern for proteins

Application of the threshold of toxicological concern (TTC) concept is recommended for ranking and prioritizing risks from exposure to chemicals for which there is a lack of toxicity data, but which may be present in food at low concentrations and for which exposure analysis can provide sound intake estimates (Kroes et al., 2004). This tool could also be applied to ranking the risks from dietary exposure to functionally active introduced proteins present at low levels in grain and seed, or at even lower levels in processed food fractions. Until now, TTC risk assessment models have excluded proteins because the threshold for exposure to food allergens necessary for sensitization and/or elicitation of a response is in most cases unknown. Recently, population threshold doses for elicitation of allergic reactions to peanut allergens were proposed based on the review of clinical records for 286 peanut-allergic patients in France (Taylor et al., 2010). The threshold dose was low for the most sensitive population (approximately 2–4 mg/person) and considerably higher for those whose allergic reactions were less severe. Thus, it may be possible in the future to establish

population elicitation thresholds for at least some allergens in food. It is unlikely that the sensitization thresholds will be known but it is hypothesized that they are higher than the elicitation thresholds.

With respect to toxicity, the final proposed decision tree omitted proteins because the databases used to derive TTC values do not include data on proteins (Kroes et al., 2004). However, since the purpose of this review is to provide guidance on the safety assessment of proteins that do not fit the profile of allergens, the TTC concept might be applicable as a risk assessment tool for proteins in GM crops (Delaney et al., 2008a).

The application of TTC proposed here requires that the introduced protein does not share any amino acid sequence similarities with known allergenic proteins (based on bioinformatics comparisons). Since allergenic proteins tend to be resistant to digestion by proteases, the introduced protein should have the potential to be degraded by proteases, as confirmed when incubated *in vitro* with digestive enzymes, or degraded when processed (e.g. heat treatment) as some allergens are heat stable (Privalle et al., 2011). Known allergens are also are frequently present in abundant amounts (many 1000s of ppm) in food crops (Thomas et al., 2009) whereas introduced proteins are normally present at low levels (0.5–400 ppm) in GM crops (Hammond & Cockburn, 2008). In addition, the levels of structurally intact and functionally active introduced proteins may be further reduced by orders of magnitude depending on how the food is processed (see the section on testing for denaturation by heat treatment).

Hammond & Cockburn (2008) illustrated how the TTC concept might be applied to proteins in GM crops that do not fit the profile of allergens. As an example, they used the CP4 EPSPS protein (Padgette et al., 1996), which imparts tolerance to glyphosate herbicide and is structurally and functionally similar to endogenous EPSPS proteins in plants, but when first introduced into food and feed, lacked a HOSU in food because it was derived from *Agrobacterium* sp. CP4. TTC levels were estimated for acute and chronic exposures based on available toxicology data for a variety of different proteins, many of which are enzymes used in food processing. TTC levels were determined following adjustments to correct for purity of enzymes in fermentation batches tested in animals and applying a 100-fold safety margin below the no-observed-adverse-effect levels (NOAELs) in animal toxicology studies. For acute exposure, the TTC was calculated to be 17.9 mg/kg BW, or 1074 mg/person/day (assuming a 60-kg BW). For chronic exposure, the TTC was 2.49 mg/kg BW, or 149 mg/person/day (Hammond & Cockburn, 2008). Comparison of these threshold levels to actual dietary exposure levels assumes a ''worst-case'' scenario in which there is no loss of the introduced protein when the GM crop is processed into food.

CP4 EPSPS is present in grain from glyphosate-tolerant maize at levels of approximately 10–14 ppm (Hammond & Cockburn, 2008). Assuming a chronic human intake of maize grain (endosperm fraction) in the United States of 270 mg/kg BW/day (Hammond & Cockburn, 2008), the intake of CP4 EPSPS would be 0.004 mg/kg BW/day. This is based on the conservative assumption that all of the maize consumed by humans is glyphosate-tolerant and that CP4 EPSPS is not denatured during food processing and is resistant to proteases in the digestive system. This estimated dietary exposure level is approximately 600-fold lower than the TTC level for chronic exposure of 2.49 mg/kg BW/day. Since the CP4 EPSPS protein is readily denatured by normal processing conditions (Tables 3 and 4), the actual dietary exposures are likely reduced by at least 100-fold (e.g. 0.00004 mg/kg BW/day), or ~60 000-fold lower than the chronic exposure TTC level. In comparison to the elicitation threshold peanut allergen levels in the most sensitive cases mentioned above (Taylor et al., 2010), the dietary exposure to CP4 EPSPS is 50 000-fold lower.

As illustrated by this example, the predicted low level of dietary exposure to structurally intact and functionally active CP4 EPSPS protein, and the close structural and functional relatedness to EPSPS proteins with a HOSU are strong indicators that CP4 EPSPS poses minimal toxicological concern. There is accordingly no scientific justification for further toxicological testing in animals based on the weight of evidence.

Potential interactions between introduced proteins in combined-trait crops

GM crops containing more than one introduced protein (i.e. combined-trait products) have been developed using traditional plant breeding methods. Varieties containing introduced traits can be crossed to produce a strain with combined traits. Alternatively, transformation vectors designed to express more than one introduced protein (i.e. molecular stacks) can be introduced into a plant variety. Questions have been raised about the potential for interactions of introduced proteins in combined-trait crops.

> ''In the case of GM plants obtained through conventional breeding of parental GM lines (stacked events), possible interactions between the expressed proteins, new metabolites and original plant constituents should be assessed. If the potential for adverse interactions is identified, feeding trials with the GM food/feed are required. (EFSA, 2009a)''

Two forthcoming ILSI International Food Biotechnology Committee (IFBiC) publications examine genome stability and protein interactions in combined-trait crops in more detail (Steiner et al., 2013; Weber et al., 2012), but a few general concepts are described below. Some of these concepts are specific to combined-trait crops produced by crossing single-trait GM crops, whereas others are applicable to any combination of newly introduced proteins.

An assessment of the potential for interactions between introduced proteins would include the following considerations:

(1) *Biochemical function of individual newly expressed proteins*. As described in previous sections, the biochemical function of each introduced protein in single-trait GM crops will have been characterized as part of the food safety assessment to confirm it is safe to consume by humans and farm animals.
(2) *Molecular function of the combined proteins.* The biochemical function of insect-control proteins derived

from *Bt*-derived commercial microbial pesticides is provided below as a case study. Combinations of different Cry proteins are being introduced into food and feed crops to expand the number of insect pests that can be controlled and to reduce the potential for the development of insect resistance to the Cry insect-control proteins (Bates et al., 2005).

The biochemical function of Cry insect-control proteins has been well described in a number of published reviews (Betz et al., 2000; Federici & Siegel, 2008; IPCS, 1999; McClintock et al., 1995; OECD, 2007; Siegel, 2001). In the target insect gut, the activated Cry proteins bind to high-affinity receptors present on cells lining the intestinal tract. The activated/bound Cry proteins insert themselves into the insect intestinal cell membrane, forming pores that lead to cell lysis; the insect stops eating and subsequently dies. Phylogenetically unrelated non-target organisms, including mammals, lack the high-affinity Cry protein receptors found on the membranes of cells lining the gastrointestinal tract of the target insect species (Griffitts et al., 2005; Hofmann et al., 1988a, 1988b; Lambert et al., 1996; Mendelsohn et al., 2003; Noteborn et al., 1995; OECD, 2007; Sacchi et al., 1986; Van Rie et al., 1989, 1990). Target larval insect pests have a glycosylating enzyme, BL2 that creates the specific sugar residues on the glycolipid microvillar receptors recognized by Cry proteins. Vertebrates lack this enzyme, which explains in part their lack of sensitivity to Cry proteins (Federici & Siegel, 2008).

Dietary exposure to either an individual Cry protein or a mixture of Cry proteins by non-target mammals and avian species has not been associated with additive or synergistic toxicity, even in cases where additive or synergistic toxicity was observed in the target organisms (i.e. insects). For example, Cry34Ab1 and Cry35Ab1 act synergistically in corn rootworm pests to achieve optimal levels of insect control; however, when tested in non-target species such as poultry or mammals (mice), no evidence of toxicity was observed (Juberg et al., 2009; McNaughton et al., 2007). Various *Bt* microbial pesticide formulations that contain different mixtures of Cry proteins have been fed to animals to evaluate their safety. There was no evidence of toxicity in these studies with individual Cry proteins or mixtures of Cry proteins (Betz et al., 2000; Brake et al., 2003; Federici & Siegel, 2008; Flachowsky et al., 2005; IPCS, 1999; McClintock et al., 1995; McNaughton et al., 2007; OECD, 2007; Scheideler et al., 2008; Siegel, 2001; Taylor et al., 2005, 2007). These results are similar to what is observed in the safety assessment of mixtures of small molecules that have been individually assessed for safety: toxic (or pharmacological, metabolic, and pharmacokinetic) effects are not observed when the individual components of the mixture are administered at doses well below their toxicity thresholds (Groten et al., 1997; Seed et al., 1995).

(3) *Potential for interaction between introduced enzymes with different catalytic functions*. The modes of action of enzymes introduced into plants that impart tolerance to various classes of topically applied herbicides are different for each enzyme. For example, in plants, the only known physiological target of the herbicide glyphosate is EPSPS. Tolerance is achieved by introduction of a modified EPSPS enzyme that is not inhibited by glyphosate (Herouet-Guicheney et al., 2009; Padgette et al., 1996). Another enzyme that imparts herbicide tolerance is PAT (phosphinothricin-N-acetyl transferase), which adds an acetyl group to the substrate L-phosphinothricin, the active isomer of the herbicide glufosinate–ammonium (Hérouet et al., 2005). Acetylation results in loss of herbicidal activity (Delaney et al., 2008a). Since the substrates of both EPSPS and PAT are chemically different, it is biochemically improbable that these enzymes would interact synergistically when present together in a combined-trait crop.

Based on the assessment of potential interactions as outlined in points 1, 2 and 3 above, confirmation of the safety of the introduced proteins in single-event GM crops should provide an adequate basis for evaluation of the food and feed safety when these proteins are combined in stacked-trait GM crops. An independent Scientific Advisory Panel of experts recently convened by the US EPA concluded that if insect-control proteins are proved to be safe individually, their effects would not need to be tested in combination for a human health assessment (US EPA, 2009). They also stated that ''sensitive species are also more likely to detect synergism than a species insensitive to the pesticidal substance(s)'' (US EPA, 2009). Therefore, confirming the lack of interaction (i.e. the proteins act independently) in insect bioassays provides a mechanism to link the existing body of evidence on non-target toxicity of the individual proteins in the previously approved single-event products. Since the newly produced proteins have been shown to act independently (no interaction), then they can be assessed independently for their food/feed safety.

Another consideration indicating that synergistic effects would be highly unlikely can be drawn from experiences assessing the biological effects of mixtures of chemicals. It is known that synergistic biological effects from chemical mixtures are unlikely to occur as long as each of the individual constituents are present at levels well below those that produce no measurable biological effects in animals (Groten et al., 1997; Seed et al., 1995). In dietary risk assessments provided to regulatory agencies, it is routinely demonstrated that the potential human dietary intake of various introduced proteins is orders of magnitude below the highest dosages administered to rodents in acute high-dose hazard toxicity studies that produced no adverse effects (Betz et al., 2000; Hammond & Cockburn, 2008). These dietary risk assessments were also based on worst-case assumptions using estimated 97.5% consumption levels (maize and soy) and assuming no loss (denaturation) of proteins during normal food processing. Analogous to the

situation with chemical mixtures, no adverse additive or synergistic effects would be predicted for a combination of proteins introduced into a transgenic crop when neither one was shown to be toxic, even when administered at doses higher than in the human diet.

Further evidence for the absence of interactions of introduced proteins can be derived from 90-day rat feeding studies with combined-trait products containing Cry insect-control proteins and enzymes that impart herbicide tolerance. Assuming a ''worst-case'' scenario in which humans only consume grain from the maize varieties tested, rats were fed 22–27 g/kg BW/day maize grain, approximately 100 times what humans might consume (based on the European and North American maize consumption Dietary Exposure Evaluation Model [DEEM] UK and DEEM US databases, Exponent, Inc; WHO GEMS, 2006), and no treatment-related adverse effects were reported. These studies have been reviewed by the EFSA and results have been published on their website for the following event combinations:

MON 810 × MON 863 (Cry1Ab × Cry3Bb1) (EFSA, 2005a)
MON 863 × NK603 (Cry3Bb1 × CP4 EPSPS) (EFSA, 2005b)
MON 810 × MON 863 × NK603 (Cry1Ab × Cry3Bb1 × CP4 EPSPS) (EFSA, 2005c)
DAS 1507 (Cry1F × PAT) (EFSA, 2005d)
DAS 59122–7 (Cry34Ab1/Cry35Ab1 × PAT) (EFSA, 2007a)
DAS 15Ø71 × DAS-59122 (Cry1F × Cry34Ab1/Cry35Ab1 × PAT) (EFSA, 2009b)

In a recent EFSA opinion on a combined-trait maize product (59122 × 1507 × NK603) expressing Cry34Ab1, Cry35Ab1, Cry1F, PAT, CP4 EPSPS, and CP4 EPSPS L214P proteins, the following conclusion was reached: ''As the composition of maize 59122 × 1507 × NK603 is comparable with that of non-GM maize varieties and the single events and also no indication for interaction between the newly expressed proteins was found, the GMO Panel is of the opinion that no additional animal safety studies are required'' (EFSA, 2009c). The same conclusions were provided for another combined-trait maize product, MON89034 ×MON88017, which contains Cry1A.105, Cry2Ab2, Cry3Bb1, and CP4 EPSPS (EFSA, 2010b).

The weight of evidence for existing products containing insect control proteins and enzymes that impart herbicide tolerance points to a very low potential for interactions in plants with combined traits. In the future, other kinds of proteins will be introduced into crops, such as transcription factors and enzymes that modify metabolic pathways, and similar questions about potential interactions will be raised. Understanding the substrates and catalytic products for enzymes will help to predict whether interactions are possible, and if needed, targeted compositional analysis of metabolites could be undertaken if interactions between enzymes are suspected. There may be a low probability that introduced transcription factors, metabolic enzymes, and insect control proteins may interact based on their differing modes of action. However, traditional tools that monitor for unintended changes such as assessing agronomic characteristics of new crop varieties (e.g. detecting ''off-types'' as is done for traditional breeding), compositional analysis, and the conduct of hypothesis-based animal feeding studies are well constructed to assess whether protein–protein interactions have occurred (Parrott et al., 2010).

Lack of scientific justification to test proteins for genotoxic potential

According to the International Conference on Harmonization (ICH) guidelines (www.ich.org) for pharmaceuticals, routine genotoxicity testing of protein is not considered necessary to confirm safety even when the protein drugs is administered parenterally, bypassing the digestive tract barriers discussed in previously in the section on current protein safety assessment considerations. Chao & Krewski (2008) also proposed that safety testing requirements for proteins introduced into GM crops do not need to include genotoxic testing, which is consistent with the scientific recommendations from Delaney et al. (2008a). Chao and Krewski added that tiered testing should be tailored to the expected exposure level and structural comparability to compounds with known toxicity profiles.

Over the years, mutagenicity studies have been carried out with many enzyme preparations produced by fermentation, based on early concerns that the fermentation organisms might potentially produce genotoxic contaminants such as mycotoxins (Hammond & Cockburn, 2008). The US Enzyme Technical Association (ETA) reported that, as of 1999, 102 bacterial mutagenesis tests and 63 mammalian chromosomal aberration mutagenesis tests had been carried out with enzyme preparations from both conventional and GM microorganisms. The vast majority of these tests found no evidence of mutagenic activity (Pariza & Johnson, 2001). For example, in the histidine reversion bacterial mutagenicity tests (Ames test), 95 out of 102 assays showed no evidence of mutagenicity. The positive results in the remaining seven tests were attributed to the presence of free histidine in the tested enzyme preparation, which gave false-positive results in the histidine reversion bacterial mutagenicity test (Pariza & Johnson, 2001). Given the absence of mycotoxin contamination in commercial strains of fermentation organisms, it was concluded that routine testing enzymes for potential genotoxicity was not necessary for safety evaluation (Pariza & Johnson, 2001).

Summary

The following characteristics would provide a reliable weight of evidence to conclude that toxicological testing of an introduced protein without a HOSU is not needed:

(1) The introduced protein is structurally/functionally similar to a family of related proteins that have a HOSU in food, based on bioinformatics analysis and literature review.
(2) The biochemical function of the introduced protein has been adequately characterized.
(3) The introduced protein is readily digested when incubated *in vitro* with simulated digestive fluids.
(4) The introduced protein is susceptible to inactivation and/or denaturation during normal processing

(e.g. cooking) of foods produced from that crop, based on either *in vitro* heat stability studies or food processing studies.

The following characteristics would indicate that an introduced protein without a HOSU would require toxicology testing:

(1) The introduced protein is shown by bioinformatics analysis to be structurally/functionally related to proteins that are known to be toxic to mammals.
(2) The biochemical function of the introduced protein is not sufficiently characterized to predict risks for mammals.
(3) There is potential dietary exposure to the functionally active protein because it is not degraded by digestive fluids when tested *in vitro*.

Acknowledgements

The authors and Task Force members thank the following individuals for participating in the review process and for providing many constructive comments and suggestions: Bruce Chassy, University of Illinois, Urbana-Champaign; Andrew Cockburn, University of Newcastle and Toxico-Logical Consulting Ltd.; Johanna Dwyer, Tufts-New England Medical Center; Karin Hoffman-Sommergruber, Medical University of Vienna; Ib Knudsen, Danish Institute for Food and Veterinary Research; Richard Lane, PepsiCo; Drew Lichtenstein, Novus International; Mark Martens, FORMAC Pharmaceuticals N.V.; William Price, US Food and Drug Administration Center for Veterinary Medicine (retired); Joseph Scimeca, Cargill; Dae Yeul Son, Department of Herbal Foodceutical Science, Daegu Haany University, Daegu, Korea; John Thomas, University of Texas and Indiana School of Medicine; Rick Tinsworth, Exponent; Hai Bin Xu, Chinese Center for Disease Control and Prevention; and Hiroshi Yoshikura, Japanese Ministry of Health Labour and Welfare.

The Task Force also wishes to thank Ms. Kate Walker, ILSI Scientific Program Manager, for her efforts in seeing this project to completion. The authors acknowledge the assistance of Virginia M. Peschke (Oakside Editorial Services) and Christina West (Editorial Services) in the preparation of this paper.

Declaration of interest

The employment affiliation of the authors is shown on the cover page. The paper was prepared during the normal course of the authors' employment. B. Hammond was employed at the time of authorship by Monsanto Company, a sustainable agriculture company that does research on and markets genetically engineered crops. J.M. Jez participated in the Task Force as an employee of his academic institution, and received an honorarium in recognition of that participation. J. Kough is employed by the United States Environmental Protection Agency; the contents of this paper reflect the thoughts and opinions of the author and do not represent an official policy statement of the United States Environmental Protection Agency or other Federal government agencies. Any mention of a product does not constitute an endorsement by the United States Federal government. C. Herouet-Guicheney is employed by Bayer SAS, a company that does research on and markets genetically engineered crops. Preparation of this manuscript was financially supported by members of the ILSI International Food Biotechnology Committee: BASF Corporation; Bayer CropScience; Dow AgroSciences; Monsanto Company; Pioneer Hi-Bred, a DuPont business; and Syngenta Biotechnology, Inc. This paper has been reviewed by individuals internationally recognized for their diverse perspectives and technical expertise. However, it must be emphasized that the content of this document is the authors' responsibility and not the reviewers', and it does not represent an endorsement by the reviewers' institutions. While the authors carefully considered the views and comments of the various reviewers and the sponsors, the final analyses, interpretations and conclusions drawn are exclusively those of the authors.

The publication of this supplement was funded by members of a task force of the ILSI International Food Biotechnology Committee: BASF, Bayer CropScience, Dow AgroSciences, Monsanto Company, DuPont, and Syngenta.

Literature Search

The literature was reviewed in a comprehensive manner to identify relevant publications addressing the safety assessment of proteins introduced into GM crops. The U.S. National Library of Medicine, National Institutes of Health online search service ''PubMed'' (www.ncbi.nlm.nih.gov/pubmed) and the Web of Knowledge (wokinfo.com) were searched for relevant publications on the safety assessment of proteins introduced into GM crops. Where citations were identified, their reference sections were reviewed to determine if other relevant publications had been overlooked. Only papers written, or translated into English were considered in this review. Annual Monitoring reports are required to be submitted by agricultural biotech companies to the European Commission for all GM crop products authorized for import or cultivation in the European Union. The Annual Monitoring reports include an analysis of peer reviewed, up-to-date relevant publications. There are several lists of categories which must be included in the searches including toxicology/allergenicity studies on the proteins introduced into GM crops. The searches utilize the Web of Science database that surveys the contents of over 12 000 top tier international and regional journals in every area of the natural sciences, social sciences, and arts and sciences.

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