

Original article

Allergenicity of varieties of soybean

Background: Soybean hulls (SHs) cause respiratory allergies. This study investigates the allergenicity of soybean varieties (SVs) by *in vivo* and *in vitro* tests. **Methods:** Ten SVs were studied: (a) five with a proved clinical relevance (SVs 1, 2, 3, 4, 5), the last four with a 'dull' phenotype; (b) five of undetermined relevance, three of them (SVs 6, 7, 8) with a 'shiny' phenotype, and two (SVs 9 and 10) with a 'dull' phenotype. Extracts from all 10 SVs were used to skin prick test (SPT) 21 subjects sensitized to SHs. Positive and negative sera pools prepared from sera of subjects sensitized or not to SHs, respectively, were utilized to perform *in vitro* experiments (specific IgE and IgG4 determinations, SDS-PAGE/IgE-Western blot, and IgE-inhibition).

Results: In this study, it was found that 52.4, 52.4, 57.1, 71.4, 80.9, 42.9, 57.1, 71.4, 52.4, and 38.1% subjects had a positive SPT with SVs 1–10, respectively (*P* NS). Specific IgE values to SVs 1–10 obtained with the positive pool are 28.3, 26.4, 29.9, 28.3, 26.8, 4.8, 13.4, 6.7, 24.7, and 17.5% total counts bound, respectively; and specific IgG4 values 0.851, 0.818, 0.721, 1.609, 0.789, 0.617, 0.662, 0.0, 1.127, and 0.934 OD units, respectively; the microgram of protein required to produce 50% inhibition are 2.5, 3.7, 4.5, 2.4, 5, 39.8, 25.2, 25.1, 4.5, and 8.9, respectively. A 7-kDa band is present in all SVs except in those with a 'shiny' phenotype.

Conclusions: The SVs with a 'shiny' phenotype contain less allergens than the other SVs studied, as determined by *in vitro* tests. However, SPT results with the SVs do not differ. Genetic screens should be devised to select plants with reduced, preferably absent, allergenicity, but with a high nutritional value, and this allergenicity should be studied utilizing *in vivo* and *in vitro* tests.

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Dust released into the air during unloading of soybeans (*Glycine max*) from ships was identified as the etiologic agent of asthma outbreaks in Barcelona, Cartagena, and Tarragona, all cities in Spain (1–3). The hull from soybean seeds was identified as the source of aeroallergens responsible for these asthma outbreaks (4), and two major allergens, Gly m1 and Gly m2, were purified (5, 6). Soybean dust was also implicated as the probable cause of epidemic asthma in New Orleans, LA, USA (7). The levels of soybean dust inhalation were also associated with the severity of symptoms of asthma and/or allergic rhinitis, skin test reactivity, and levels of specific IgE and IgG4 to soybean allergens in subjects from rural areas in Argentina (8).

There are more than 200 varieties of soybean, and soybean seeds vary greatly in surface texture and

appearance. During seed maturation, membranous inner epidermis of the endocarp detaches from the pericarp, or pod wall, to cover the seed influencing the luster of the seed surface (9). Different seed luster phenotypes have been described, including 'shiny', 'intermediate', 'dull', 'bloom', and 'dense bloom', and previous work performed by Gijzen et al. (9) suggested that the Gly m1 content is associated with 'dull-seeded' phenotypes, as demonstrated by sodium-dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) and Southern blot analysis. The purpose of this study is to investigate the allergenicity of such different varieties of soybean by *in vivo* and *in vitro* tests.

Materials and methods

Varieties of soybean

The following varieties of soybean were studied: (a) variety 1, which was responsible for an asthma outbreak in Barcelona, Spain; (b) varieties 2–5, transgenic and glyphosate-resistant, with a 'dull' phenotype, associated with sensitization to soybean dust in rural

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HR, histamine rate; mAb, monoclonal antibody; NC, nitrocellulose; PBS, phosphate buffered saline; RIA, radioimmunoassay; SDS-PAGE, sodium-dodecyl sulfate polyacrilamide gel electrophoresis.

Table 1. Characteristics of the varieties of soybean studied

Variety	Source	Variety name	Seed luster	Amount of Gly m1
1	Barcelona	Unknown	Unknown	Unknown
2	Argentina	A-5409-RG	Dull	Unknown
3	Argentina	A-6401-RG	Dull	Unknown
4	Argentina	A-5634-RG	Dull	Unknown
5	Argentina	A-5435-RG	Dull	Unknown
6	Canada	CH-720	Shiny	Trace
7	Canada	A-96-591033	Shiny	Trace
8	Canada	1A-2036	Shiny	Trace
9	Canada	X-785	Dull	Abundant
10	Canada	Harovinton	Dull	Abundant

subjects from Argentina; (c) varieties 6–8, from the Agriculture and Agri-Food Canada collection (Ontario, Canada), grown under controlled conditions, with a 'shiny' phenotype; and (d) varieties 9 and 10, also from the Agriculture and Agri-Food Canada collection, with a 'dull' phenotype. The latest five varieties contained a certain amount of the allergen Gly m1 as determined by SDS-PAGE and Southern blot analysis (9). Varieties with a 'dull' phenotype showed strongly stained protein bands in SDS-PAGE gels, and genomic DNA blots produced strong hybridization signals. On the contrary, varieties with a 'shiny' phenotype showed faint bands and low hybridization signals. Therefore, the 'dull' and 'shiny' phenotypes we associated with 'abundant' and 'trace' content of Gly m1, respectively (9) (Table 1).

Study subjects

Twenty-one Argentinean subjects with IgE-mediated allergic asthma and/or rhinitis (10), exposed to different levels of soybean dust inhalation of different varieties planted locally, with a 'dull' phenotype, and with a positive skin prick test (wheal soybean hulls/wheal histamine ≥ 0.5) and specific IgE to a soybean hull allergen extract from a local variety are included in this study. These subjects participated in a previous epidemiological study, and their clinical characteristics have been previously described (8). Briefly, level of exposure was classified as: (a) direct, subjects with an occupational exposure to soybean dust, which includes farmers, grain elevator workers and individuals working in soybean processing factories; (b) indirect exposure, subjects living in rural areas (less than 2000 inhabitants) near grain elevators or soybean fields; and (c) urban exposure, subjects living in cities or towns without a known source of soybean dust inhalation. These subjects were selected for this study based on their availability to participate. Their inclusion was approved by the University of Rosario Institutional Review Board, and all subjects gave informed consent.

Sera pools

The following sera pools were studied: (a) positive pool, obtained by mixing equal volumes of sera from six subjects from Argentina with IgE-mediated allergic asthma and/or rhinitis and sensitive to soybeans as determined by the skin prick test, exposed to soybean dust from varieties planted locally with a 'dull' phenotype; (b) negative pool, obtained by mixing equal volumes of sera from six atopic subjects from Argentina with IgE-mediated allergic asthma and/or rhinitis not sensitized to soybeans. These sera samples were obtained in the previous epidemiological study described above (8), and were kept frozen at -20°C until tested. Selection of sera samples

to prepare both pools was based on the availability of a certain volume of sera to prepare the pools.

Preparation of soybean hull allergen extracts

Hulls from the 10 varieties of soybean were removed from the seed, ground with a tissue homogenizer, and extracted 1 : 20 (w/v) with 0.2 M ammonium bicarbonate, pH 9, overnight at 4°C . The supernatant was obtained by filtration under vacuum using a $0.44\text{-}\mu\text{m}$ pore size cellulose nitrate filter (Nalgene Company, Rochester, NY) and sterilized through a $0.22\text{-}\mu\text{m}$ pore size filter. Total protein content was measured by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Skin tests

Skin tests with the 10 varieties of soybean were performed on all 21 subjects by the prick technique. Extracts were applied on the forearm and a prick lancet (Hollister Stier) was used to prick the skin. Negative (saline solution) and positive (histamine chloride, 1 mg/ml) controls were used. Each skin test was assessed at 15 min. The measured averages of the largest and orthogonal wheal diameters obtained with the soybean extracts and with the positive control were recorded. The histamine rate (HR) was calculated as the ratio of soybean wheal diameter compared to the histamine wheal diameter. Results were considered positive when the HR was greater than or equal to 0.5.

Immunoglobulin determinations

Specific IgE (radioimmunoassay, RIA) and IgG4 (enzyme-linked immunosorbent assay, ELISA) to the 10 varieties of soybean were performed as previously described (11).

Inhibition experiments

Assays were performed essentially as that for specific IgE determination. A solid phase was prepared by coating the wells with $1\text{ }\mu\text{g}$ of the extract obtained from variety 1, a variety with proved clinical relevance, which was made to react with a mixture of $50\text{ }\mu\text{l}$ of the positive pool diluted to 1 : 4 v/v along with twofold serial dilutions of the 10 soybean allergen extracts. A curve obtained with each diluted extract was plotted and compared with the curve obtained with the extract from variety 1. Results were expressed as the microgram of protein to produce 50% inhibition.

SDS-PAGE/Western blot

SDS-PAGE was carried out in a 5–20% polyacrylamide gradient gel (Ready Gels, BIO-RAD Laboratories, Richmond, CA) according to the discontinuous buffer system of Laemmli (12). Ten micrograms of protein was mixed with $10\text{ }\mu\text{l}$ of 10% mercaptoethanol and boiled for 5 min. A set of molecular weight markers (Prestained SDS-PAGE Standards, low range, BIO-RAD Laboratories) was loaded in a lane adjacent to the hull extract. Electrophoresis was carried out in a Mini Protean II Dual Slab Cell (BIO-RAD Laboratories) at 200 V until the tracking dye reached the bottom of the gel. After electrophoresis, the proteins were immunoblotted.

Transfer of the proteins to $0.2\text{-}\mu\text{m}$ nitrocellulose (NC) membranes (BIO-RAD Laboratories) was carried out at 150 mA constant power for 3 h in a Mini-Trans-Blot Cell (BIO-RAD Laboratories), according to the method of Towbin et al. (13). After the transfer,

the NC membranes were blocked overnight with phosphate buffered saline (PBS)/0.01% Tween 20/3% bovine serum albumin (BSA) (blocking buffer), washed four times with PBS/0.01% Tween 20 (washing buffer), and incubated with both sera pools diluted 1 : 2 with PBS/0.01% Tween 20/1% BSA (diluent buffer) overnight. The NC membranes were washed four times with washing buffer and then incubated overnight at room temperature with a rabbit ¹²⁵I-anti-human IgE (Hycor Biomedical, Garden Grove, CA), diluted to 1 : 20 in a diluent buffer. The NC membranes were again washed, dried and exposed to an X-ray film (Kodak X-Omat, Sigma Chemical Co., St Louis, MO) for 3 days at -80°C. The IgE-binding protein bands were scanned and analyzed using the Kodak Digital Science 1D Image Analysis Software, version 3 (Eastman Kodak Company, Rochester, NY).

Statistical analysis

The chi-square test was used to check the association between positive skin test and seed luster phenotype. An ANOVA was performed to compare mean values of the histamine index obtained with the varieties of soybean. A *P* value <0.05 was considered significant.

Results

In vivo tests

Eleven of 21 (52.4%), 11/21 (52.4%), 12/21 (57.1%), 15/21 (71.4%), 17/21 (80.9%), 9/21 (42.9%), 12/21 (57.1%), 15/21 (71.4%), 11/10 (52.4%), and 8/21 (38.1%) subjects have a positive skin prick test with the allergen extracts prepared from varieties 1–10, respectively (*P* = NS). The histamine indexes obtained with varieties 1–10 are 0.65 ± 0.151 , 0.59 ± 0.158 , 0.64 ± 0.168 , 0.71 ± 0.205 , 0.72 ± 0.260 , 0.73 ± 0.255 , 0.68 ± 0.147 , 0.70 ± 0.189 , 0.65 ± 0.186 , 0.66 ± 0.192 , respectively (*P* = NS).

Twelve of the 21 (57.1%) subjects reacted to five varieties or more. The pathway of reactivity of the 21 individuals to the 10 varieties of soybean is illustrated in Table 2.

In vivo tests

The soybeans with the lowest specific IgE and IgG4 bindings are varieties 6–8 (shiny phenotypes) (Table 3). The amount of protein to produce 50% inhibition is higher for these three varieties than for the other seven, indicating their lower potency (Table 4).

Western blot results (Fig. 1) demonstrate that a 7-kDa band (a), which is probably Gly m1, is present in all varieties of soybean except in those with a 'shiny' phenotype. A 8-kDa band (b), which is probably Gly m2, is present in variety 4 and both varieties with a 'dull' phenotype (varieties 9 and 10). One or two (c and d) additional bands are present in varieties 4 and 10. The three varieties of soybean with a 'shiny' phenotype (varieties 6–8) do not bind specific IgE in the positive pool of sera by Western blot. One of the transgenic

Table 2. Pathway of reactivity of the 21 individuals to the 10 varieties of soybean

Subject	Variety									
	1	2	3	4	5	6	7	8	9	10
1	+	+	+	+	+	+		+	+	+
2	+	+	+	+	+	+	+	+	+	+
3		+	+	+	+		+	+	+	+
4		+		+	+			+		
5					+			+		
6	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
8				+	+			+		
9	+	+	+	+	+	+	+		+	
10	+	+	+	+	+	+	+			
11					+		+			
12	+	+	+	+		+	+		+	
13								+		+
14				+	+					
15								+		
16				+	+		+	+	+	
17										
18	+					+	+	+	+	
19	+				+	+	+	+	+	+
20	+			+	+			+		
21	+	+	+	+	+		+	+	+	+

Table 3. Specific IgE and IgG4 determinations to the 10 varieties of soybean

Variety	IgE (% TCB)		IgG4 (OD)	
	(+) Pool	(-) Pool	(+) Pool	(-) Pool
1	28.3	0.5	0.851	0
2	26.4	0.3	0.818	0
3	29.9	0.4	0.721	0
4	28.3	0.5	1.609	0
5	26.8	0.4	0.789	0
6	4.8	0.5	0.617	0
7	13.4	0.5	0.662	0
8	6.7	0.4	0	0
9	24.7	0.4	1.127	0
10	17.5	0.6	0.934	0

%TCB, % total counts bound to soybean allergens.

OD, optical density.

(variety 4) contains the largest amount of Gly m1 and Gly m2 allergens. The negative pool of sera did not bind to any protein band on the immunoblot (Fig. 2).

Discussion

Soybean is the most important bean in the world economically, representing one of the richest and cheapest sources of protein. Modern agriculture produces large quantities of soybeans, and numerous ways of handling, from harvest to final consumption, produce much grain dust. Asthma outbreaks in different cities of the world, with many deaths, were caused by release of such soybean

Table 4. Inhibition experiments using the Barcelona soybean variety as a solid phase and the 10 varieties as fluid phases

Variety	µg protein to produce 50% inhibition
1	2.5
2	3.7
3	4.5
4	2.4
5	5
6	39.8
7	25.2
8	25.1
9	4.5
10	8.9

dust during the unloading of soybeans from ships in the city harbors (1–3), until measures to avoid the release of soybean dust in the air were implemented (14). However, low levels of soybean allergens are still present in the air and sensitize individuals (14). Soybean hull allergens also cause IgE-mediated allergic hypersensitivity (10) in rural subjects exposed to different levels of soybean dust (8). Therefore, soybean dust exposure is a health hazard, affecting large populations, and the prevention of soybean-induced asthma is an important public health problem.

The demonstration in a previous study that fresh soybeans are less allergenic than stored soybeans suggested that new soybean allergens are created by increases in temperature upon storage and transportation (15). Thus,

it would be possible to reduce soybean allergenicity and prevent soybean respiratory allergies by transporting and storing soybeans under controlled temperature and humidity conditions. As this is cost prohibitive, another strategy to reduce soybean allergenicity could be to create hypoallergenic soybean varieties using bioengineering techniques.

This study is one among the few that compare the allergenicity of plant varieties of different phenotypes utilizing both *in vitro* and *in vivo* tests. The results of this study indicate that the ‘shiny’ soybean phenotype has a lower total allergen content and potency than the ‘dull’ phenotype and the variety associated with an asthma outbreak. Western blot analysis demonstrates that different varieties of soybean contain different amounts of a particular allergen with a molecular weight of 7 K, which is probably Gly m1, as well as other allergens. Although the varieties with a ‘shiny’ phenotype are still allergenic, as shown by skin prick test and specific IgE determinations, no IgE-binding proteins were detected by Western blot, which confirms that Western blot is less sensitive than other solid-phase assays. These *in vitro* test results confirm previous work performed by Gijzen et al. (9), which suggested that the Gly m1 content is associated with ‘dull-seeded’ phenotypes, as determined by SDS-PAGE and Southern blot analysis. However, the *in vivo* test results, performed in 21 subjects, do not support these findings. Skin test results indicate that extracts from the 10 varieties studied caused a similar wheal and flare reaction in subjects sensitized to soybeans, which suggests that

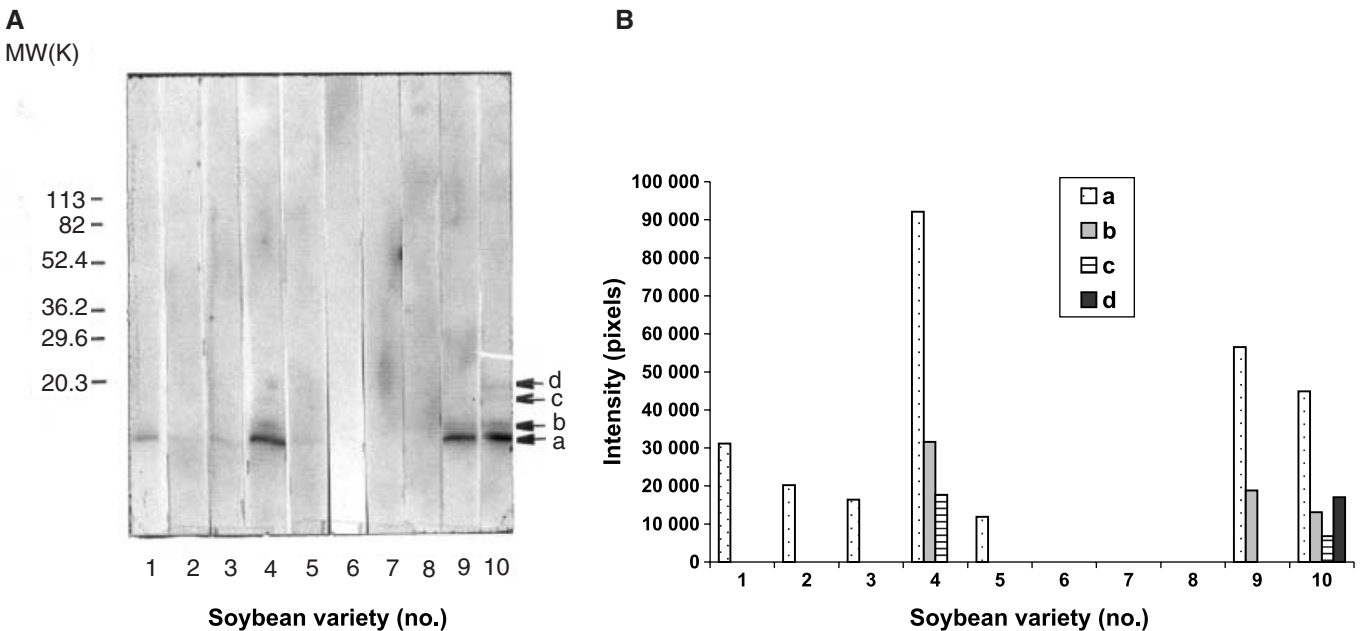


Figure 1. SDS-PAGE/Western blot of the 10 varieties of soybean incubated with the positive sera pool (A) and scanning of the IgE-binding protein bands (B).

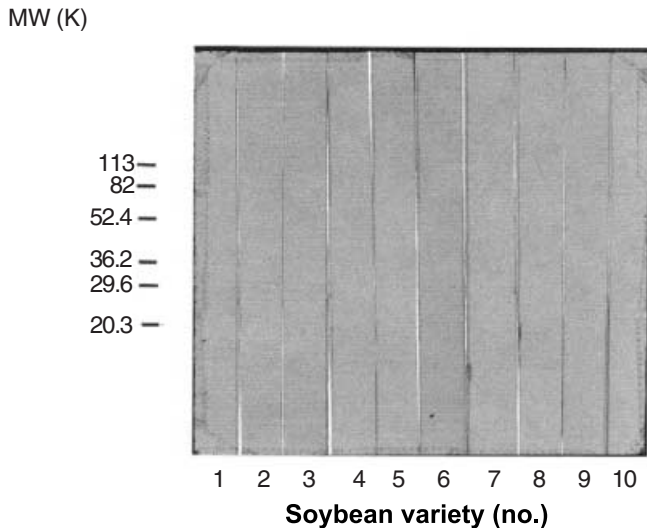


Figure 2. SDS-PAGE/Western blot of the 10 varieties of soybean incubated with the negative sera pool.

even a small amount of allergens could potentially elicit an allergic reaction in individuals sensitized to them. Perhaps the differences in percentage of positive skin test results to the soybean varieties studied obtained in this study would become significant in a larger sample size. Therefore, this study should be repeated in a larger population of individuals with IgE-mediated allergic asthma and/or rhinitis to soybean hulls, and skin prick tests should be performed at least in two different parts of the body in each subject. Another limitation of this study is that the skin test was performed in subjects sensitized to soybean dust from local varieties with a 'dull' phenotype, and it is not known if they had been exposed to varieties with a 'shiny' phenotype.

A study that investigated the allergenicity of 16 cultivars of olive trees by *in vivo* and *in vitro* tests using 20 subjects sensitized to olives indicated differences in allergenicity as demonstrated by skin prick test and specific IgE determination (16). However, the authors did not compare the percentage of positive results to the different varieties using statistical methods.

The soybean allergens responsible for food allergy are different than those responsible for respiratory allergies. The most important soybean food allergen is a protein termed P34, which is abundant in the seeds and other parts of the plant. A study of soybean cultivars indicated that this protein is present in significant amounts in all the cultivars studied (17), suggesting that it may not be possible to eliminate this protein from soybeans by breeding, and that biotechnological approaches might be necessary to obtain hypoallergenic soybeans. In addition, genetic selection of cultivars with reduced

allergenicity should include studies of the nutritional value of these varieties.

Bioengineering techniques are being utilized to create hypoallergenic plant varieties. Tada et al. (18) obtained rice transgenic lines that produced lower amounts of a 14–16 kDa allergen compared to the wild type, as demonstrated by immunoblot using an allergen-specific monoclonal antibody (mAb). However, these authors did not perform any *in vivo* or *in vitro* testing with subjects allergic to rice, and mAbs do not necessarily react with IgE-binding epitopes, so that reactivity suggests only common structural features. Another study with the allergen Ara h2, a major allergen from peanut, indicated that altering IgE-binding epitopes of Ara h2 could reduce peanut allergenicity, as demonstrated by *in vitro* testing using sera from subjects allergic to peanut (19).

Recombinant DNA technology is being extensively used in agriculturally important crops to improve their resistance to disease, herbicides, and insects; to delay ripening; or to increase major components of the plant. As the traits introduced results from the expression of proteins that are not necessarily part of the recipient's original genome, there is concern about the safety of transgenic foods, and one main concern is the potential allergenicity of these transgenic proteins. Therefore, the allergenicity of these genetically altered foods is being assessed by *in vitro* immunological assays such as RIA- or ELISA-inhibition or Western blot (20–22). However, a problem is to obtain a sufficient number and volume of serum samples from individuals whose clinical sensitivity has been previously confirmed. The results of the present study indicate that not only *in vitro* tests but *in vivo* tests also should be performed to assess the allergenicity of different plant varieties.

In conclusion, some varieties of soybean have a low content of the allergens responsible for soybean respiratory allergies, as determined by *in vitro* tests. However, this low allergen content may not reflect clinical sensitivity. Research should focus on selecting plants with reduced, preferably absent, allergenicity, but with a high nutritional value, and this allergenicity should be studied utilizing *in vitro* and *in vivo* testing.

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