

## Allergenicity assessment

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### 1 Introduction<sup>3</sup>

Allergenicity is defined in this chapter as the capacity to elicit an IgE immune response upon animal or human immunisation or exposure. All newly expressed proteins<sup>4</sup> in genetically modified plants that could be present in the final food or feed destined for human or animal consumption should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply carries the risk to cause allergic sensitisation and to induce allergic reactions in some individuals. The necessity to test for allergenicity of genetically modified organisms destined for animal consumption is supported by the possibility to find back the transgenic protein in animal-derived products for human consumption, such as milk or eggs.

At present, there is no single definite test that can be relied upon to predict allergic responses in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case-by-case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

The endpoint of the assessment is a conclusion as to the likelihood of the protein for being a food allergen. The decision tree as enclosed in annex 1 to this chapter will be helpful to determine the endpoints but is not to be strictly followed.

### 2 Assessment Strategy

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known

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<sup>3</sup> The expert consultation group 'Allergenic aspects of genetically modified foods/feeds' of the Biosafety Council formulated the Belgian comments to the Ad Hoc Open-Ended Working Group on Allergenicity which convened in Vancouver in September 2001. This Working Group was established by the Codex Ad Hoc Intergovernmental Task Force on Foods derived from Biotechnology in order to develop detailed guidelines for the assessment of potential allergenicity of genetically modified foods (FAO/WHO, 2002). The text, as described below, is based on these guidelines which have been further elaborated and completed by the expert consultation group of the Biosafety Council.

<sup>4</sup> This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or acid treatment.

As there is no single test that can predict the risk of human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the genetically modified plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and/or biochemically equivalent to that produced in the genetically modified plant. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

The level of a protein in a food cannot be taken as a criterion for assessment of allergenicity. The first reason is that there is no scientifically determined minimal level of exposure for an allergic reaction. Moreover, nobody can ascertain that the level of expression of a transgene-encoded protein cannot be increased under certain circumstances (climatic, soil,...) so that it becomes life-threatening for the allergic individuals.

### **3 Initial Assessment**

#### **3.1 Source of the Protein**

As part of the data supporting the safety of foods derived from genetically modified plants, information should contain any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated allergy is available after inhalation, ingestion or skin contact with any part of the organism. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and incidence of allergic reactions, prevalence of occupational allergy (inhalation/worker exposure); physicochemical (structural characteristics and amino acid sequence) and immunological properties (when available) of known allergenic proteins from that source.

#### **3.2 Amino Acid Sequence Homology**

The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is homologous in sequence to a known allergen (food, respiratory or any other type). This information may suggest whether that protein has an allergenic potential. Sequence homology searches of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural homologies. Strategies such as stepwise contiguous identical amino acid segment

searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results<sup>5</sup>. Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% homology in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding specifically with IgE antibodies. Most of B cell epitopes of soluble proteins, and in particular epitopes recognized by IgE antibodies, are made of amino acid residues located at distance on the protein and brought together by the tertiary conformation of the molecule. The only way to determine which amino acids are involved in the epitopes is by elucidating the 3-D structure through crystallography and X-ray structure. The number of allergens that have been crystallized today is very limited and, apart from phospholipase A2, Der p 2 and Bet v1, crucial information is therefore lacking.

A negative sequence homology result based on the findings of a less than 50% homology between sequences of 6 to 8 amino acids of the newly expressed protein and of known allergens, indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein carries the risk to be an allergen. If the product is to be considered further, it should be submitted to serum screening using serum from individuals sensitized to the homologous allergen.

### 3.3 Pepsin Resistance

Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential<sup>6</sup>. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen and that pepsin digestion might reveal allergenic epitopes. Moreover, the wide use of proton-pump inhibitors to reduce gastric acidity, and thereby the efficiency of pepsin digestion, further reduces the relevance of pepsin

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<sup>5</sup> It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

<sup>6</sup> The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood et al. 1996).

resistance assays in the evaluation of the allergenicity potential of newly expressed proteins.

Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Additional protocols may be used where adequate justification is provided.

## 4 Serum Screening

For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed.

Sera from individuals with a clinically validated allergy to the source of the protein or to the allergen with sequence homology can be used to test the specific binding to IgE class antibodies of the protein in a specific serum screen test. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result.

The search for the presence of IgE antibodies to the protein of interest can be carried out for instance by dot blotting. This consists in first applying drops of the protein solution onto a membrane and let the protein bind to it. Next, protein dots are incubated with the sera (one dot for one serum to be tested), and bound IgE are detected by using a system of labelled IgE-specific antibodies. Demonstration of IgE binding indicates that the target carries the risk to be an allergen. The assay procedure needs to be set up on a case-by-case basis to produce a valid test result with limited risk of false negative and false positive reactions. Critical parameters are the amount of protein, the volume of serum, and the detecting system of antibodies. Serum screening should be performed on the purified transgene-encoded protein. If the protein cannot be purified from the transgenic plant and the protein is glycosylated in its natural form, a recombinant form obtained in another high-producing system than bacteria, for example yeast, allowing glycosylations, should be considered. The purification procedure should not exclude any glycovariant of the target protein. It will also be required to compare intact, pepsin digested and heat denatured proteins for IgE binding.

As for the number of sera that should be used for testing, twenty-four different well documented sera is statistically enough to detect an allergen with a 99% confidence interval. One positive serum out of at least 24, if available, is enough to declare that the protein carries the risk to be allergenic.

For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, as well as in the case of a negative outcome of a specific serum screening, targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of allergens) may be considered.

In the case of a newly expressed protein derived from a known allergenic source, a negative result in in vitro immunoassays may not be considered sufficient, but should prompt additional testing. The search for the presence of specific IgE antibodies can be completed by a search for the capacity to activate human basophils, using, for instance, whole blood assays. Additionally, a passive cutaneous anaphylaxis test (PCA) can be carried out, in which the serum containing the putative IgE antibodies is injected into the abdominal skin of rats, which are later challenged by i.v. injection of the protein under scrutiny and a dye to visualize the skin extravasation reaction.

Alternatively, a rat basophil leukaemia (RBL) cell line that is transfected with the alpha chain of the human high-affinity receptor for IgE could be used to avoid the necessity to prepare fresh basophils from the peripheral blood of human subjects. In such a system, RBL are passively sensitized by incubation with human serum containing putative IgE antibodies. RBL are then washed and incubated with different concentrations of the protein under scrutiny. Activation of RBL is followed by measuring the production of  $\beta$ -hyaluronidase. A positive result in such tests would indicate a potential allergen.

## 5 Additional tests

After the first two screening steps which are the essential components of the assessment strategy for possible allergenicity, a number of other analyses characterising the properties of the protein should be recommended which further document and strengthen the status of “non-allergenic” proteins.

### ***T-cell epitope search***

It may be important to determine the possible sharing of T-cell epitopes between transgene-encoded proteins and allergens. Recent evidence shows that, on the one hand, the T-cell receptor (TCR) recognizes more a conformation than an actual sequence and, on the other hand, that tightness and duration of contact between the TCR and the peptide are more important than recognition itself. The lack of sequence homology between a new transgene-encoded protein and a known allergen offers no valuable information to determine as to whether a new transgene-encoded protein presents a risk of being allergenic. Recent databases nevertheless offer the possibility to explore in more details the interaction between peptides and MHC class II molecules and are based on virtual matrices in which the contribution of each amino acid with each pocket of the MHC molecule is quantified (see for instance Hammer et al, 1994 and the TEPITOPE database).

### ***Animal models***

The use of animal models does not seem to be useful to identify allergenic proteins in IgE-mediated allergy because MHC restrictions of immune responses preclude any conclusion. Nevertheless, if animal models for the identification of protein allergens are further developed and validated, the use of animal models can be considered as an enhancing step in the weight-of-evidence approach.

For the other types of sensitivity, animal tests should be considered along with the information provided in Chapter III, Section 3.4.5.

### ***Testing of the whole genetically modified plant***

Another problem that should be considered in the allergenicity assessment of genetically modified foods/feeds is that the insertion of a new gene might also increase the level of expression of proteins naturally present in the conventional plant. Therefore, if the host contains allergenic proteins, the expression level of such allergens might be increased and such plants in general might become more allergenic. In this case the whole genetically modified plant or crop should be assessed for allergenicity.

## 6 Recommendations

Sequence homology searches should be performed by an independent organisation (possibly designated by the organisation in charge of the safety evaluation).

Serum screening and purification of the transgene-encoded protein should preferentially be carried out by an independent laboratory.

To allow serum screening, steps should be taken and funding should be raised to organise an international serum bank, linked to a facility that also will be able to perform the testing. Banks could possibly be raised on regional scale (South- and North-America, Europe, Africa, Asia, etc.) within a framework. The advantage of an international input of serum samples is the increased likelihood of containing IgE antibodies against a wide variety of proteins, to which people in certain areas of the world are more (or even selectively) exposed.

## 7 Conclusions

Proteins that are positive in the sequence homology search or serum screen test should be considered as allergenic or at least as carrying the risk to be allergenic. Transgenic crops of which the newly expressed proteins are allergenic or at least carry the risk to be allergenic should not be approved for marketing.

When sequence homology analysis and serum screening tests are negative, the protein can be considered as being probably non-allergenic. However, this can never mean that the protein is definitively considered as such, especially as many of these proteins have never been inhaled or ingested by humans before. Post-marketing surveillance on the occurrence of allergy should therefore be strongly supported.

If a protein shows pepsin resistance, or contains T-cell epitopes cross-reacting with epitopes of known allergens, post-marketing surveillance should be exerted.

## 8 References

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## Annex 1

### Decision Tree for the assessment of possible allergenicity (proteins) (adapted FAO/WHO 2001 Decision Tree)

