

Application of the IFBC/ILSI Allergenicity Assessment Decision Tree An Industry Perspective

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ABSTRACT: Three case studies are described; each represents a specific application of the IFBC/ILSI decision tree as prescribed by Metcalfe *et al.*¹ to assess the allergenic potential of foods derived from genetically engineered crop plants. The case studies include high methionine soybean, high oleic soybean and Herculex TM 1 Insect Protected Maize, the results of which support/validate the use of the IFBC/ILSI decision tree for the prescribed applications.

KEYWORDS: allergenicity assessment; Cry1F protein; genetically engineered crop plants; high-methionine soybean; high-oleic acid soybean

INTRODUCTION

The International Food Biotechnology Council (IFBC) and Allergy and Immunology Institute of the International Life Sciences Institute (ILSI) published a report in 1996 that included a proposed “decision tree” or step-wise strategic approach for assessing the allergenic potential of foods derived from genetically engineered crop plants; specifically those mediated by type I IgE immunologic reactions. The scientific basis for the development of, and details pertaining to, the decision tree (shown in FIG. 1) are described in Metcalfe *et al.*¹

This presentation has two objectives: (1) To provide a description of the results of three case studies. Each case study represents a specific application of the IFBC/ILSI decision tree as prescribed by Metcalfe *et al.*¹ (2) Provide an opinion of the validity of each application of the decision tree based on the case study results.

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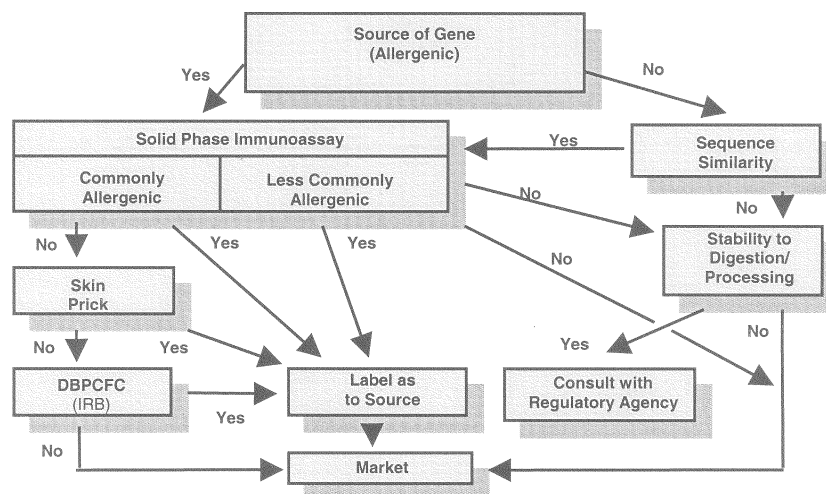


FIGURE 1. IFBC/ILSI assessment decision tree for potential food allergens. (From Metcalfe *et al.*¹ Used with permission.)

CASE STUDIES

Three case studies will be described. The product and specific application of the IFBC/ILSI decision tree are as follows:

- High-methionine soybean: This case study describes application of the decision tree to a food containing a gene derived from a commonly allergenic food.
- High-oleic acid soybean: This case study describes application of the decision tree to a food from a host plant that is known to contain endogenous allergens.
- Cry1F protein: The active ingredient in Herculex™ 1 Insect Protected Maize. This case study describes application of the decision tree to a food containing a gene derived from a source that has no history of allergenicity.

HIGH-METHIONINE SOYBEAN

Soybean (*Glycine max*) is deficient in the essential amino acid methionine; diets based on soybean meal must be supplemented with synthetic methionine or other protein sources containing this amino acid in order to achieve op-

timum growth and development in domestic animals. Brazil nut (*Bertholletia excelsa*) seeds contain high levels of sulfur amino acids, and protein extracts from this source have been used as methionine supplements in human and animal diets.^{2,3} In order to improve the methionine content of soybean meal specifically for use in poultry and swine diets, the gene for the Brazil nut 2S seed storage albumin protein (contains ~18% methionine) was introduced into soybean; this gene encodes a 17-kDa precursor polypeptide that is processed to 9-kDa and 3-kDa subunits via a 12-kDa intermediate. Analysis of the high-methionine soybean (HMS) seed showed that the 2S albumin comprised 4–8% of the salt-soluble protein fraction, representing an ~26% gain in available methionine.

Tree nuts represent one of the commonly allergenic food groups,¹ and allergic reactions specific to Brazil nut have been reported.^{4,5} The assessment procedure prescribed by the IFBC/ILSI decision tree for genes from a source of this type was followed. Initial studies employed radioallergosorbent (RAST) inhibition assay and SDS-PAGE/Western blot procedures. Sera for these studies were obtained from subjects with a history of allergic symptoms to Brazil nut and positive responses in RAST and skin-prick tests with Brazil nut protein and extracts, respectively. Control serum was obtained from subjects with no history of food allergy. In the RAST inhibition assay, serial dilutions of Brazil nut, control (nontransgenic) soybean, and HMS extracts were incubated with Brazil nut protein in the solid phase and a serum pool from Brazil nut-allergic subjects. After overnight incubation, the solid phase was washed, incubated overnight with anti-human IgE labeled with iodine-125, rewashed, and counted for radioactivity. The SDS-PAGE/Western blot procedures involved separation of Brazil nut, control soybean and HMS extracts, and Brazil nut 2S albumin using gels with gradients of 10 to 20%; transfer of the proteins to nitrocellulose; and incubation of the blots with control serum or serum from Brazil nut-allergic subjects. IgE binding was detected using autoradiography after incubation with anti-human IgE labeled with iodine-125. The *in vitro* tests were followed by the conduct of a skin-prick test in human volunteers: three with a history of sensitivity to Brazil nut and no sensitivity to soybean and three with a history of no sensitivity to Brazil nut or soybean. All subjects gave informed consent as required by the review boards of the institutions involved in study conduct. Serial dilutions of sterile Brazil nut, control soybean, and HMS extracts were employed with all subjects. Details of materials and methods are fully described by Nordlee *et al.*⁶

The results of the RAST inhibition assay showed that the HMS extract was able to compete effectively with Brazil nut protein extracts bound to a solid phase for binding to IgE from Brazil nut-sensitive subjects. The slope of inhibition was similar for the HMS and Brazil nut extracts (FIG. 2). No inhibition was observed with control soybean extracts. The results from the SDS-PAGE/Western analysis showed that sera from subjects allergic to Brazil nut recognized Brazil nut 2S albumin. Individual sera also reacted predominantly

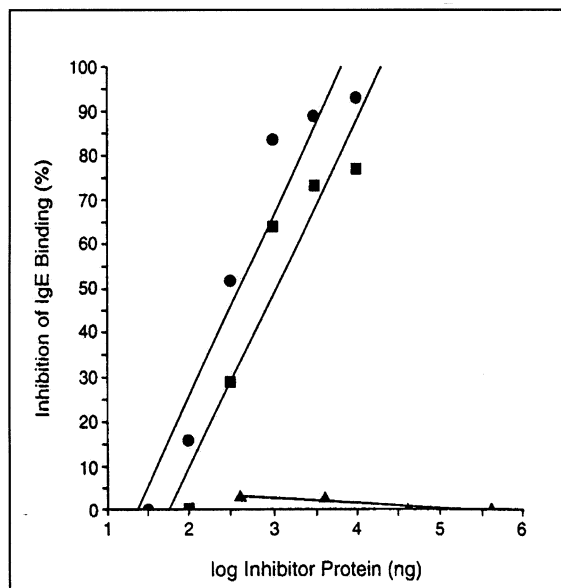


FIGURE 2. Results of RAST inhibition assays with extracts of control soybean (▲), HMS (■), and Brazil nut (●). (From Nordlee *et al.*⁶ Used with permission.) (Identification of a Brazil-nut allergen in transgenic soybeans, Nordlee *et al.* Copyright ©1996, Massachusetts Medical Society. All rights reserved.)

with a 9-kDa protein in both Brazil nut and HMS extracts that comigrated with the Brazil nut 2S albumin protein and that was absent from control soybean extracts (FIG. 3). Sera from nonatopic control subjects did not react with Brazil nut or soybean extracts (data not shown).

The three subjects with histories of hypersensitivity to Brazil nut and no hypersensitivity to soybean all exhibited positive reactions to skin-prick testing of Brazil nut and HMS extracts. All three subjects exhibited positive responses to histamine and negative responses to diluent and control soybean extract (TABLE 1).

The three control subjects did not react to extracts of Brazil nut, HMS, control soybean, or diluent. All had a positive response to histamine (data not shown). Full details of the results are described by Nordlee *et al.*⁶

On the basis of the results of these studies, it was concluded that the 2S albumin from Brazil nut was likely a major allergen. As a consequence, product development of HMS was discontinued; that is, it was not commercialized.

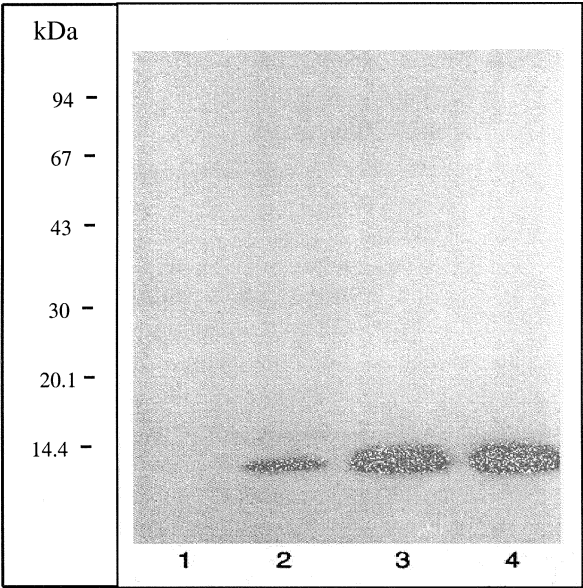


FIGURE 3. Western blots of control soybean (*lane 1*), HMS (*lane 2*), Brazil nut (*lane 3*), and 9-kDa Brazil nut 2S albumin protein (*lane 4*). (From Nordlee *et al.*⁶ Used with permission.) (Identification of a Brazil-nut allergen in transgenic soybeans, Nordlee *et al.* Copyright ©1996, Massachusetts Medical Society. All rights reserved.)

TABLE 1. Results of skin-prick tests in subjects allergic to Brazil nut

Subject number	Diluent	Histamine	Control soybean extract	HMS extract ^a	Brazil nut extract ^a
1	0/0∇ ^b	6/10	2/5	15/35	10/30
2	1/0	7/19	2/0	9/29	7/26
3	0/0	5/20	2/5	14/41	8/54

^aDilution of extract: 1:1000, 1:10,000, and 1:1,000,000 for subjects 1, 2, and 3, respectively.
^b∇ Refers to maximal wheal diameter (mm)/maximal flare diameter (mm).
SOURCE: From Nordlee *et al.*⁶ Used with permission. (Identification of a Brazil-nut allergen in transgenic soybeans, Nordlee *et al.* Copyright ©1996, Massachusetts Medical Society. All rights reserved.)

HIGH OLEIC ACID SOYBEAN

Soybean oil has poor oxidative stability as the result of high levels of poly-unsaturated fatty acids (linoleic [18:2] and linolenic [18:3] acids). In order to improve the functional performance (i.e. oxidative stability) of soybean oil for food-processing applications, the gene (*GmFad 2-1*) for soybean δ-12 de-

saturase was introduced into soybean with the objective of coordinately silencing gene expression (i.e. "cosuppression") of the introduced and endogenous *GmFad 2-1* genes. δ -12 desaturase catalyzes polyunsaturated fatty acid synthesis via the conversion of oleic acid (18:1) to linoleic acid (18:2).⁷ The *GmFad 2-1* gene is only expressed in developing seed^{8,9}; a soybean seed-specific promoter was therefore employed to control expression of the introduced gene. Oil from high-oleic acid (HOS) lines had increased oleic acid content (82–85%; control soybean level ~23%), decreased linoleic and linolenic acids content, and increased oxidative stability versus that from control (nontransgenic) lines.

Metcalf *et al.*¹ propose that the key determinant for the analysis of the host plant for endogenous allergens is the nature of the genetic modification. The seed-specific promoter introduced into HOS was that for the soybean α' subunit of β -conglycinin. Promoter co-suppression has been reported for other gene/plant combinations.¹⁰ β -Conglycinin (7S globulin) and glycinin (11S globulin) represent the two major seed storage proteins in soybean¹¹ and have been identified as endogenous soybean allergens.¹ β -conglycinin comprises various combinations of three subunits denoted α , α' , and β . Glycinin comprises various combinations of five subunits; each subunit is composed of one acidic and one basic polypeptide linked by a disulfide bond. Analysis of soybeans occurring either naturally or produced via induced mutations has shown that lines lacking the α' subunit of β -conglycinin have low levels of the α and β subunits of β -conglycinin, and a negative correlation exists between low β -conglycinin levels and glycinin content.^{12,13} Because protein products derived from HOS were expected to enter food (i.e., as isolates/concentrates) and feed (i.e., as meal) channels, host plant analysis was performed.

The β -conglycinin and glycinin content of HOS and control (nontransgenic) soybean was determined using densitometry measurements of SDS-PAGE gels and Western blots; β -conglycinin and glycinin IgG-detecting antibodies were employed for the latter. A RAST inhibition assay and SDS-PAGE/Western blot procedures were employed to assess the overall allergenic activity of HOS and control soybean. Sera for these studies were obtained from subjects with a history of allergic symptoms to soybean and positive responses in RAST and/or skin-prick tests with soybean extracts. Control serum was obtained from subjects with negative RAST and/or skin-prick tests to soybean extract and with total IgE levels similar to those sera from soybean-sensitive subjects. All subjects contributing serum gave informed consent as required by the review boards of the institutions responsible for sample collection. Individual sera from seven subjects were pooled for the RAST inhibition assay; all sera had IgE reactivity to soybean extracts >20% in RAST. Serial dilutions of HOS and control (nontransgenic) soybean extracts were incubated with control soybean protein in the solid phase and the serum pool from soybean-allergic subjects. Following overnight incubation, the solid phase was

TABLE 2. β -Conglycinin and glycinin levels in control soybean and high oleic acid soybean

β -Conglycinin			Glycinin		
Subunit	Control soybean ^a	HOS ^b	Subunit	Control soybean ^a	HOS ^b
α	5.4	ND	Acidic	34.9	36.2
α'	8.8	0.5	Basic	23.6	28.3
β	3.6	5.7	Precursor	2.9	7.1
Total	17.8	6.2	Total	61.4	71.6

NOTE: Percent total protein per lane determined by densitometer measurements of Coomassie blue R stained gels (total protein: 20 μ g/lane).

^aLine A2396.

^bMean of lines G94-1, G94-19, and G168. HOS = high oleic acid soybean.

ND: not detected.

washed, incubated overnight with anti-human IgE labeled with iodine-125, rewashed, and counted for radioactivity. The SDS-PAGE/Western blot procedures involved separation of serial dilutions of HOS and control soybean extracts using SDS-PAGE, transfer of the proteins to nitrocellulose, and incubation of the blots with control serum or serum from soybean-allergic subjects. IgE binding was detected using autoradiography after incubation with, in the following order, anti-human IgE conjugated to alkaline phosphatase, Nitroblock[®] chemi-luminescence enhancer, and CSPD.

Densitometry measurements of SDS-PAGE gels of HOS and control soybean showed that β -conglycinin levels were reduced in HOS (6.2% of total protein [TP]) versus control soybean (17.8% of TP). This reduction was related completely to changes in the levels of the β -conglycinin α and α' subunits, which were not measurable and reduced to almost zero (0.5%), respectively. The level of the β -conglycinin β subunit was increased slightly in HOS (5.7% of TP versus 3.6% of TP in control soybean). Glycinin levels were increased in HOS (71.6% of TP) versus control soybean (61.4% of TP); this increase was related predominantly to elevations in glycinin basic and precursor proteins (TABLE 2). The decrease in β -conglycinin was almost entirely compensated for by the increase in glycinin. Western blot analysis confirmed the above results; using this more sensitive technique, the levels of the β -conglycinin α and α' subunits were shown to be reduced 11- to 100-fold in HOS lines versus control soybean. The levels of β -conglycinin and glycinin observed in control soybean were comparable to those previously reported.¹¹ The observed levels of β -conglycinin and glycinin in HOS have also been documented in one soybean variety (Altona) developed by conventional breeding.¹⁴

The results of the RAST inhibition assay showed that the HOS and control soybean extracts competed similarly with control soybean protein extracts

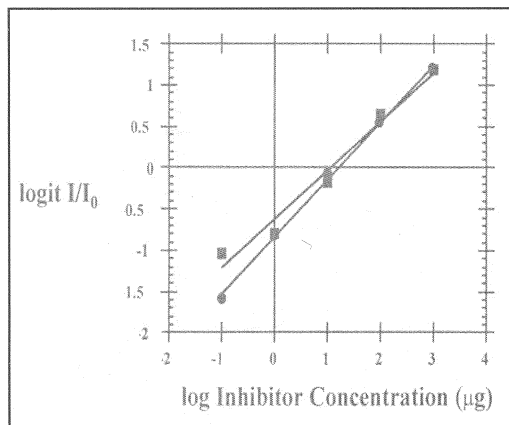


FIGURE 4. Results of RAST inhibition assay with extracts of control soybean (■) and HOS (●).

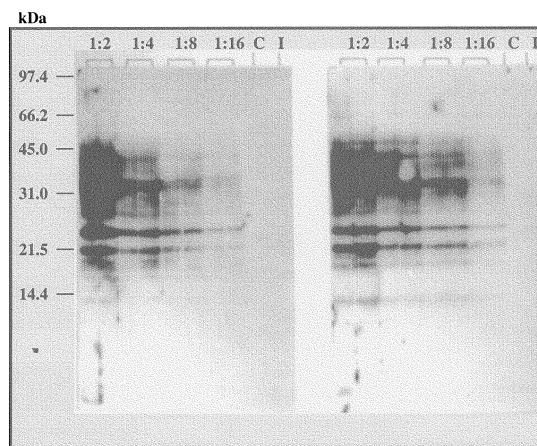


FIGURE 5. Western blot of control soybean (*left*) and HOS (*right*). Dilution series 1:2 → 1:16. C = negative control; I = positive stain (India ink).

bound to a solid phase, for binding to IgE from soybean-sensitive subjects. The slope of inhibition was similar for the HOS and control soybean extracts (FIG. 4). The results from the SDS-PAGE/Western analysis showed that sera from subjects allergic to soybean produced no material differences in the number of bands recognized or the intensity of recognition between HOS and control soybean extracts (FIG. 5). Sera from nonatopic control subjects did not react with either soybean extract (data not shown).

On the basis of the results of these studies, it was concluded that the ratio of β -conglycinin to glycinin was altered in HOS; the observed decrease in β -conglycinin was compensated for by an equivalent concomitant increase in glycinin, and similar levels of the two proteins have been documented in soybean varieties produced by conventional breeding. RAST inhibition assay and Western analysis results employing sera from subjects allergic to soybean showed no demonstrable differences in the overall allergen content between HOS and control soybean.

CRY1F PROTEIN

Maize (*Zea mays*) has limited natural resistance to the European corn borer, a major lepidopteran insect pest for this crop. Control of this pest can be achieved via the use of insecticides, which requires extensive scouting and timely applications to be effective; however, this is largely limited to the first of multiple generations. In order to provide full season *in planta* control, the gene for Cry1F, derived from *Bacillus thuringiensis* var. *aizawai*, was introduced into maize; this gene expresses an insecticidal protein, the active ingredient in Herculex™ 1 Insect Protected Maize. European corn borer, southwestern corn borer, black cutworm, and fall armyworm are effectively controlled with this product.

The assessment procedure prescribed by the IFBC/ILSI decision tree for genes from a source of this type (i.e., those with no history of allergenicity) was followed. An *in silico* comparison of the amino acid sequence of Cry1F to known allergenic proteins for eight contiguous amino acid matches was conducted using the FASTA algorithm¹⁵ following retrieval of allergen sequences from public domain databases. An *in vitro* simulated gastric fluid (SGF) assay of microbial Cry1F was performed.¹⁶ The microbial Cry1F was biochemically and biologically equivalent to plant-derived Cry1F. The degree of Cry1F degradation was examined after incubation times of 0, 1, 2, 4, 10, 15, 30, and 60 minutes using SDS-PAGE. The thermolability of Cry1F was assessed by incubating microbial Cry1F at temperatures of 4°, 60°, 75°, and 90°C for 30 minutes; the purpose of this experiment was to provide an initial indirect assessment of one processing factor on Cry1F activity. Following treatment, Cry1F activity was determined using a tobacco budworm neonate larva bioassay. Glycosylation of plant-derived Cry1F was examined using a Bio-Rad® Immuno-Blot kit following SDS-PAGE and transfer to nitrocellulose. Ovalbumin was used as a positive control.

The Cry1F protein had no amino acid sequence identity with known allergens and was found to be nonglycosylated. The Cry1F protein degraded completely in less than one minute in an *in vitro* SGF assay, and activity toward

TABLE 3. Summary of experimental results obtained in studies of the Cry1F protein

Experiment endpoint	Result	Comments
Homology to known allergens	No	NA
Glycosylated	No	NA
Digestible, <i>in vitro</i> (SGF)	Yes	< 1 minute
Thermolabile	Yes	Activity eliminated after treatment at $\geq 75^{\circ}\text{C}$ for 30 minutes

tobacco budworm neonate larvae was eliminated following heat treatment at $\geq 75^{\circ}\text{C}$ for 30 minutes (TABLE 3).

On the basis of the results of this “weight-of-evidence” assessment, it was concluded that the Cry1F protein does not share the general genetic and physicochemical properties associated with food allergens. An independent assessment of this information concluded that the potential for the Cry1F protein to be a food allergen was minimal.¹⁷

Overall, the results of these studies support/validate the proposed approach for food allergenicity assessment prescribed by Metcalfe *et al.*¹ for the specific applications described herein.

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REFERENCES

1. METCALFE, D.D., J.D. ASTWOOD, R. TOWNSEND, *et al.* 1996. Crit. Rev. Food Sci. Nutr. **36(S)**: S165–S186.
2. ATUNES, A.J. & P.J. MARKAKIS. 1977. Agric. Food. Chem. **25**: 1096.
3. TAO, S-H. *et al.* 1987. Fed. Proc. **46**: 891.
4. GILLESPIE, D.N., M.D.S. NAKAJIMA & M.D. GLEICH. 1976. **57**: 302.
5. ARSHAD, S.H., E. MALMBERG, K. KRAFT & D.W. HIDE. 1991. Clin. Exp. Allergy **21**: 373.
6. NORDLEE, J.A., S.L. TAYLOR, J.A. TOWNSEND, *et al.* 1996. N. Engl. J. Med. **334**: 688–692.
7. KINNEY, A.J. 1994. Curr. Opin. Biotechnol. **5**: 144–151.
8. OKULEY, J.T., J. LIGHTNER, K. FELDMAN, *et al.* 1994. Plant Cell. **6**: 147–158.
9. HEPPARD, E.P., A.J. KINNEY, K.L. STECCA & G-H. MIAO. 1996. Plant Physiol. **110**: 311–319.

10. BRUSSIAN, J.A. & E.M. TOBIN. 1995. *Plant Mol. Biol.* **27**: 809–813.
11. MURPHY, P.A. & A.P. RESURRECCION. 1984. *J. Agric. Food Chem.* **32**: 911–915.
12. TAKAHASHI, K., H. BANBA, A. KIKUCHI, *et al.* 1994. *Breeding Sci.* **44**: 65–66.
13. KITAMURA, K. 1995. *Jpn. Agric. Res. Q.* **29**: 1–8.
14. NORDLEE, J.A. 1995. Masters thesis, University of Nebraska, Lincoln, Nebraska.
15. PEARSON, W. & D. LIPMAN. 1988. *Proc. Natl. Acad. Sci. USA* **85**: 2444.
16. UNITED STATES PHARMACOPOEIA. 1990. The National Formulary, USP XXII, NF XVII. U.S. Pharmacopoeial Convention, Inc., Mack Printing Company. Easton, PA. p. 1788.
17. UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. 2001. Bt Plant Incorporated Protectants, Biopesticides Registration Action Document. U.S. E.P.A. Washington, DC. pp. 1–50.

