11 The Safety Assessment of Proteins Introduced into Crops Developed through Agricultural Biotechnology: *A Consolidated Approach to Meet Current and Future Needs*

Bruce Hammond and Andrew Cockburn

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11.1 INTRODUCTION

The concluding chapter of this book distills information from previous chapters to consolidate an overall risk and safety assessment strategy appropriate for proteins introduced into biotechnology-derived food and feed crops. The strategy builds on the information from safety assessments of proteins used in food production (enzymes and animal somatotropins), proteins used as therapeutic agents, proteins that are components of microbial pesticides applied to agricultural crops, and proteins introduced into biotechnology-derived crops. The safety assessment scheme adopts the well-established dietary exposure procedures used for low-molecular-weight chemicals added to foods, but differs fundamentally in some respects regarding the overall hazard identification. These differences are a consequence of unique structural, functional, and biochemical properties of proteins that differ in many respects from low-molecular-weight chemicals used as food additives or pesticides. These differences have a profound impact on the hazard potential of proteins screened for introduction into food crops, which is generally less than that of many low-molecular-weight chemicals that enter the human food chain. There are, of course, proteins known to be toxic to humans or pharmacologically active in man, but they have intentionally not been selected for introduction into food and feed crops.

This chapter will also look into the future to explore the anticipated use of proteins to develop new and improved food and feed crops. The proposed risk assessment strategy is considered to be relevant to both existing and new proteins that will ensure that future improved food and feed crop varieties are safe for consumption. Potential hazards that might result from an unexpected or unintended change to the plant from the introduction of the protein are not the focus of this chapter but are nevertheless addressed in subsequent discussions.

11.2 BIOCHEMICAL DIFFERENCES BETWEEN PROTEINS AND LOW-MOLECULAR-WEIGHT CHEMICALS: IMPACT ON SAFETY ASSESSMENT OF PROTEINS

As pointed out in the first chapter in the book, there are some fundamental structural and biochemical differences between proteins and low-molecular-weight chemicals. Examples are as follows:

Low-Molecular-Weight Chemicals

1. Chemical structures vary considerably and may be novel (not found in nature) or related to biochemicals found in nature. For example, the chemical structure of the insecticide chloropyriphos would be considered novel, whereas the herbicide glyphosate is structurally related to the amino acid glycine. Examples of food additives with novel structure could include the artificial sweetener saccharin, whereas another artificial sweetener, aspartame, is structurally related to the amino acid dipeptide aspartate-phenylalanine.
2. Low-molecular-weight chemical food additives and contaminants have molecular weights generally ranging from approximately 200–800 MW.
3. Absorption from the gastrointestinal (GI) tract varies depending on the structural properties of the low-molecular-weight chemical. For example, lipid solubility

can significantly enhance systemic absorption from the GI tract. Approximately 47% to 69% of an oral dose of two different lipophilic low-molecular-weight chemical insecticides were absorbed intact from the GI tract of the rat within an hour of oral dosing.[1] Other more polar low-molecular-weight chemicals that are ionized at the pH of the intestinal tract or are more water-soluble are less likely to be absorbed systemically, such as glyphosate (~30% absorbed).[2] Plants also metabolize foliar- and soil-applied pesticides to more polar derivatives that are much less likely to be absorbed systemically than the parent compound. A case in point is the herbicide acetochlor, which is absorbed systemically at > 80% when fed to rats. Its two major plant metabolites, t-ethane sulfonic acid metabolite and t-oxanilic acid metabolite, which are more polar than acetochlor, are less readily absorbed, up to 12% and 39%, respectively.[3]

Proteins

1. Virtually all proteins are polymers composed of different combinations and permutations of the same 20 common amino acid monomers. There are millions of proteins of diverse structure and function found in nature and they are made up of some or all of these 20 amino acids. Amino acids per se have low oral toxicity and are essential to human life and nutrition (Chapter 1).
2. Molecular weight (MW) of proteins can vary from 10,000 (~50 amino acids) to more than a million (> 3000 amino acids, see Chapter 1). Proteins are orders of magnitude larger than low-molecular-weight chemicals, which greatly reduces their potential systemic absorption across GI cell membranes.
3. Ingested proteins are subjected to degradation to polypeptides, peptides, and amino acids by the combined action of low pH and pepsin in the stomach and assorted proteases secreted into the intestinal tract. Loss of quaternary and tertiary structure of the protein during digestion results in loss of structural integrity and usually loss of biochemical function.
4. Proteins produced in mammalian cells can have important physiological and pharmacologic effects when injected intravenously for therapeutic applications, but these effects are not generally apparent when these proteins are ingested due to rapid denaturation and degradation within the GI tract (Chapters 6, 10).

As a consequence of the fundamental structural and size differences between proteins and low-molecular-weight chemicals, the probability for systemic absorption of the majority of intact proteins from the GI tract is exceedingly low when compared to low-molecular-weight chemicals. The need for toxicological assessment of low-molecular-weight chemicals is largely driven by observations of pharmacological or toxic responses in oral dosing studies.

As will be shown later, the vast majority of proteins involved in food use that have been selected and subjected to safety testing do not cause systemic toxicity. There is a long history of safe consumption of plant and animal proteins in the diet. As discussed above, dietary proteins are generally degraded and thus poorly absorbed intact from the GI tract (see discussion below); hence, there is very low systemic exposure. Thus, the safety evaluation of proteins intentionally selected and subsequently introduced into food generally requires less toxicology testing than that carried out for low-molecular-weight chemicals in food or feed where systemic absorption of biologically active parent compound or metabolite(s) generally occurs with the potential for end-organ toxicity prior to and or during excretion/elimination.

11.3 ABSORPTION OF PROTEINS FROM THE GI TRACT

A study of the systemic absorption of peptides (3 to 51 amino acids in length) found that peptides greater than 10 amino acids in length were poorly absorbed intact from the GI tract.[4] Others have reported that gastric absorption is inversely related to the size of the molecule so that small molecules are more readily absorbed than large ones.[5] A number of animal feeding studies with biotechnology-derived crops have investigated the digestibility and potential systemic absorption of intact introduced proteins in various tissues and blood samples using sensitive immunological assays.[6–15] These published reports confirm that proteins, including those introduced into biotechnology-derived crops, are digested and have negligible oral bioavailability.

It is recognized that for proteins stable to digestion, minute quantities can be taken up intact by Peyers patches lining the GI tract, or may pass through intestinal cells via phagocytosis or permeation between epithelial cell junctions. An example is the egg allergen ovalbumin, which is stable to digestion in simulated gastric fluid for at least 60 minutes. Most common plant proteins, in contrast, are digestible in less than 15 seconds in simulated gastric fluid (SGF).[16] Egg ovalbumin was administered to rats as an oral bolus dose (50 mg/rat). Bolus dosing increases the potential for absorption due to administration of a concentrated solution straight into the stomach. As a result, higher peak blood levels are achieved compared to lower doses resulting from consumption of albumin as a component of food in the diet. Nevertheless, even after bolus dosing of the stable egg ovalbumin protein, only 0.007% to 0.008% of the administered dose was absorbed from the GI tract.[17]

Similar results were reported for other protein allergens that are also stable to digestion, such as the soybean allergen Gly m Bd 30 k, where only approximately 0.004% of a large bolus dose was absorbed.[18] There are also human studies reporting very low blood levels (generally less than 0.0001% of ingested protein) of stable food proteins such as ovalbumin, ovomucoid, and β-lactoglobulin after consumption of foods containing these proteins.[19–21] These proteins are all highly abundant allergenic proteins in foods that are comparatively stable to digestion.[16] For proteins that are not stable to digestion, the potential for systemic absorption of intact protein would be expected to be orders of magnitude lower than the very low levels of absorption for stable proteins alluded to earlier. This general lack of systemic bioavailability from the GI tract for intact proteins would minimize any potential for toxicity compared with single low-molecular-weight chemical substances following oral administration.

11.4 SUMMARY OF SAFETY ASSESSMENTS ON PROTEINS

As discussed earlier, the oral bioavailability of digestible proteins is negligible, thus their potential to exert systemic adverse effects, if such activity were to be characteristic, is also very low. As a consequence, there is not normally the scientific case to subject proteins screened for introduction into food and feed crops to the same extensive battery of safety tests required for low-molecular-weight chemicals that end up in food or feed. As discussed in preceding chapters, no systemic toxic effects have been identified in the many dietary toxicity studies that have been carried out with proteins of variable structure and function that are used in food production.

A list of acute and subchronic oral toxicity studies conducted with these proteins is presented in Tables 11.1 and 11.2. These tables list the "no-observed-adverse-effect-levels" (NOAELs) which, for all the proteins listed, represents the highest dosages that were tested. Many of these proteins are enzymes that have been produced by microbial fermentation and are used in food processing. It has been a regulatory requirement that these enzyme preparations be tested for potential acute and subchronic toxicity. As discussed in Chapter 5, this testing has not been undertaken to resolve questions about safety of the enzymes themselves. Rather, testing has been

TABLE 11.1
Summary of NOAELs in Acute High-Dose Studies with Different Proteins

Protein	Function	NOAEL[a,b]	Reference
Cry1Ab	Insect control	4000 mg/kg	22
Cry1A.105	Insect control	2072 mg/kg	23
Cry1Ac	Insect control	4200 mg/kg	22
Cry2Aa	Insect control	4011 mg/kg	22
Cry2Ab	Insect control	1450 mg/kg	22
Cry3A	Insect control	5220 mg/kg	22
Cry3Bb	Insect control	3780 mg/kg	22
Cry1F	Insect control	576 mg/kg	24
Cry34Ab1	Insect control	2700 mg/kg	25
Cry35Ab1	Insect control	1850 mg/kg	25
Vip3a	Insect control	3675 mg/kg	26
ACC deaminase	Enzyme	602 mg/kg	27
Alkaline cellulase	Enzyme	10,000 mg/kg	28
Dihydrodipicolinate-synthase (cDHDPS)	Enzyme	800 mg/kg	29
β-galactosidase	Enzyme	20,000 mg/kg	30
Enolpyruvyl-shikimate-3-phosphatesynthase (CP4-EPSPS)	Enzyme	572 mg/kg	31
β-glucanase	Enzyme	2000 mg/kg	32
Glutaminase	Enzyme	7500 mg/kg	33
Hexose oxidase	Enzyme	2000 mg/kg	34
Laccase	Enzyme	2700 mg/kg	35
Lactase	Enzyme	10,000 mg/kg	36
Lactose oxidase	Enzyme	900 mg/kg	37
Lipase	Enzyme	2000 mg/kg	38
Lipase	Enzyme	5000 mg/kg	39
Neomycin phosphotransferase	Enzyme	5000 mg/kg	40
Phosphinothricin acetyl transferase	Enzyme	2500 mg/kg	41
Phosphomannose isomerase	Enzyme	3030 mg/kg	42
Pullulanase	Enzyme	10,000 mg/kg	43
Xylanase	Enzyme	239 mg/kg	44
Xylanase	Enzyme	2000 mg/kg	45

[a] Highest dosage tested that caused no adverse effects.
[b] Actual delivered dosage may be lower based on the purity of the enzyme preparations tested.

TABLE 11.2
Summary of NOAELs in Subchronic Feeding Studies with Different Proteins

Protein	Function	Study	NOAEL[a]	Reference
Bovine somatotropin	Hormone	13 weeks	50 mg/kg	46
Dipel Bt microbial Cry protein mixture	Insect control	13 weeks	8400 mg/kg	22
Dipel Bt microbial Cry protein mixture	Insect control	2 years	8400 mg/kg	22
Teknar Bt microbial Cry protein mixture	Insect control	13 weeks	4000 mg/kg	22
Bt Berliner microbial Cry protein mixture	Insect control	5 days (human)	1000 mg/adult	22
Cry1Ab	Insect control	28 days	0.45 mg/kg/day	22
Amylase	Enzyme	90 days	17.5 mg/kg/day	47
Amylase	Enzyme	90 days	890 mg/kg	48
Amyloglucosidase	Enzyme	14 days	1640 mg/kg	49
Amino peptidase	Enzyme	90 days	2000 mg/kg	50
Arabinofuranosidase	Enzyme	14 days	103 mg/kg	49
Chymosin	Enzyme	90 days	1000 mg/kg	51
Chymosin	Enzyme	90 days	11.9 mg/kg	51
β-galactosidase	Enzyme	6 months (rat) 30 days (dog)	4000 mg/kg 1000 mg/kg	30
Glucanase	Enzyme	90 days	1258 mg/kg	52
Glutaminase	Enzyme	90 days 365 days	9000 mg/kg/day (yeast CK)1200 mg/kg/day (yeast CKD10)10,000 mg/kg/day (yeast TK) 13,000 mg/kg(yeast CK)	33
Hexose oxidase	Enzyme	90 days	5000 HOX units/kg	34
Laccase	Enzyme	90 days	1720 mg/kg	35
Lactase	Enzyme	28 days	1540 mg/kg	36
Lactose oxidase	Enzyme	90 days	900 mg/kg	37
Lipase	Enzyme	90 days	658 mg/kg	39
Lipase	Enzyme	90 days	1680 mg/kg	38
Lipase G	Enzyme	90 days	1516 mg/kg	53
Lipase AY	Enzyme	90 days	2500 mg/kg	54
Pectin methylesterase	Enzyme	14 days	133 mg/kg	49
Phosphodiesterase	Enzyme	28 days	165 mg/kg	55
Phospholipase-A	Enzyme	90 days	1350 mg/kg	49
Phytase	Enzyme	90 days	1260 mg/kg	49
Pullulanase	Enzyme	28 days	5000 mg/kg	56
Tannase	Enzyme	91 days	660 mg/kg	57
Xylanase	Enzyme	90 days	1850 mg/kg	49

TABLE 11.2 (CONTINUED)
Summary of NOAELs in Subchronic Feeding Studies with Different Proteins

Protein	Function	Study	NOAEL[a]	Reference
Xylanase	Enzyme	90 days	4095 mg/kg	49
Lactoferrin (human)	Iron transport	90 days	2000 mg/kg/d	58
Lactoferrin (bovine)	Iron transport	90 days	2000 mg/kg/d	59
Silkworm pupae protein	Not defined	30 days	1500 mg/kg/d	60
Thaumatins	Sweetner	90 days	2696 mg/kg/d	61
Ice-structuring protein	Cryo preservation	90 days	580 mg/kg/d	62

[a] In all cases, the NOAELs were the highest dose tested.

considered necessary to confirm the absence of possible toxic contaminants (mycotoxins, bacterial toxins) from the fermentation medium that might be present in the enzyme preparation. Such testing, also applied to protein based vaccines, is also known as "freedom from abnormal toxicity" (FAT) testing.

These studies confirm the absence of oral toxicity even when the protein preparations were administered at very high dosage levels. The studies listed in Tables 11.1 and 11.2 have been published, but there are many others that have been completed and have not been published. According to a recent review,[63] as of 2001 almost 800 toxicity tests have been conducted on approximately 180 enzymes by member companies of the European Association of Manufacturers and Formulators of Enzyme Products (AMFEP). According to AMFEP, these studies raised no issues of toxicological concern.[63] Given the history of safe use for certain microorganisms to make enzyme preparations, it has been proposed that routine toxicology testing of highly characterized specific enzyme preparations prepared from these microorganisms is no longer scientifically justified and is inhumane because of its unnecessary use of laboratory animals for toxicology testing.[63]

Although the vast majority of subchronic feeding studies with food enzymes have consistently found no evidence of treatment-related adverse effects in test animals, a couple of studies reported local irritation to the stomach caused by feeding high levels of protease enzymes to rats. Such effects might be anticipated due to proteolytic effects of the enzymes on the stomach mucosa at high exposures.[64] A few other subchronic feeding studies reported adverse effects usually limited to the highest dosages tested, and at lower dosages no adverse effects were reported. Since lower dosages were still many times higher than potential human dietary exposures, a very large safety margin existed for the use of these enzymes in food production. The adverse effects were not attributed to the enzymes themselves, but rather to other constituents in the enzyme preparation. For example, enzyme preparations with high levels of ash (salts and minerals) from the fermentation medium produced nephrocalcinosis[43] or increased water consumption in rats.[64] Other effects, such as slight anemia[32] or reduced urine pH, found in other studies were either not correlated with any microscopic evidence of pathologic changes or were not reproducible

(salivary gland enlargement when rats were fed the enzyme in the diet but not by stomach tube).[65] At a recent (2005) European Toxicology Forum conference on the safety assessment of food enzymes, a European regulator was asked whether he had ever seen evidence of adverse effects in submitted subchronic toxicology studies that were directly attributable to the enzyme fed to rats.[66] He responded that in his many years of experience, he had not.

No evidence of pre-neoplastic microscopic changes have been reported in the tissues of laboratory animals fed proteins (enzymes, etc.) in subchronic feeding studies. As discussed in Chapters 5 and 6, proteins are not considered to be capable of mutagenic interactions with DNA, and this would be even less likely for proteins consumed in the diet. Mutagenicity studies have been carried out with many enzyme preparations to confirm they did not contain genotoxic contaminants (e.g., mycotoxins) from the fermentation medium. Members of the United States 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 that were from conventional and genetically modified microorganisms.[67] The vast majority of these tests found no evidence of mutagenic activity; the few tests that had positive results were considered to be largely attributable to artifacts in the test system (e.g., presence of free histidine in the enzyme preparation gave false positive results in the histidine reversion bacterial mutagenicity tests).[67] It was concluded that testing enzymes for potential genotoxicity was not necessary for safety evaluation.[67]

Similar conclusions were stated in Chapter 6 regarding International Conference on Harmonization (ICH) guidelines for safety testing of protein pharmaceuticals. The ICH guidelines for genotoxicity testing comment that biologicals (which include protein therapeutics) are not expected to interact directly with DNA. They are degraded to peptides and amino acids which are not considered to have genotoxic potential. Routine genotoxicity testing of protein pharmaceuticals is not considered necessary to confirm safety.

There are a few published examples of enzyme preparations being tested in rat teratology and/or one generation rat reproduction studies to confirm the absence of fermentation contaminants that might exert adverse effects. No evidence of adverse effects attributable to the enzymes on progeny development or reproductive performance were reported in these studies.[28,30,64,68]

A few chronic feeding studies have been carried out with protein preparations produced by fermentation.[22,69] This was done to determine whether there were any chronic adverse effects attributable to potential contaminants from the microorganisms used in the fermentation production. These studies did not report that protein preparations caused cancer in laboratory animals. There is no evidence to that proteins directly induced cancer, birth defects, or mutagenic effects when fed in the diet of laboratory animals.[67]

In the 1980s there was some controversy regarding the chronic effects of trypsin inhibitor proteins on the rat pancreas and the relevance of these findings to humans. Trypsin inhibitors are considered to be antinutrients and members of a larger family of protease inhibitors found naturally in a variety of food crops such as legumes, cereals, and potatoes.[70] As the name implies, trypsin inhibitors block the protease activity

of trypsin in the gut, interfering with protein digestion. Protease inhibitors may play a role in plant defense by interfering with insect digestion and reducing insect feeding on the crop. The safety controversy began in the UK when rats that had been fed a diet containing raw (unprocessed) soybean meal were dosed with azaserine, a low-molecular-weight chemical that induces pancreatic cancer.[71] Soybean meal must be subjected to thermal processing to inactivate trypsin inhibitors before the meal is used as food/feed or the trypsin inhibitors will interfere with protein digestion. The aforementioned study found that trypsin inhibitors in soybeans promoted the development of pancreatic cancer induced by azaserine. In addition, control animals that had not been treated with azaserine, but maintained chronically on unprocessed soybean meal also developed hypertrophic and hyperplastic changes in the pancreas.

It was subsequently shown that this response was not due to a direct effect of trypsin inhibitors on the pancreas but, rather, to negative hormone feedback by cholecystokinin (CCK), a hormone produced in the stomach. CCK is released in response to undigested protein and feeds back on the pancreas to increase production of proteases for release into the digestive tract to increase protein digestion. The continued presence of trypsin inhibitor prevented protein digestion; more CCK was released to stimulate the pancreas and the cycle continued. Rats chronically fed unprocessed soybean meal had very high levels of blood CCK levels due to impaired protein digestion, resulting in chronic stimulation of pancreatic growth which eventually led indirectly to the development of tumors.[72]

Questions were raised about the relevance to human food safety[72–74] since it was reported that the average adult intake of trypsin inhibitors from consumption of normal foods in the UK diet was approximately 330 mg/person/day.[74] Feeding studies with raw soybean meal in other species (dog, pig, calf) did not demonstrate hypertrophic or hyperplastic changes in the pancreas,[74] suggesting that rats were more sensitive than other species and may not be a relevant model for humans. It was recognized that trypsin inhibitors mediated their effects on the rat pancreas through the endocrine system. Moreover, according to Gumbmann et al. in 1986, "[T]here is no evidence of absorption from the gastrointestinal tract, direct neoplastic action or tumor induction, genotoxicity, interaction with cellular genetic material or epidemiological indication of a potential risk in man."[75] It was ultimately concluded that "humans are not at increased risk for pancreatic neoplasia for foods containing natural trypsin inhibitor activity."[72] Thus, the earlier observation of lack of evidence for direct carcinogenic effects of proteins fed in the diet remains true.

As discussed in Chapter 2, certain proteins are known to be toxic to humans.[76] Some of these toxins are produced by pathogenic bacteria that elaborate the toxins in the GI tract when ingested. Some pathogenic bacteria are present in food and form protein toxins in food. Understanding each step in the life cycle of protein toxins can help to define their mode of action and explain why some are toxic when ingested and others are not (Chapter 2). There are also protein antinutrients, such as protease inhibitors and lectins, that are naturally present in a number of foods that are traditionally consumed (legumes, grain, potatoes, etc.).[70,77] Although there is a history of safe consumption to many of these proteins, a few of them are toxic, particularly when the food is not properly cooked to inactivate the toxin (e.g., kidney bean lectin).[78] The are other examples, such as the castor bean plant, which is not consumed for food but its oil has

been used as a cathartic. Castor plants produces ricin, a highly toxic lectin that causes poisoning in humans and animals that accidentally consume the bean.[79]

Lastly, there is the example of a unique class of proteins known as prions that are components of mammalian neurons. Prion structure can be modified by spontaneous mutations in the prion gene to form stable, pathogenic forms that cause neurodegenerative diseases. The modified prions cause unmodified prions in neurons to assume the altered structural configuration that induces neuropathologic changes. Modified prions can contaminate surgical equipment or blood and be transmitted to others. Ruminants with bovine spongioform encephalopathy (BSE) caused by modified prions may "infect" those who consume meat from these animals.[80] Modified prion proteins are unusually stable as they are resistant to proteases, standard sterilization, and disinfection agents.

As will be discussed below, developers of improved crop varieties initially screen the proteins that are being considered for introduction into agricultural crops for a range of attributes. In particular, the efficacy of the trait to be conferred (e.g., insecticidal activity), and they do not have properties that would pose a risk to consumers or farm animals. Subsequently, following selection and first proof of concept, they undergo systematic bioinformatics, *in vitro* and *in vivo* testing on a case-by-case basis. To date, none of the proteins introduced into agricultural crops has shown any evidence of adverse effects, confirming the rigorousness of the screening system that has been developed.

11.5 SAFETY ASSESSMENT STRATEGY FOR PROTEINS INTRODUCED INTO FOOD/FEED CROPS

In Chapter 10, a safety testing approach was outlined for proteins introduced into biotechnology-derived crops. This strategy was based on guidelines provided by the Organisation for Economic Co-operation and Development (OECD), the World Health Organization (WHO), the European Food Safety Authority (EFSA), etc. The basic elements of this testing strategy are:

History of Safe Use (HOSU): Proteins introduced into biotechnology-derived crops that have a history of safe use/consumption in food, or are structurally and functionally related to proteins with a HOSU, are generally considered safe to consume. The HOSU concept is widely used in a regulatory context to provide guidance on the level of familiarity with respect to probable safety of chemicals or proteins in food. Safety testing guidelines developed by EFSA state, "The studies required to investigate the toxicity of a newly expressed protein should be selected on a case-by-case basis, depending on the knowledge available with respect to the protein's source, function/activity and history of human/animal consumption. In the case of proteins expressed in the GM plant where both the plant and the new proteins have a history of safe consumption by humans and animals, specific toxicity testing might not be required."[81]

11.5.1 Mode of Action and Functionality

Understanding the mode of action and/or biological function of the introduced protein will inform the safety assessment so that appropriate testing can be undertaken

to address any safety concerns that may exist. If the mode of action is specific for a certain biological function (for example, enzymatic conversion of substrate A to product B) and the products of the enzymatic reaction pose no safety concerns, then no additional safety testing may be warranted beyond the bioinformatics and digestibility assessments previously discussed in Chapter 10.

If the mode of action is not established (control insect pests by an unknown mechanism) or the function is related to the mode of action of known mammalian protein toxins or pharmacologically active proteins [antifungal protein (AFP) example, Chapter 10], then additional safety testing is warranted to assess whether the protein can be safely used.

11.5.2 Bioinformatics

The protein introduced into biotechnology-derived crops should not show amino acid sequence similarity to known mammalian toxins, allergens, or pharmacologically active proteins. If similarity to those proteins is found, additional safety evaluations will be needed to determine whether these proteins can be safely consumed in the diet.

11.5.3 Digestibility

Proteins that are readily digested *in vitro* using simulated gastric and/or intestinal fluids would normally be capable of being digested or degraded when consumed in the diet. As discussed in Chapter 10, digestible proteins would, in the majority of cases, be less likely to act as food allergens which are generally more stable to digestion.

11.5.4 Confirmatory Safety Studies

As discussed in Chapters 3 and 10, high-dose acute toxicology studies are required by the U.S. Environmental Protection Agency (EPA) to assess the potential hazards of plant-incorporated protectants (PIPs). This testing requirement is based on the need to demonstrate that the toxic mechanism of the plant protectant is not relevant to animals and man. For example, the knowledge that existing commercial insecticidal Cry proteins (derived from *Bacillus thuringiensis* bacteria) act through acute mechanisms at low doses to control insect pests (Chapter 3) and that does not occur in man is important and reassuring from the safety perspective. The EPA requires that PIPs be tested at high dosage levels (generally g/kg body weight where feasible) to confirm their safety. Further, although most consumed proteins are not toxic, those that are toxic generally exert their effects through acute modes of action.[82]

The procedures for carrying out high-dose acute testing of proteins were presented in Chapter 10. To date, no treatment-related adverse effects have been observed up to the highest dosages tested (Table 11.1). As will be shown later, the high dosages of proteins administered to mice are orders of magnitude higher than potential human dietary exposures from consuming food from biotechnology-derived crops. For PIPs that have a history of safe use and defined mode of action, the EPA does not require additional toxicology testing beyond acute oral maximum hazard dose testing.[22]

Acute toxicology studies are generally conducted via the oral route because the diet is the most likely route of human exposure to the proteins introduced into

biotechnology-derived crops. Mice are generally used instead of rats as they are approximately 1/10 the body weight of rats and require much less protein for dosing. Mice are also known to be sensitive to the adverse effects of known protein toxins and are most commonly used to assess their toxic effects.[83]

Intravenous (IV) dosing has also been used to assess the intrinsic safety of proteins introduced into biotechnology-derived crops.[41] Generally, low dosages (~10 mg/kg) of the introduced protein are administered as it is assumed that only small amounts of ingested proteins could be absorbed intact, and IV dosing poses the most conservative test of potential toxicity. However, dosing by this route may not simulate what occurs locally in the GI tract, and thus its relevance to dietary exposure could be questioned. For example, the potential toxicity of antinutrient proteins that interfere with protein digestion and uptake (protease inhibitors, lectins) may not be manifest in the same way if they were administered intravenously instead of by the oral route. For IV dosing, proteins produced in bacteria would need to be highly purified to remove bacterial/fermentation contaminants (e.g., lipopolysaccharides) that are themselves toxic when administered parenterally.[84] If there was evidence of toxicity following IV dosing of the protein, acute oral toxicology studies would still need to be conducted to resolve whether these effects were relevant to dietary exposure. Repeat IV dosing is also not recommended as plant-derived proteins would be recognized as foreign to rodents, leading to the development of neutralizing antibodies in the blood that would confound interpretation of study findings. This phenomenon is well documented for the repeated administration of protein-based pharmaceuticals that are not native to the test species (Chapter 6).

EFSA guidelines for testing the safety of biotechnology-derived crops do not recommend acute high-dose testing for insecticidal proteins or for other nonpesticidal proteins.[81] Rather, EFSA proposes a case-by-case assessment of the safety of introduced proteins, and if the biological profile/activity of the protein raises questions about safety or the protein is considered to be "novel," then a 28-day feeding study with the protein is recommended. This recommendation is appropriate for certain classes of potentially toxic proteins such as lectins or protease inhibitors whose toxicity is manifest after a short-term feeding study.[85–86] The characteristics that define an introduced protein as novel have not been elaborated and are best determined on a case-by-case assessment.

It may not be possible to carry out repeat-dosing studies for certain membrane-bound enzymes if they are considered to be novel. Purification and isolation of certain membrane-bound enzymes can lead to their immediate inactivation as membrane lipids and the cofactors needed for catalytic function of the enzyme are removed during purification.[87] As a practical matter, there could be negligible dietary exposure to functionally active membrane-bound enzymes in foods if solvent extraction and heat processing (e.g., foods derived from soybeans) results in their inactivation. This may obviate the need for confirmatory safety testing of proteins in animals, given the negligible potential for human and animal dietary exposure.

When an introduced protein is functionally or structurally related to proteins that are toxic to mammals (AFP example, Chapter 10), then an acute high-dose toxicity study may not be sufficient to confirm safety. Other hypothesis-driven studies (based on knowledge of the protein's mode of action) may be necessary, as outlined for the

AFP example. These studies could include a 28-day dietary study with the purified protein in rodents, assuming it could be prepared in sufficient quantities to test.

Not all introduced proteins have pesticidal properties, as some impart other desired traits into crops such as herbicide tolerance, virus resistance, improvements in nutrient content, etc. Often these proteins are enzymes that catalyze specific biochemical reactions. Based on their known mode of action, specificity, lack of functional or structural similarity to protein toxins, digestibility, history of safe use, etc., the weight of evidence would suggest these proteins would not raise food safety concerns. However, in certain countries outside the United States or Europe, regulators have requested high-dose acute studies to provide further confirmation of safety, and proteins that have been so tested are also listed in Table 11.1 (see also Chapter 10). As with the case of PIPs, there has been no evidence to date of adverse effects in mice dosed with high levels of nonpesticidal proteins.

Proteins introduced into biotechnology-derived crops are also components of grain or seed that are formulated into diets and fed to rats for approximately 90 days to confirm the lack of any unintended effects in the biotech crop. Thus, their safety is tested as a component of the grain/seed fed to rats. Other studies, such as molecular characterization of the gene insert, the nutrient/antinutrient composition of food/feed, the phenotypic and agronomic characteristics of the plant grown in different environmental conditions, and animal performance studies with feed will also have been carried out to assess the potential for unintended effects.

The study design for a 90-day rat feeding study is adapted from OECD 408 guidelines for subchronic studies that include measurement a comprehensive battery of toxicology parameters. Commercial rodent diets used by toxicology testing facilities often include processed soybean meal and corn meal in diet formulations as a source of dietary protein. When new biotechnology-derived corn or soybean crops are developed, they can be incorporated into commercial rodent diets to substitute for conventional corn grain or processed soy meal, and their safety can be assessed. Since the rats are fed levels of corn grain approximately 100 times higher than humans would consume in Europe (assumes conservatively that 100% of the corn grain is derived from the biotechnology-derived crop), these studies can provide confirmation of an acceptable safety margin for the biotechnology-derived crops including the introduced protein(s). If triggered, for example, by results from compositional analysis or differences in phenotypic or agronomic performance, subchronic feeding studies may be conducted to determine whether the biotechnology-derived food is "as safe as" conventional, nonbiotech comparators in accordance with the general principles of substantial equivalence.[88–90]

Subchronic feeding studies are often required to obtain registration of the biotechnology-derived crop in the EU even though the aforementioned triggers did not occur. It was recently acknowledged in a draft EFSA guideline[91] that "In the situation where molecular, compositional, phenotypic and agronomic analysis have demonstrated *equivalence* between the GM plant derived foods/feed and their near isogenic counterpart, except for the inserted trait(s), and do not indicate the occurrence of unintended effects, the performance of 90-day feeding trials with rodents or with target animal species would be considered to add little if anything to the overall safety assessment. … These studies did not show any indication for the occurrence

of unintended effects." This has been demonstrated in 90-day rat studies conducted to date, some of which have been published in peer-reviewed journals.[92–96]

11.6 DIETARY RISK ASSESSMENT

Risk assessments are routinely performed to assess the safety implications for the intentional or unintentional presence of low-molecular-weight chemicals in food and feed. The procedures and mathematical models used to predict risk have evolved over the years and have been extensively reviewed.[97–99] The dietary assessment includes both acute and chronic exposure assessments. Acute exposure assessments address short-term exposures using approximately 95th- or 97.5th-percentile food consumption data (where available) and acute toxicity data generated with the low-molecular-weight chemical. Some, however, may question the use of acute dietary risk assessments for proteins when there is no evidence that they are acutely toxic. Chronic exposure assessments use mean (50th-percentile) food consumption data and use the lowest no-effect level from the battery of toxicology studies to establish an acceptable daily intake (ADI) for the low-molecular-weight chemical added to food. Calculation of an ADI has not been considered necessary for certain proteins such as the Cry insecticidal proteins. Cry proteins, whether introduced into biotech food crops, or sprayed on food crops as components of commercial microbial pesticide formulations, have generally been exempted from the requirement of a tolerance.

The same procedures have been used for preparing dietary risk assessments for proteins introduced into biotechnology-derived food and feed crops. The dietary intake of the introduced protein can then be estimated by multiplying the intake estimates by the concentration of the introduced protein in the food. Chapter 9 provides lists of food consumption databases that are available for various countries. Some food consumption data is based on the annual disappearance of food within the borders of the country, which is divided by the overall population to estimate daily intake of the food commodity. These databases overestimate daily intake of the food by adults. The more accurate consumption databases are based on survey information of individuals over 24 to 48 hours. This information can be collected for both adults and children. There is a need for countries to develop more comprehensive food survey data on their respective populations so that dietary risk assessments can be more accurately performed. At present, 95th- or 97.5th-percentile food consumption data are only available for certain countries such as the United States, the UK, and Australia. However, as shown in Chapter 9, a number of countries have been carrying out food consumption surveys and it is hoped that this will be more publicly available for those that have a need for this information to carry out dietary risk assessments. An example for a dietary risk assessment for YieldGard® Cornborer (Monsanto Technology, LLC.), an insect-protected, biotechnology-derived crop is provided below.

Cry1Ab protein derived from *Bacillus thuringiensis* (Bt) was introduced into corn plants to provide protection against corn borer pests that damage both the stalk and ears. The levels of Cry1Ab protein in leaf and stalks is around 12 ppm, and in grain, 0.3 ppm.[100] As shown in Table 11.1, mice were dosed up to 4000 mg/kg with Cry1Ab protein and experienced no adverse effects.

1. Acute Dietary Exposure Assessment

- The 97.5th-percentile corn endosperm* fraction consumption in the UK for adults is 113 g/person/day ÷ 70 kg body wt/person = 1.6 g/kg.
- The 97.5th-percentile adult dietary intake of Cry1Ab protein would be: 1.6 g/kg/day × 0.3 μg/g corn = 0.48 μg/kg for an adult (0.00048 mg/kg).
- The margin of safety for acute exposure to Cry1Ab protein is 4000 mg/kg ÷ 0.00048 mg/kg = 8,333,333 X.

Put another way, a 70-kg-body weight human adult would need to consume > 900,000 kg (900 metric tonnes) of grain in one day to attain the same acute dosage (4000 mg/kg) of Cry1Ab protein given to mice which produced no adverse effects.

2. Chronic Dietary Exposure Assessment

- The average (50th-percentile) corn consumption in the UK for adults is ~16 g corn/person/day ÷ 70 kg body wt/person = 0.23 g/kg.
- The average adult dietary intake of Cry1Ab protein would be: 0.23 g/kg/day × 0.3 μg/g corn = 0.07 μg/kg for an adult (0.00007 mg/kg).
- The average rat dietary intake of Cry1Ab protein in a 90-day feeding study is 25 g corn/kg BW × 0.3 μg/g corn = 7.5 μg/kg
- The margin of safety for chronic dietary exposure to Cry1Ab protein is 7.5 μg/kg divided by 0.07 μg/kg = 107 X

This dietary exposure assessment makes some very conservative assumptions. It assumes that 100% of the corn consumed in the diet is YieldGard® Cornborer that contains the Cry1Ab protein. In reality, many varieties of corn are sold commercially, so that YieldGard® Cornborer represents only a fraction (~20%) of the total corn varieties consumed in the diet (as of 2002).[101] It also assumes that the Cry1Ab protein is not denatured by thermal processing of corn grain into food products. Soybeans are both heat-processed to inactivate trypsin inhibitors and solvent-extracted to remove oil. Processing denatures proteins like CP4 EPSPS, which have been introduced into soybeans to impart tolerance to glyphosate herbicide.

The dietary risk assessment shown above uses corn consumption data for adults in the UK. If a dietary risk assessment was prepared for Central America, the safety margin would be somewhat lower, as corn consumption is hundreds of grams per person per day.[102] However, the safety margin would still be very large since the level of Cry1Ab in corn grain is very low. Thus, risk assessments can be tailored for individual countries when there are accurate food consumption data available.

11.7 THRESHOLD OF TOXICOLOGICAL CONCERN

Introduced proteins are generally present at low levels in the grain/seed of biotechnology-derived crops commercialized to date (Table 11.3). One could assume that the presence in food of low levels of introduced proteins poses minimal risks and should not require comprehensive safety assessment. There is a regulatory mandate in most

* Human dietary exposures are estimated using the corn endosperm fraction. This fraction contains most of the protein which would include the introduced protein. Other corn fractions such as bran, sweeteners, and oil contain very little protein. It also assumes that the Cry1Ab protein has not been introduced into sweet corn. Data derived from the *DEEM*-UK database (Exponent, Inc.).

TABLE 11.3
Levels of Introduced Proteins in the Grain/Seed of Biotechnology-Derived Crops

Crop	Introduced Protein	Concentration[a](ppm)	Reference
Corn			
Roundup Ready®	CP4 EPSPS	10–14	103
YieldGard® Cornborer	Cry1Ab	0.3	100
YieldGard® Rootworm	Cry3Bb1	70	94
YieldGard® Plus	Cry3Bb1	20 (range 15–26)	104
	Cry1Ab	0.38 (range 0.2–0.47)	
YieldGard® Rootworm Plus	Cry3Bb1	32 (range 22–48)	105
	Cry1Ab	0.56 (range 0.48–0.67)	
	CP4 EPSPS	9.6 (range 7–14)	
Herculex 1® Insect Protection	Cry1F	71–115	106
Lysine Maize	Dihydrodipicolinate-synthase (cDHDPS)	24 (range13–43)	107
Cotton			
Roundup Ready®	CP4 EPSPS	47–117	108
Bollgard®	Cry1Ac	1.62	106
Bollgard II®	Cry2Ab2/Cry1Ac	34–60/1.3–1.6	109
Roundup Ready Flex®	CP4 EPSPS	67–580	110
Soy			
Roundup Ready®	CP4 EPSPS	186–395	

[a] fwt, fresh weight.
® Registered trademark, Monsanto Technology, LLC.

countries to assess the safety of the many substances found in food, whether they occur naturally or are added in some manner to food. Without some means to prioritize all substances that need further evaluation, regulators would be utilizing scarce resources to assess safety for many substances that may not require a comprehensive safety evaluation. Moreover, without prioritization, the costs would be enormous to carry out indiscriminate safety testing and many research animals would be used unnecessarily. There is a growing demand to reduce animal experimentation where possible.[112]

A risk assessment strategy has been proposed for evaluating low-level exposure to low-molecular-weight chemicals in the diet. If adequate safety margins exist for human exposure to these substances, then no further safety testing would be required. This would enable regulators to focus resources on higher-priority food safety issues.[112] This risk assessment strategy is described as the threshold of toxicological concern (TTC).[112–114] According to Kroes et al., the TTC "is a pragmatic risk assessment tool that is based on the principle of establishing a human exposure threshold value for chemicals, below which there is a very low probability of an appreciable risk to human health. This concept…is inherent in setting acceptable

daily intakes (ADIs) for chemicals with known toxicological profile."[113] This concept could also be applied to proteins introduced into food and feed crops.

The TTC values for low-molecular-weight chemicals are as low as 1.5 μg/person/day for those that have not been tested for carcinogenicity but have structural properties (alerts) similar to known chemical carcinogens. Exposures below the 1.5 μg/person/day level are considered to pose a very low risk (< 1 in a million) of producing cancer in man. Other low-molecular-weight chemicals that do not have structural properties or alerts that raise questions about potential toxicity have TTC levels much higher, ranging up to 1800 μg/person/day in the diet.[113]

Proteins were not initially included in determining TTC levels because, again citing Kroes et al., "[T]here are insufficient dose–response data regarding allergenicity of proteins and low-molecular-weight chemicals, on which a TTC (or any other assessment) can be based."[113] However, as discussed in Chapter 8, developers of biotechnology-derived crops rigorously avoid intentionally introducing potentially allergenic proteins into foods, for obvious reasons. As indicated in Chapter 8, there is a battery of tests undertaken to confirm that introduced proteins do not fit the profile for known allergens. Based on the very low probability that proteins introduced into biotechnology-derived crops pose an allergenic risk, the TTC risk assessment tool could be applied to low-level exposure to introduced proteins in biotechnology-derived food crops.

One fundamental difference between proteins introduced into foods and low-molecular-weight chemicals is the general lack of evidence for toxic effect levels in animal safety studies with selected proteins (Tables 11.1 and 11.2). For low-molecular-weight chemicals, TTC values were calculated using the 5th percentile of the distribution of the NOELs (based on animal toxicology studies) divided by an uncertainty factor of 100, and assuming an average human body weight of 60 kg.[114] Low-molecular-weight chemicals were divided into three different classes based on the relatedness of their chemical structures to those that either posed minimal safety concerns or those that suggested potential for toxicity. Proteins could likewise be catalogued into three structural divisions based on their relatedness, or lack thereof, to proteins known to be toxic. Relatedness is already evaluated by bioinformatics searches, as discussed in Chapter 10. The most toxic proteins to humans are generally those derived from microorganisms that cause food poisoning, and these could represent one class. The next class of proteins could include those generally found in plants that act as antinutrients (lectins, protease inhibitors). As a practical matter, proteins with potential mammalian toxicity are obviously not considered for addition to food or feed crops, although there is a history of consumption to many endogenous antinutrient proteins found in food (lectins, protease inhibitors, etc.). The last category of proteins would include proteins being introduced into food and feed crops that are structurally and functionally related to those currently present in food or have been safely used in food production (e.g., Cry proteins from *Bacillus thuringiensis* microbial sprays and food processing enzymes).

As an exercise, NOAELs for all of the non-toxic proteins listed in Tables 11.1 and 11.2 were averaged for either acute or subchronic toxicity. Since the enzyme concentration present in fermentation preparations can vary from 2% to 70%,[63] an arbitrary assignment of 10% enzyme concentrate was applied to all NOAELs for those enzymes prepared by customary fermentation techniques (some publications listed the concentration of enzyme in the preparation, whereas many others did not). This 10%

correction factor was applied to all the NOAELs presented in Tables 11.1 and 11.2. The adjusted NOAELs were used in determining the overall averages for acute and subchronic toxicity studies. The mean values were divided by a 100-fold uncertainty factor to estimate TTC levels for acute and chronic exposures.

For acute exposure, the average NOAEL (always the highest dosage tested) across 30 acute studies was 1790 mg/kg, and when divided by a 100-fold uncertainty factor, would provide a TTC of 17.9 mg/kg, or 1074 mg/adult person/day for acute dietary exposure (assumes adult body weight of 70 kg). For chronic exposure, the average NOAEL (always the highest dosage tested) across 40 subchronic studies was 249 mg/kg, which divided by a 100-fold uncertainty factor would provide a TTC of 2.49 mg/kg, or 149 mg/adult person/day.

The chronic dietary exposures to various introduced proteins have been calculated in publications for three biotechnology-derived corn products [Roundup Ready® corn; YieldGard® Rootworm corn, and YieldGard® Cornborer corn; (Monsanto Technology, LLC.)] that were fed to rats in subchronic toxicology studies.[92–94] The intake of introduced proteins was 0.27 mg/person/day for CP4 EPSPS protein, 1.3 mg/person/day for Cry3Bb1 protein, and 0.005 mg/person/day for Cry1Ab protein. These dietary exposures were based on the very conservative assumptions that 100% of the corn consumed was derived from each biotech variety that was tested, and there was no loss of the introduced proteins during thermal processing of corn grain into food products. Even at the 95th-percentile U.S. corn consumption level (which is approximately 4× the mean dietary exposure), the mg/person/day intakes would still be far below the TTC (149 mg/person/day) for chronic dietary exposure to introduced proteins. For parts of Mexico and Africa, where the per capita corn consumption is approximately 20 times that in the United States, the mg/person/day intakes would still be well below the calculated TTC level.

The levels of the aforementioned introduced proteins in the grain from three biotechnology-derived corn products are quite low: 14 ppm (CP4 EPSPS), 70 ppm (Cry3Bb1), and 0.3 ppm (Cry1Ab). To achieve a level of protein consumption equivalent to the 149 mg/person/day TTC level, and using a 50th-percentile daily U.S. adult corn endosperm consumption figure of 0.27 g/kg/day (*DEEM* database, Exponent, Inc.), the levels of an introduced protein would have to be approximately 7800 ppm in the grain for dietary consumption to reach the TTC level. If the dietary exposure for an introduced protein exceeded the TTC, this would not mean that there was a safety concern. Appropriate toxicology studies could be done to assess safety at dietary levels above the TTC, as discussed previously. Adoption of the TTC concept for risk assessment would mean that dietary exposures to proteins below the TTC would not require confirmatory animal safety testing based on the following conditions: (1) the source of the protein raises no safety concerns; (2) the mode of action of the protein is known and poses no safety concerns; (3) the protein is not structurally or functionally related to proteins that are known mammalian toxins or antinutrients; (4) the protein is digestible; and (5) the protein does not fit the profile of known food allergens.

11.8 THE FUTURE

As the next generation of biotechnology-derived crops approaches commercialization, it is important to confirm whether the existing safety assessment paradigm is appropriate for these new products. The safety assessment paradigm for introduced

proteins presented earlier in this chapter is aligned with existing internationally accepted approaches provided in numerous publications.[115–122] A discussion of the new kinds of introduced proteins that are being developed and the efficacy and utility of the existing safety testing paradigm to confirm their safety will be presented below.

11.8.1 Applications of Protein Engineering for Food-Processing Enzymes

The advent of biotechnology has made it possible to modify proteins to increase their existing functional activity, or to impart new functional properties for a desired application. Protein engineering includes changing amino acids at key positions in the molecule that can modify their structural and/or functional properties. The first applications have focused on the engineering of food enzymes to improve their stability under food-processing conditions. For example, protein engineering has been used to modify proteases by changing key amino acids to increase their stability to high temperatures and pH — conditions that can occur during food processing.[123] Another example is the modification of α-amylases to increase thermostability for production of sweeteners from corn starch.[124] Biotechnology has also made it possible to identify and produce enzymes from thermophillic and psychrophilic microbes that exhibit unique thermostable properties, as the organisms that produced them live in extreme environmental conditions (e.g., volcanic heated pools or vents).

A recent review by Spok discusses other tools used to improve enzyme performance: "Combinatorial approaches of rational protein design and directed evolution methods turn out to efficiently alter the properties of enzymes, enzyme stability, catalytic mechanism, substrate specificity and range, surface activity, folding mechanisms, cofactor dependency, pH and temperature optima, and kinetic parameters have been successfully modified."[63] Other techniques such as protein shuffling can increase the variability of enzymes that can be produced and may yield enzymes that can carry out catalytic activities that were heretofore not possible with existing enzymes.[63]

Biotechnology is being used to reduce the potential for contamination of enzyme concentrates with toxic impurities, which can benefit the consumer. It is now possible to introduce the gene coding for food enzymes into microorganisms that have been well characterized and have an established history of safe use because they do not make toxic impurities.[63] Given this scenario, it is probably not necessary to continue carrying out 90-day rat safety studies when the fermentation organisms are known to not produce toxic contaminants and the enzyme is fully characterized.

11.8.2 Modification of Insect Control Proteins to Improve Potency or Broaden Selective Activity against Targeted Pests

A wide range of activity of Cry proteins against several orders of insects has resulted from a naturally occurring recombination and sequence diversity.[125] Generally, Cry proteins have a defined spectrum of insecticidal activity within a particular insect order.

Cry proteins are composed of several functional domains that have highly conserved areas between the classes.[126] For example, Cry1A proteins are highly conserved in domains I, II, and III. Sequence identity can indicate similarity in biological function, i.e., activity toward a similar spectrum of insects. These functional domains have been shown to determine the specificity of Cry proteins: domains I,

II, and III form the toxin portion (tryptic core), and a C-terminal protoxin domain is cleaved upon entry into the insect midgut.[126] Domain I is involved in membrane insertion and pore formation and domain II is involved in specific receptor recognition and binding, as shown by mutagenesis studies. Domain III plays a role in receptor binding. The combination of domains I and II has been shown to determine insect specificity. The C-terminal protoxin domain plays a role in crystal formation. Domain swapping is a well-known mechanism for generating diversity. Mutagenesis and domain swapping is widely used in research in order to better understand function of each domain and have been described previously.[125,127]

The safety assessment of future Cry insecticidal proteins with enhanced insecticidal properties developed through domain swapping or other techniques can be confirmed using existing toxicological study designs. This would include the standard bioinformatics, *in vitro* digestibility, and high-dose rodent acute toxicity test required by the EPA for registration of PIPs. If indicated, confirmation of safety would also be possible through a 90-day rat feeding study with grain or seed containing the insecticidal protein. Other environmental toxicity tests, as outlined in Chapter 4, would also be needed to confirm selectivity toxicity against targeted insect pests and absence of toxicity to nontarget organisms, as exists for conventional Cry proteins. If the mode of action for the insecticidal protein is not well characterized, or raises questions about safety for consumers (such as the AFP example discussed earlier), then targeted toxicity tests designed to resolve safety questions may be needed based on a case-by-case assessment.

11.8.3 Introduction of Transcription Factor Proteins to Modify Endogenous Plant Metabolic Pathways

Modulation of regulatory control proteins and regulatory processes has occurred during plant domestication through both natural and selected breeding of improved crop varieties.[128–131] For example, the changes responsible for improved wheat yields as part of the "green revolution" involved selection for mutant *Reduced height-1* genes through conventional breeding.[132] The proteins encoded by these genes are regulators of endogenous gene transcription that make wheat plants insensitive to giberellin, a plant growth regulator, thus making the plants shorter and protecting them from collapsing under their own weight.[132] As a consequence, yield is increased at harvest. Wheat domestication also involved the *Q* gene, an AP-2-like transcription factor that confers free-threshing character and reduces fragility, enabling more efficient grain harvesting.[133] The domestication of maize from its ancestral form, teosinte, has involved selection for enhanced expression of the *teosinte branched 1* transcription factor[134] and regulatory changes in the maize allele of the *teosinte glume architechture* transcription factor.[135] Another example of the impact of transcription factors in corn breeding is a mutation in the *opaque 2* transcription factor. This mutation led to the generation of Quality Protein Maize (QPM), an improved nutrition maize variety (high in lysine content) that was the winner of the World Food Prize in 2000.[136] Reduced grain shattering resulting from a single base pair mutation in the DNA binding domain of the putative transcription factor *sh4* has been thought to be a key event in the domestication of rice.[137] Tomato hybrid cultivars with a mutant transcription factor yield fruit with a longer shelf life.[138]

We are now learning that the domestication and breeding of modern crops with beneficial traits carried out over the past centuries has involved selection for changes in proteins regulating endogenous plant gene expression. Transcription factors have played a prominent role in these processes. These crop varieties produced as a result of altered transcription factor expression have an established history of safe consumption as they are staples in the human diet. This demonstrates that plants with alterations in endogenous gene expression of proteins that modulate other endogenous plant genes have been safely consumed.

Profiling technologies such as genomics, proteomics, and metabolomics have facilitated identification of genes that regulate endogenous plant processes and the phenotypic effects elicited by their protein products.[139] Therefore, proteins that affect endogenous pathways are among the likely targets to improve the next generation of biotechnology-derived crops. During the last few years, there has been a growing number of biotechnology-derived plants with modifications in endogenous transcriptional regulatory processes.[140–142]

A fundamental principle to consider when evaluating the safety of these biotechnology-derived crops is that the transcription factor proteins operate through regulation of endogenous plant processes. Thus they are unlikely to produce novel metabolites not previously present in plants. These proteins will be structurally or functionally homologous to endogenous plant transcription factor proteins. They could also be obtained from the same crop into which they will be reintroduced through biotechnology.

During the growing season, plants are normally subjected to a variety of biotic and abiotic stress conditions. In response to these environmental conditions, a variety of transcription factor-mediated changes in endogenous plant gene expression occur. Humans and animals consume food or feed from crops that contain the cumulative gene expression changes that occur in plants grown under variable stress conditions.

There is a history of consumption of transcription factors as they are present in all eukaryotic cells, some of which are consumed as food. Out of an estimated 59,000 genes in the rice genome, approximately 1600 (~3%) are predicted to encode transcription factors.[143] The soybean genome is predicted to contain approximately 1300 transcription factors out of an estimated 63,500 genes, representing about 2% of the genome.[144] Questions concerning the safety of food or feed derived from crops containing introduced transcription factors should be considered in the context of the history of safe consumption of food and feed derived from plants containing these naturally and regularly occurring changes in transcriptional profiles.

An additional exposure consideration for many regulatory proteins is that they usually have a small number of specific targets. Moreover, although transcription factors are expressed in every cell, they are generally present in low levels in plant and animal tissues. In *Arabidopsis,* for example, the number of mRNAs encoding an individual transcription factor has been reported to range from 0.001 to 100 copies per cell, illustrating the relatively low level of these transcripts in plant cells.[145] The wide range in potential levels for a given transcription factor may result from spatial (cell type), temporal (cell cycle), and developmental (life cycle) regulation of gene expression.[141] Transcription factor proteins also tend to be present at very low

amounts in plant tissue. For example, only 50 µg (80 pmol) of KAP-2 transcription factor was obtained from 6 kg of bean cells, corresponding to about 8 ng of transcription factor protein per gram of tissue.[146]

Even with large uncertainties in available estimates, it is apparent that transcription factors represent only a tiny fraction of total plant proteins, and their concentrations (~ppb) are likely to be several orders of magnitude lower than proteins introduced into biotechnology-derived crops (ppm) to date (Table 11.3) or typical food proteins that might constitute 1% (10,000 ppm) or more of the total protein present in the food.[16] Total protein levels in food crops can range from 10% for maize to 40% for soybeans.[147] Tissues consumed from food animals also provide a dietary source of transcription factors and other regulatory control proteins as they are ubiquitous in the cells of animals, albeit at low levels. If levels of these transcription factors or other regulatory control proteins are elevated in food or feed beyond that normally observed in the plant product, this information would also be used in the evaluation of the history of safe consumption of related proteins.

The assessment of potential oral activity for introduced transcription factors needs to take into consideration the following factors:

1. The lack of a specific transport system for regulatory control proteins may provide an explanation, in part, as to how GI tract epithelia are continuously exposed to these proteins from dietary sources (plant- and animal-derived foods) without any evidence of biological response in mammals.
2. Transcription factors and many other proteins that regulate gene expression function in the nucleus. In order for ingested regulatory control proteins to be active in the consuming organism, the protein would thus need to not only survive digestive barriers, gain access to the systemic circulation, and be transported to a target tissue, but would also have to undergo cellular uptake, evade cytoplasmic degradation, and would require subsequent transport across the nuclear membrane and into the nucleus. Selective import of proteins across the nuclear membrane requires the presence of a nuclear localization signal within the protein sequence.[148] Whether an exogenous transcription factor or other regulatory control protein would enter the nucleus would depend partly on the interaction between that protein and nuclear import machinery in cells of the consuming organism. The specificity required for such interactions adds yet another barrier to function of dietary proteins that regulate gene expression.

Based on all of the aforementioned considerations, one can conclude that the existing risk assessment procedures used to assess safety of proteins introduced into biotechnology-derived crops are also applicable to transcription factors.

Since endogenous metabolic pathways may be modified to achieve the desired plant improvement, the agronomic performance and phenotypic appearance of the plant will be examined under a variety of environmental conditions to confirm that there are no deleterious unintended changes. The composition of grain or seed will also be analyzed to confirm that endogenous nutrients or antinutrients have not changed, unless the intended technical effect results in changes in levels of

endogenous nutrients. In this case, the safety and nutritional impact of those changes will be evaluated independently.

If there is evidence of significant unexpected/unintended molecular, compositional, agronomic, and/or phenotypic changes that could be adverse, then the safety implications of these changes would require further study before a decision could be made whether the crop could be safety used. This safety assessment process which is aligned with international guidelines discussed previously is considered to be fully adequate to confirm the safety of food/feed derived from plants whose metabolic pathways are modified to achieve intended improvements in the crop.

11.9 CONCLUSION

A consolidated risk assessment strategy is proposed for the introduction of proteins of diverse structure and function into food and feed crops. The strategy is based on, and aligned with, international guidelines and recommendations and can be adapted to evaluate the safety of new and improved varieties of biotechnology-derived crops that are under development. Based on the overall weight of evidence from assessing the safety of proteins of diverse structure and function used in food production and processing, as well as those introduced into biotechnology-derived crops, it is clear that introduced proteins can be safely used in the production of food and feed. The safety assessment tools are in place to and will continue be used as needed to ensure that food and feed derived from new varieties of biotechnology-derived crops can be safety consumed.

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