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# Prediction of the potential allergenicity of novel proteins

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## Abstract

The potential allergenicity of novel proteins expressed in genetically modified organisms is an important item in the safety assessment of these organisms. The various components of the allergenicity assessment include the source of the protein, sequence similarity with known allergenic proteins, *in vitro* digestibility, sera binding tests, animal models and clinical tests. Recent developments in the field of bioinformatics studies on the sequence similarity of novel proteins with allergenic proteins, as well as *in vitro* digestibility, are discussed.
**Keywords**: genetically modified organisms; transgenes; novel proteins; allergy; protein allergens; safety assessment; international harmonization; Codex alimentarius; bioinformatics; sequence alignment; proteolytic stability; sera binding tests; clinical testing; animal experiments

## Introduction

Since the first large-scale introduction of commercially grown genetically modified (GM) crops in 1996, their cultivation area has grown steadily, reaching a global 67.7 million hectares in 2003. For comparison, this area is twice as much as the national area of Finland. Prospects are that this area of GM crops will continue to grow. While the major part of the GM-crop areas are located in the United States, Canada, Argentina, Brazil, South Africa and China, GM crops in the European Union (EU) are reportedly grown in Spain and Germany. In addition, countries that do not grow GM crops themselves, such as some EU countries, may import them as feed and food products from countries growing these crops (James 2003).

In most nations, the marketing of GM products requires pre-market approval from the authorities. The assessment of the safety for humans, animals and/or the environment of a GM product destined for food and feed purposes usually is part of the approval procedure. While the regulations pertaining to GM products may differ from one country to another, the underlying principles for the safety assessment are similar and based upon an international approach. This approach has been the result of consensus-building activities of international organizations, such as the Food and Agriculture Organization (FAO), World Health Organization (WHO), Organisation for Economic Co-operation and Development (OECD), and the International Life Sciences Institute (ILSI), which started long before the first large-scale introduction of GM crops took place (reviewed by Kuiper et al. 2001). In 2003, this consensus culminated into the guidelines for the safety assessment of foods derived through

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modern biotechnology of the FAO/WHO Codex alimentarius committee (FAO and WHO 2003). This committee has regulatory capacities, since its codes and standards, such as those for residues of pesticides, contaminants and veterinary drugs, should be implemented in the legislation of its member states (most nations). In case of trade disputes over the safety of internationally traded foodstuffs, the World Trade Organization will use Codex standards and codes as reference.

The international safety-assessment approach is based on the principle of the comparative safety assessment, where the specific requirements are based upon the 'substantial equivalence' of a genetically modified organism (GMO) to a conventional counterpart with a history of safe use. For GM crops, this usually entails a comparison of genotype, phenotype (for example, shape, height), field behaviour, and composition. In addition, a detailed description of the molecular characteristics of the newly inserted genetic material is provided. Based upon the identified differences, it is further decided which tests will be required for safety testing. The issues that are commonly assessed include potential toxicity, allergenicity and nutritional value of the GM product, as well as the potential of gene transfer from the newly inserted DNA and unintended effects as a consequence of the genetic modification. Below, we will discuss the assessment of the potential allergenicity of novel proteins expressed in GM products, in particular the analysis for sequence similarity and *in vitro* digestibility.

## Allergenicity of GM products

Two scenarios for potential allergenicity of a GM product may be assessed. First, a novel protein expressed in a GM organism should be assessed for the potential to cause new allergies or to cross-react with other allergens in sensitized patients. Second, the intrinsic allergenicity of the modified host organisms may have been altered by the genetic modification. In fact, some experimental crops, so-called 'hypo-allergenic' crops, have deliberately been modified to express lower levels of a specific, intrinsic allergenic protein.

The strategy recommended by the Codex alimentarius for assessing the potential allergenicity of a food derived from a GMO includes the following items:

- Source of the gene: is the introduced genetic material derived from an allergenic source?

For example, a well-known case was that of a methionine-rich protein from Brazil nut that had been introduced into soybean in order to increase the nutritional value for animals. Brazil nut has allergenic properties and it was observed that extracts of the GM soybeans reacted positively with antisera from patients allergic to Brazil nut, as well as in skin prick tests (Nordlee et al. 1996). By contrast, non-modified soybean did not cause such reactions. Apparently, the allergenic properties of Brazil nut had been transferred to the soybean by means of the methionine-rich protein. The development of the experimental GM soybean was therefore halted and it did not come to the market.

- Sequence similarity between the novel protein and known allergenic proteins

The amino-acid sequences of a great number of allergenic proteins have been determined and data on these sequences have been stored in publicly accessible databases. Using special computer programs, the amino-acid sequence of a novel protein can be 'aligned' to these sequences. In case sufficient similarity is found, this indicates potential cross-reactivity between the novel protein and the allergenic protein. Two types of sequence similarity are considered. First, short identical

stretches consisting of contiguous amino acids above a specified number (generally six or eight) are identified, since these may constitute so-called 'linear' or 'continuous' IgE-binding epitopes. Second, partial identity in a larger segment of the amino-acid sequence, which reflects 'overall' similarity of the protein structure, is identified. For example, the FAO/WHO Expert Consultation on the allergenicity of GM foods, which was held in preparation of the Codex alimentarius guidelines, recommended 35% identical amino acids in 80-amino-acid segments as threshold for comparison (FAO and WHO 2001).

- Serum screening with sera from allergic patients

Depending upon the outcomes of the previous items, two types of screening with sera from allergic patients may be considered. If the introduced gene is derived from an allergenic product, or if the amino-acid sequence of the novel protein is similar to that of a known allergen, specific screening with sera from patients who are allergic to these particular items is recommended. Targeted screening with sera from patients that are sensitive to allergenic products that are broadly related to the source of the foreign gene can be performed if the gene source itself is not known as an allergen. The predictive value of sera screening will, among other things, depend on the availability of sera against certain allergens. Because some allergies are more prevalent than others, the availability of sera and therefore the number of assays that can be done varies.

- *In vitro* digestibility and degradability

Novel-food proteins that are stable towards processing during food manufacture and digestion may reach the immune system of the intestinal wall as intact proteins. These proteins may subsequently prime the immune system for an allergic reaction or trigger such a reaction in patients that have already been sensitized by previous contacts with the particular protein or other cross-reactive proteins. Stability towards processing and digestion is therefore considered a predisposing factor to potential allergenicity. However, some cases are known where digestible proteins cause allergic reactions, such as for apple allergens after they have come into contact with oral tissues in the so-called 'Oral Allergy Syndrome'. In addition, epitopes known as 'neo-epitopes' may be formed or become exposed during processing or digestion of a protein. The Codex alimentarius recommends the *in vitro* digestibility assay in simulated gastric fluid. In this assay, the test protein is added to simulated gastric fluid, which consists of the protein-degrading enzyme pepsin dissolved in dilute hydrochloric acid. After certain time periods, samples are taken from the incubation mixture, and the integrity of the novel protein is determined, for example by gel electrophoresis.

- Animal studies

Predictive animal models are currently being developed to test allergenicity of substances that may be administered intraperitoneally, intratracheally or through feed, depending on the model. One of the promising models is that of the Brown Norway rat, which is an IgE-hyperresponder. In fact, the sensitivity of these animals requires that they be reared on allergen-free diets for several generations. Subsequent challenges of these rats with allergens do not require 'booster' injections, contrary to other immunological models. Other models include Balb/c mice, dogs and swine. In the Codex alimentarius guidelines, animal models are not part of the main recommended tests, but it is recognized that they may be included as knowledge evolves. Several recent articles review laboratory animals used for testing allergenicity (Kimber et al. 2003; Ladics et al. 2003).

- Clinical studies

In addition to the serum tests discussed above, other clinical tests may be considered in further testing, for example, if previous tests have shown negative outcomes. These clinical trials may include skin prick testing, in which the patient receives small aliquots of the test substance and controls underneath its skin. In case of positive reaction, red wheals will develop at the site of the skin prick. Another type of test is the food challenge. Double-blind placebo-controlled food challenges are the method of choice here. However, it should be realized that ethical concerns may be raised over these types of tests as they may expose patients to harmful substances that may elicit adverse reactions. In addition, the Codex alimentarius guidelines do not mention these types of tests.

The Codex alimentarius guidelines describe a 'weight of evidence' approach, in which no single outcome of the recommended tests alone is sufficient to predict allergenicity, but rather the total picture that emerges from the combined results.

Below, we will discuss sequence similarity and *in vitro* digestibility in more detail.

## Sequence alignments *in silico*

As described above, the amino-acid sequence of a novel protein can be compared with the sequences of allergenic proteins with the aid of computer programs. The aim of this comparison is to determine whether the structure of the novel protein shows similarity with structures of allergenic proteins. Sequences of allergenic proteins can be retrieved from general protein databases, such as SwissProt (http://www.expasy.org/cgi-bin/lists?allergen.txt). In addition, dedicated websites provide data on allergenic protein sequences, such as WHO/IUIS (www.allergen.org) and Dr. S. Gendel (http://www.iit.edu/~sgendel/foodallr.htm). For the alignments of protein sequences, well-known algorithms such as FASTA and BLAST can be used. Some websites offer the facility to carry out alignments of a given input sequence against database sequences using such algorithms. The FASTA algorithm aligns sequences 'from head to tail', from the beginning to the end of each sequence, possibly with the beginnings shifted with respect to each other. The BLAST algorithm, on the other hand, allows for local alignments between sequences, irrespective of their position within the whole length of the sequence. In addition, other algorithms, such as Wordmatch or Wordsearch, may be used to find small identical sequences of a given size ('word length').

The Codex alimentarius guidelines provide, among other things, recommendations for the sequence alignment between a novel protein and allergenic proteins. Two types of identity between protein sequences are considered. First, short identical sequences that may constitute linear epitopes. For example, the FAO/WHO Expert Consultation recommended a minimum threshold size of six amino acids. The Codex alimentarius guidelines do not mention a specific number and recommend that the threshold for identical stretches should be chosen to exclude false positives and false negatives as much as possible. Second, overall structural similarity may indicate potential cross-reactivity between the novel protein and the allergen with similar structure. The FAO/WHO Expert Consultation recommended a threshold of 35% identical amino acids in 80-amino-acid segments of the novel protein that are each aligned to the allergen sequences in a given database. The rationale for this particular threshold is not specified in the proceedings of the Expert Consultation. Other literature sources indicate that 35% sequence identity is a reliable lower threshold for identifying

members of the same protein family (Rost 1999). The 80-amino-acid segment size may pertain to the size of a separate domain within the quaternary protein structure.

The Allermatch website (www.allermatch.org) allows users to carry out the bioinformatic procedures recommended by the Codex alimentarius and the FAO/WHO Expert consultation. After an input amino-acid sequence has been entered, this sequence is compared to sequences of 303 allergenic proteins, which have been stored in a database specifically constructed for this website. FASTA alignments are carried out with the full input sequence or 80-amino-acid segments that are automatically generated from the input sequence by the website. In addition, 'word matches' of short identical stretches between the input sequence and the database sequences can be searched for.

**Short identical stretches**

With regard to the search for short identical stretches, it has been common practice until recently to apply an 8-amino-acids threshold for the assessment of GM products. The question may arise what would happen if this threshold would be lowered to 6 amino acids. In fact, several authors searched for sequence similarity of short identical stretches between novel proteins expressed in genetically modified crops and allergenic proteins (Gendel 1998; Hileman et al. 2002; Kleter and Peijnenburg 2002). In all these studies, the maximum identical stretch size was 7 contiguous amino acids.

For example, Kleter and Peijnenburg (2002) tested 33 novel proteins from GM crops for identical stretches of six or more contiguous amino acids. It was observed that two-thirds (22) of the test proteins gave positive results with 6-7 amino-acids-long identical stretches. Because many of these positive outcomes are likely false positive, the authors recommended applying a selective 'filter' to these results in two ways. First, literature data on IgE antibody-binding epitopes should be consulted to determine whether the identical sequence is part of an epitope. In case of positive outcomes, these should be further verified by clinical testing. Second, antigenicity prediction should be applied to determine whether the identical stretch is a site of high antigenicity, in other words a probable binding site for antibodies. The antigenicity prediction method used was that of Hopp and Woods (1981), using a 6-amino-acid window. Within this window, each amino-acid residue is assigned a hydrophilicity value, which is fixed for that particular amino acid. The values for the 6 residues are then averaged and the resulting average value is assigned to the middle point of the window, while all the middle points are incorporated into a hydrophilicity plot. The highest point in this plot is considered the most likely binding site for antibodies. In case this peak coincides with the identical stretch, the outcome of the second method is positive for that stretch. This second method is particularly useful for allergenic proteins for which comparatively few data are available on IgE-epitopes, such as recently identified allergenic proteins. Positive outcomes of the second method should be further verified by serum-binding tests with the pertinent peptides, using sera from patients who are allergic to the allergen showing similarity with the novel protein. In case the serum-binding tests are positive, further clinical tests may be considered.

By applying the two methods of literature search for epitopes and antigenicity prediction, these authors identified comparatively few cases that warranted further investigation. For example, it was found that the glyphosate oxidoreductase enzyme, which is expressed in some glyphosate-resistant crops, shares a short identical stretch of 6 amino acids (LAEEAD) with tropomyosin allergens from seafood and a fish parasite. This short stretch was also part of a 9-amino-acid peptide from shrimp tropomyosin described in literature that was bound by IgE-sera from shrimp-allergic

patients. Another interesting identical stretch that was predicted to be antigenic was a 6-amino-acid sequence (EKQKEK) that was identical between Papaya Ringspot Virus coat protein and the nematode (roundworm) allergen ABA-1.

In a second publication, these authors applied the same methodology to 16 novel proteins expressed in conventional crops (Kleter and Peijnenburg 2003). These novel proteins were derived from two sources. First, open reading frames that code for novel proteins and that have been created by natural recombination in mitochondrial DNA have been identified in cytoplasmic male-sterile plants that are used for commercial hybrid breeding. Second, some crops have been found to contain double-stranded, self-replicating RNA (dsRNA), which is probably derived from a viral ancestor and has lost its infective capacities. Several protein sequences encoded by open reading frames on these RNA molecules have been described. These types of open reading frames created by recombination or from exogenous sources may be considered natural forms of genetic engineering.

As previously observed for the proteins expressed in genetically modified crops, the novel proteins from mitochondrial DNA and dsRNA share identical stretches of 6 and 7 contiguous amino acids with allergenic proteins. Four cases of identity were selected that were considered to be of special interest. For example, the ORF138 protein from the mitochondria of *Brassica* species contains three stretches of the sequence KEEKKE. One copy of this sequence is also present in the grass-pollen allergen Phl p 13. The KEEKKE sequence was found to be the antigenic determinant site of both proteins as predicted by the Hopp and Woods antigenicity prediction method. In addition, the KEEKKE sequence is present as multiple copies within ORF138. If KEEKKE were truely a linear IgE-epitope of Phl p 13, ORF138 might be able to bind multiple IgE antibodies and cross-link them on the surface of mast cells, which would trigger an allergic reaction in grass-pollen-allergic patients. The KEEKKE sequence also corresponds to the KXEE/KEXE (X=empty, T, or A) -motif sequence that is recognized by IgE-antibodies from the sera of mice immunized with the latex allergen Hev b 5. Similar to the study on the proteins from transgenic crops, it was recommended that positive results with the mitochondrial and dsRNA-derived proteins should be further confirmed by clinical tests (Kleter and Peijnenburg 2003).

The authors also discussed the implications of these findings for the safety assessment of conventional crop plants compared to the assessment of their transgenic counterparts. It was recognized, among other things, that many mutations in conventional crops that may give rise to novel proteins remain unknown. Therefore, a holistic approach should be followed to identify potential novel allergens, such as the use of proteomics with protein detection by sera from allergic patients. Interestingly, holistic approaches for identification of potential novel allergens are currently explored within the EU-funded project GMOCARE.

**Similarities of larger segments**

Several recent studies have focused on the similarity of larger segments of test proteins with allergenic proteins. As noted above, the Codex alimentarius guidelines recommend a 35% threshold for identical amino acids in 80-amino-acid segments of a novel protein’s sequence aligned to that of an allergenic protein.

In his review on structural characteristics of allergenic proteins, Aalberse (2000) mentions, however, that allergic cross-reactivity of proteins is rare below 50% identity of their amino-acid sequences. Hileman et al. (2002) compared the outcomes of FASTA alignments of allergenic sequences with the sequences of 6 Cry proteins (from *Bacillus thuringiensis*, comparable to proteins expressed in GM crops), 3 dietary

proteins and 50 randomly selected proteins from maize. Interestingly, the authors concluded that no significant structural homologies were found between the Cry proteins and allergens, whereas the maize proteins generated a number of positive outcomes that would warrant further clinical testing.

Some authors have described methods that would further improve the FASTA alignment recommended by the Codex alimentarius. Zorzet et al. (2002)), for example, developed a bioinformatic learning approach based on the FASTA algorithm coupled to a neural-network algorithm. By training with FASTA alignments of allergenic and non-allergenic proteins, as well as different subgroups of allergenic proteins, a system was created that successfully identified the 2S albumin from Brazil nut as an allergen. Stadler and Stadler (2003) created 50-amino-acid fragments, which are smaller than protein domains, from allergenic protein sequences and screened these fragments for motifs that were shared by the allergens. Most of these motifs could be related to allergenic protein families, such as pathogenesis-related proteins and profilins.

## *In vitro* digestilibity

As noted above, the lack of *in vitro* digestibility of proteins is considered a factor that may predispose proteins to become an allergen. The Codex alimentarius guidelines recommend an *in vitro* incubation of a novel protein with simulated gastric fluid. This fluid contains diluted hydrochloric acid with the proteolytic enzyme pepsin. Samples from the incubation mixture may be taken after certain incubation periods and analysed for the integrity of the protein. The guidelines also mention that a lack of resistance to digestion alone does not exclude the potential allergenicity of a novel protein.

Recently, a report by Fu et al. (2002) compared the *in vitro* digestibility of allergenic proteins (for example, alpha-lactalbumin) with that of proteins with unproven allergenicity (for example, spinach-leaf rubisco). They observed that degradation of the proteins in simulated gastric fluid did not predict their allergenicity, neither could it predict the prevalence of allergies against the tested allergenic proteins. Interestingly, these authors also observed that the pepsin:protein ratio could greatly influence the outcomes of the observed periods of stability. These authors therefore called for standardization of the digestibility assay. In fact, the Codex alimentarius guidelines also call for a consistent and well-validated protocol for the simulated gastric fluid assay. The article by Fu et al. (2002) was criticized by others, among other things because of the choice of proteins used in their study (Taylor 2003; Fu, Abbott and Hatzos 2003).

Takagi et al. (2003) also performed a study on the *in vitro* digestibility of allergenic proteins and proteins with unproven allergenicity (including a protein that is expressed in GM crops) in simulated gastric fluid after heat treatment. It was found that previous heat treatment markedly influenced the digestibility of some proteins. In addition, the proteins with unproven allergenicity were more digestible than the allergenic proteins. These authors conclude that information about food-processing conditions may be necessary to assess the potential allergenicity of proteins.

Another recent study describes kinetics studies of the degradation of two Cry-proteins (Cry34Ab1 and Cry35Ab1) from *Bacillus thuringiensis* in simulated gastric fluid (Herman et al. 2003). The intensity of the stained bands of the Cry-proteins on electrophoresis gels of samples taken at various time intervals was determined. Based on these time series, the half-lives and times till 90% degradation were determined

according to first-order decay kinetics. This methodology would allow for comparison of the relative stabilities of different proteins.

TNO Food and Nutrition Research has developed an instrument that mimics the whole gastrointestinal tract. This instrument simulates peristaltic movements and is filled at specific sites with simulated fluids. In addition, at specific sites of the tract samples may be taken for analysis. The model can be adapted to resemble digestion in humans and various animals of interest. Such models may prove useful in providing insight into the gastro-intestinal behaviour of test compounds without the use of laboratory animals.

## Conclusions

No single analysis at the moment can be completely predictive of the allergenicity of a novel protein. A 'weight of evidence' approach is recommended by the Codex alimentarius guidelines to assess the risk of allergenicity of GM foods. Current research activities focus on the improvement and refinement of the various components of this weighted-evidence approach, including bioinformatics, digestibility and animal models.

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