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Research Article

Serum testing of genetically modified soybeans with special emphasis on potential allergenicity of the heterologous protein CP4 EPSPS

Michael Hoff[1], Dae-Yeul Son[2], Michaela Gubesch[1], Kangmo Ahn[3], Sang-Il Lee[3], Stefan Vieths[1], Richard E. Goodman[4], Barbara K. Ballmer-Weber[5] and Gary A. Bannon[6]

[1] Department of Allergology, Paul Ehrlich Institute, Langen, Germany
[2] Department of Oriental Medicine Biofood Science, Daegu Haany University, Korea
[3] Department of Pediatrics, Sungkyunkwan University School of Medicine, Samsung Medical Center, Korea
[4] Food Allergy and Research Program, University of Nebraska, Lincoln, NE, USA
[5] Department of Dermatology, Allergy Unit University Hospital, Zurich, Switzerland
[6] Product Characterization Center, Monsanto Company, St. Louis, MO, USA

Roundup Ready soy contains the CP4-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein. Serum IgE from two distinct populations of soy-allergic patients were recruited to determine their IgE-binding specificity. One population consisted of 10 adult patients from Europe, whose primary diagnosis was soy food allergy with some also having mite allergy. In addition, 6 primarily mite-allergic, 6 food-allergic (celery, carrot, milk, shrimp, walnut, and apple), and 5 non-allergic patients were tested. Another population consisted of 13 children from Korea, whose primary diagnosis was atopic dermatitis and secondarily soy and egg sensitization. In addition, 11 non-allergic patients were tested. Each patient population was extensively characterized with respect to clinical symptoms, specific IgE (CAP) scores, and total IgE. Immunoblots and ELISA assays were developed using serum IgE from these patients and soy extracts, CP4 EPSPS, rice extract, ovalbumin, rubisco, purified major peanut allergen Ara h 2, the putative soy allergen Gly m Bd 30k and mite allergen Der f 2 proteins as the intended targets. Immunoblot results indicated that soy-allergic patients bound soy extracts but did not specifically bind rubisco or CP4 EPSPS. ELISA results were in general agreement with the immunoblot results except that rubisco bound significant quantities of serum IgE from some patients. These results indicate that the CP4 EPSPS protein does not bind significant quantities of IgE from two geographically distinct sensitive populations and there is no evidence for an increased allergenic potential of this biotech protein.

Keywords: Atopic / CP4 EPSPS / Food allergy / IgE / Soy allergy

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1 Introduction

Genetically modified crops have been in the food supply around the world for almost 10 years and are currently planted on 222 million acres in 21 different countries [1]. In 2005, Roundup Ready soybean was planted on approximately 89% of the US Acreage (USDA-NASS, 2005. http://usda.mannlib.cornell.edu/reports/nassr/field/ [Accessed: January 11, 2006]) and 60% of the global soybean areas [1], and is the most cultivated biotechnology product to date. Roundup Ready soybeans contain the *cp4 epsps* gene from *Agrobacterium spp*. strain CP4, a common soil-borne bacterium that produces the CP4-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein [2]. The CP4 EPSPS protein produced in Roundup Ready plants is functionally identical to endogenous plant EPSPS enzymes with the exception that CP4 EPSPS naturally displays reduced affinity for glyphosate relative to endogenous plant EPSPS [2]. The EPSPS enzyme is part of the shikimate pathway that is involved in the production of aromatic amino acids and other aromatic compounds in plants [3]. When conventional plants are treated with the herbicide glyphosate, the

Correspondence: Dr. Gary A. Bannon, Regulatoy Sciences, Monsanto, 800 N. Lindbergh Blvd., St. Louis , Missouri, 63167, United States
E-mail: gary.a.bannon@monsanto.com
Fax: +1-314-634-8619

Abbreviations: **CP4 EPSPS**, CP4-enolpyruvylshikimate-3-phosphate synthase; **GM**, genetically modified

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plants cannot produce the aromatic amino acids needed to survive.

All proteins introduced into commercial genetically engineered plants have undergone a rigorous safety assessment to ensure that there is a reasonable certainty of no harm prior to their being placed on the market for food and feed use. Protein safety is assessed by assays that are based on recommendations from a variety of regulatory agencies including CODEX, US-FDA, and EFSA. Evaluation of protein safety includes assessments of a protein's history of safe use, expression level, mode of action, allergenicity, and acute toxicity. Results from these assessments are used in a weight of evidence approach to determine if a protein is likely to pose a risk to human or animal health.

The primary allergy risk to consumers from genetically modified crops may be placed into one of three categories. The first, that represents the highest risk to the allergic consumer is the transfer of a known allergen or IgE cross-reacting protein into a food crop. The second category is the potential for an increase in the endogenous allergenicity of an already allergenic crop [4]. The last category involves expression of novel proteins that may become allergens in man [5]. In order to mitigate the three categories of potential allergy risk associated with biotech crops, all genes introduced into food crops undergo a series of tests that includes determination of the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and known allergens; its susceptibility to enzymatic degradation; and serum screens using documented sera from allergic individuals if the protein is similar to known allergens or comes from an allergenic source [6]. While there is detailed guidance on how to perform bioinformatic analyses [7] and pepsin digestive fate studies [8], there is no consensus in the scientific community on the details of how to perform serum studies for regulatory agencies. IgE-binding assays such as radioallergosorbent tests (RAST; [9, 10]), ELISA [11], or immunoblotting assays are available for this type of testing. All these assays use IgE fractions of serum from appropriately sensitized individuals. Sensitized individuals should be allergic to the food from which the transferred gene was derived as shown by a convincing clinical history [12] or positive responses in double-blind, placebo controlled food challenges [9, 13].

In this study, our investigation focused on evaluating whether at-risk individuals, those with food allergy to soybeans and plausible exposure to genetically modified (GM)-soybeans, have developed IgE specific to the introduced CP4 EPSPS protein. Two different patient populations were used: children with atopic dermatitis secondarily diagnosed with soybean and other food allergies (Korean patients) and adult individuals with clinically documented soy allergy (European patients). For this purpose, immunoblot and ELISA assays for specific IgE were developed and validated, and control proteins were included in the screening to correlate the results obtained with CP4 EPSPS to immune responses to known allergenic and non-allergenic proteins.

2 Materials and methods

2.1 Patient sera

2.1.1 Europe

A total of 22 sera of food- and mite-allergic patients as well as 5 normal human sera were collected from European patients (see Table 1). Ten patients (21–57 years old at the time of sampling) with soy allergy were positive in double blind placebo controlled food challenge (DBPCFC) or an open soy challenge, or had a convincing history of anaphylaxis after ingestion of soy protein. Sera from other patients (18–60 years old at the time of sampling) with documented allergies to mite (6 patients), celery, carrot, apple, milk, shrimp and walnut (1 patient each) were utilized as atopic controls in this study. At the time of assessment, allergic patients had total IgE levels ranging from 21.5 to 2440 kU/L as measured by using the Phadia CAP System (Phadia, Uppsala, Sweden).

2.1.2 Korea

A total of 13 sera of food-allergic patients and 11 normal human sera were collected from Korean patients (see Table 2 for details). Ten patients (2–3 years old) exhibiting severe atopic dermatitis with accompanying soy allergy had a convincing history of allergic reactions to soy protein and high IgE levels to soy protein in the Phadia CAP system. Sera from 3 egg-allergic patients (4–8 years old) also exhibiting severe atopic dermatitis were utilized in this study. At the time of the assessment, allergic patients had total IgE levels ranging from 13.8 to 9250 kU/L as measured by using the Phadia CAP System.

2.2 Test proteins and extracts

2.2.1 Soy and rice extracts

Full-fat flour was extracted from four different commercial varieties of soybean (two Roundup Ready soy varieties and two non-transgenic soy varieties) using 1 X PBS (NaCl 0.138 M, KCl 0.0027 M, with 0.05% Tween 20, PBST) containing a protease inhibitor cocktail (Roche Cat. # 1697498, 1 tablet/50 mL extraction buffer) using a ratio of 1 g of flour to 10 mL of buffer. The suspensions were mixed with slow agitation in closed sealed tubes at 4°C for 2 h. The extracts were clarified by high-speed centrifugation (10 000 × or more). The clarified supernatants were passed through a 0.20-µm-sterile membrane filter. The protein content of the extracts was determined by a commercial dye-binding assay (Roth, Karlsruhe, Germany) and aliquots were frozen at –20C until use. The rice extracts were prepared in similar manner.

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Table 1. European patients: chinical and serological characteristics

Patient	DOB	Allergy	Clinical symptoms[a)]	DBPCFC[b)] (+/–)	CAPS (kU/L)			CAPS (kU/L)	Total IgE (kU/L)
					Soy	Mite	Other	Gly m 4	
		Soy							
1	07/10/70		OAS,N,F,BP	–	1 (0.54)	3 (3.98)		3 (3.98)	137
2	09/10/60		U	+	1 (0.63)	0 (< 0.35)		0 (< 0.35)	72.2
3	02/01/82		OAS,D,R	+	1 (0.51)	3 (10.4)		3 (10.4)	21.5
4	12/20/71		OAS,BI	+	2 (1.59)	3 (9.34)		3 (9.34)	175
5	08/11/80		OAS,D,N,E,AE	+	3 (9.91)	0 (< 0.35)		0 (< 0.35)	161
6	10/27/46		U,AE,BP,K	+	3 (5.05)	6 (100)		6 (100)	2440
7	11/08/71		OAS,AE,D,E,BP	–	2 (2.10)	0 (< 0.35)		0 (< 0.35)	284
8	04/13/77		OAS,U	+	1 (0.65)	2 (0.92)		2 (0.92)	50.7
9	08/19/71		OAS	+	2 (2.12)	5 (77.7)		5 (77.7)	1435
10	11/28/81		OAS	+	2 (1.34)	4 (31.23)		4 (31.23)	1695
		Mite							
11	07/27/85				0 (< 0.35)	3 (8.46)			55.1
12	08/09/73				1 (0.52)	6 (>100)			1245
13	02/24/62				0 (< 0.35)	3 (6.10)			300
14	07/18/79				0 (< 0.35)	6 (>100)			1217
15	08/09/53				0 (< 0.35)	4 (21.3)			222
16	04/29/85				0 (< 0.35)	4 (35.6)			321
17	05/15/58	**Celery**	OAS	+	1 (0.52)		3 (4.77)		75.8
18	04/03/41	**Carrot**	OAS	+	2 (0.83)		3 (10.3)		954
19	10/21/69	**Milk**	BP	–	0 (< 0.35)		4 (19.9)		200
20	01/25/77	**Shrimp**	D,BP	–	0 (< 0.35)		3 (12.2)		131
21	1968	**Walnut**	U,GI	–	0 (< 0.35)		4 (45.0)		283
22	1973	**Apple**	OAS	–	0 (< 0.35)		2 (2.93)		362
		NA							
23	01/04/63				0 (< 0,35)				7.31
24	05/15/76				0 (< 0,35)				221
25	04/18/43				0 (< 0,35)				4.36
26	07/29/70				0 (< 0,35)				6.98
27	09/29/73				0 (< 0,35)				4.05

a) AE, angioedema; BI, blisters of the oral mucosa; BP, blood pressure drop; D, dyspnea; DOB, date of birth; E, Emesis; F, flush; GI, gastrointestinal distress; K, conjunctivitis; N, nausea; NA, not allergic; OAS, oral allergy syndrome; R, rhinitis; U, urticaria.
b) Double blind placebo controlled food challenge.

2.2.2 CP4 EPSPS proteins

Plant- and *Escherichia coli*-produced CP4 EPSPS proteins were obtained from Monsanto Company (St. Louis, MO). The plant- and *E. coli*-produced proteins were characterized and compared to each other with respect to their identity, function, glycosylation status and purity. For every criterion, the plant- and *E. coli*-produced proteins met pre-set acceptance criteria for equivalence. The purity of the plant-produced protein was 85% and the purity of the *E. coli*-produced protein was 97% as assessed by SDS-PAGE.

2.2.3 Der f 2 protein

The purified Der f 2 protein was purchased from Indoor Biotechnologies (Charlottesville, VA). This protein was purified from mites by affinity chromatography using an anti-mite Group 2 mAb. The purity of the Der f 2 protein was >95% according to the specifications provided by the manufacturer.

2.2.4 Rubisco and ovalbumin

The rubisco and ovalbumin proteins were obtained from Sigma-Aldrich (St. Louis, MO). Rubisco was prepared from spinach leaves and contained both the large and small subunits, and was shipped as a partially purified powder.

2.2.5 Ara h 2 and P34 (Gly m Bd 30 k) proteins

The peanut allergen Ara h 2 was prepared according to the methods of Sen *et al*. [14]. *E. coli*-produced soy allergen P34 protein was obtained from Monsanto Company.

2.3 SDS-PAGE and Western blotting

The extracts and purified proteins were separated by SDS-PAGE according to Laemmli [15]. Extracts and proteins were reduced by heating with 2x SDS-PAGE reducing gel loading buffer (Laemmli Buffer, Bio-Rad cat. # 161-0737; mixed 1:20 with 2-mercaptoethanol, Bio-Rad cat. # 161-

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Table 2. Korea patients: chinical and serological characteristics

Patient	DOB	Allergy	Clinical symptoms[a)]	DBPCFC (+/−)	CAPS (kU/L)			Total IgE (kU/L)
					Soy	Egg	Milk	
		Soy						
258	12/03/01		AD,E,I	–	>100	>100	>100	9250
259	07/17/02		AD,E,I	–	27.1	>100	74.4	2454
377	06/20/02		AD,E,I	–	0.91	22	2.3	639
466	09/29/01		AD,E,I	–	14.4	65.9	1.96	424
537	08/16/02		AD,E,I	–	2.45	15.3	0.75	91.7
571	05/27/02		AD,E,I	–	65	>100	44.6	2072
622	10/17/01		AD,E,I	–	>100	51.2	58.9	721
664	11/23/02		AD,E,I	–	4.99	7.89	0.46	331
733	04/18/03		AD,E,I	–	7.14	0	0	13.8
815	06/21/02		AD,E,I	–	>100	63.4	73.8	2495
		Egg						
86	03/18/97		AD,BA	–	0	4.74	0	1569
208	04/14/01		AD	–	0	>100	38	699
414	01/30/00		AD	–	0	>100	0	381
		NA						
13	03/01/01				0			
14	08/03/00				0			
17	10/25/03				0			
20	07/10/89				0			
21	09/02/03				0			
22	01/30/00				0			
25	08/30/02				0			
26	01/07/04				0			
27	10/10/03				0			
30	09/02/97				0			
31	04/26/91				0			

a) AD, atopic dermatitis; BA, bronchial asthma; DOB, date of birth; E, erythema; I, itching; NA, not allergic.

0710) and loaded into each well of a gradient gel (10–20% Tris-Glycine gels, 1.0 mm × 15 wells, Novex pre-cast gel; Invitrogen cat. # EC61355BOX). Precision Plus Protein Standards (Bio-Rad, product # 161-0374) were used as molecular weight markers to estimate protein size. The separated proteins were transferred onto 0.45-µm pore-sized PVDF (polyvinylidene fluoride) membrane (Sigma, P-2813) by means of the tank-blotting method using a Hoefer transfer system with a cooling unit (Hoefer® TE22, Amersham Bioscience).

Immunoblots were pre-incubated for 1 h at room temperature with blocking reagent (PBST with 2% non-fat dry milk, NFDM) and then incubated overnight with allergic and non-allergic sera diluted 1:10 v/v in blocking reagent. Unbound IgE was removed by repeated rinses with PBST. For detection of bound IgE the membranes were incubated with biotin-labeled goat polyclonal IgG-anti-human IgE (KPL, Gaithersburg, MD), followed by washing and then incubation in NeutrAvidin-HRP (Pierce Biotechnology, Rockford, IL). Detection was achieved using ECL (enhanced chemiluminescence; Amersham, or Supersignal from Pierce), with multiple exposure times per membrane to provide optimal signal to noise ratio on the X-ray films. As a control, one membrane was incubated with the secondary antibody and ECL detection, but without human serum to evaluate the specificity of the detection system.

In some cases, inhibition experiments were performed by incubating serum samples with soluble inhibitor for approximately one hour before adding to pre-blocked membranes. Inhibitor proteins were diluted in 2% NFDM so that the final concentration of inhibitor, as incubated with 1:10 diluted serum, would be 50 µg of inhibitor/mL for CP4 EPSPS, rubisco and Ara h 2 and 500 µg of inhibitor/mL when using non-GMO or Roundup Ready soy extract. Immunoblot incubation and detection were performed as described above.

2.4 IgE ELISA assay

Extracts and target proteins (pool of the two Roundup Ready soy extracts, rubisco, ovalbumin and CP4 EPSPS for the Korean population; for the European sera, ovalbumin was replaced with Der f 2) were diluted in pH 9.6 carbonate buffer to coating concentrations of 10 µg/mL for extracts and 2 µg/mL for pure proteins and incubated in microtiter plates overnight at 4°C in a humidity chamber to coat the wells. The coating solution was removed by washing with PBST and nonspecific binding was blocked with 2%

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NFDM in PBST. Serum samples were diluted 1:20 v/v in blocking buffer (at room temperature) 160 min prior to addition to aspirated sample wells. Plates were incubated for 2 h in a humidity chamber at room temperature. Wells were washed three times with PBST before the addition of biotinylated goat anti-IgE (KPL) which was diluted 1:4000 v/v in PBST. Plates were incubated for 160 min at room temperature and then washed three times with PBST. NeutrAvidin-HRP (Pierce Biotechnology) was diluted 1:8000 v/v in PBST, added to sample wells, and incubated for 60 min at room temperature. Plates were washed three times with PBST before addition of 100 µL substrate tetramethylbenzidine (TMB) from KPL at room temperature. Reactions were stopped after 10 min of incubation at room temperature by addition of 50 µL 6N sulfuric acid and the OD were read at 450 nm by a microplate reader.

3 Results

3.1 Patients and sera characteristics

The soy-allergic patients tested in this study represent two distinct populations that have very different clinical presentations. The European patient population consisted of adults ranging in age from 18–60 years old at the time of sampling that presented with a wide variety of clinical symptoms after ingestion of soy (Table 1). Their total IgE levels ranged from 21.5–2440 kU/L with a population mean of 647 kU/L. The soy CAP values for these patients ranged from 1–3 with a population mean of 2.4 kU/L. This low soy CAP value for soy-allergic patients is, at least partly, due to the exclusion of a major soy allergen from the CAP assay that these patients recognize (Gly m 4) [16, 17]. When tested on recombinant Gly m 4 CAP, seven out of ten patients were clearly positive with specific IgE levels of up to 100 kU/L and a mean value of 33.4 kU/L for the positive patients (Table 1).

In contrast, the Korean patient population consisted of children ranging in age from 2–3 years old at the time of sampling that presented with clinical symptoms of atopic dermatitis that were secondarily diagnosed with soy allergy from clinical histories (Table 2). Their total IgE levels were much higher than in the European patient population ranging from 13.8–9250 kU/L with a population mean of 1849 kU/L. The soy CAP values for these patients were also much higher than the European patient population ranging from 0.91->100 kU/L with a population mean of 42.2. In addition, many of the Korean patients showed significant CAP values to both egg and milk allergens (Table 2).

The control for the European population represented three different patient groups. One group consisted of 6 patients with mite allergy, 6 patients with food allergy but not soy-allergic, and 5 non-allergic patients. Total IgE levels for the mite-allergic patients ranged from 55.1–1245 kU/L, for the food but not soy-allergic patients from 75.8–954 kU/L, and the non-allergic patients from 4.05–221 kU/L (Table 1). The control for the Korean population represented two different patient groups. One group consisted of 3 patients with atopic dermatitis that were secondarily diagnosed with egg allergy from clinical histories and 11 non-allergic patients. Total IgE levels for the atopic dermatitis patients secondarily diagnosed with egg allergy ranged from 381–1569 kU/L. Total IgE was not determined for the non-allergic patients (Table 2).

3.2 IgE immunoblot analysis

A goat anti-CP4 EPSPS antibody was used to probe PVDF membranes loaded with *E. coli* and plant expressed CP4 EPSPS, the mite allergen Der f 2, the major peanut allergen Ara h2, rubisco, ovalbumin, extracts from two Roundup Ready soy varieties and two non-transgenic varieties, as well as rice extract (Fig. 1). As expected, the CP4 EPSPS protein was detected in the transgenic soy extract lanes and those lanes loaded with CP4 EPSPS protein standard. The immuno-detectable level of CP4 EPSPS in GM soybean was approximately equivalent to the signal visible in the 40 ng/lane sample of *E. coli* produced protein and markedly below the 400 ng/lane sample (Fig. 1), demonstrating the relevance of loading purified sample at 40 ng as representative for soybean loading for human IgE assays. An example of the IgE immunoblot results is indicated in Fig. 2. In all cases, the patient sera reacted against positive control extracts and allergens as expected. For example, the European soy-allergic patients had IgE that recognized protein bands in soy extracts that ranged in molecular weight from <10 kDa to >75 kDa. There was also significant cross-reactivity with rice extract proteins observed (Fig. 2). Similarly, the Korean patients with atopic dermatitis secondarily diagnosed with soy allergy also recognized a wide range of soy proteins (<10–75 kDa) with significant cross-reactivity noted for a variety of other proteins including ovalbumin, rubisco and rice extracts (data not shown). No significant difference in binding to extracts of wild-type versus GM soybean was observed with any of the allergic patient sera.

One European soy-allergic patient serum (#8) and 2 Korean atopic dermatitis patient secondarily diagnosed with soy allergy (537, 622) initially reacted with the pure transgenic protein, CP4 EPSPS (Fig. 3) on long exposures of the film. The intensity of binding was not strong (based on 40 ng loading equivalence to soybean samples) and was obvious in serum that bound to several proteins in soy and rice extracts. In order to determine if these sera contained IgE specific to CP4 EPSPS protein, a Western blot inhibition experiment was performed (Fig. 4). In this experiment the CP4 EPSPS protein, rubisco, non-transgenic soy extract and transgenic soy extract were used separately as inhibitors to determine their effect on IgE binding. However, in these experiments, and several others, these sera failed to repro-

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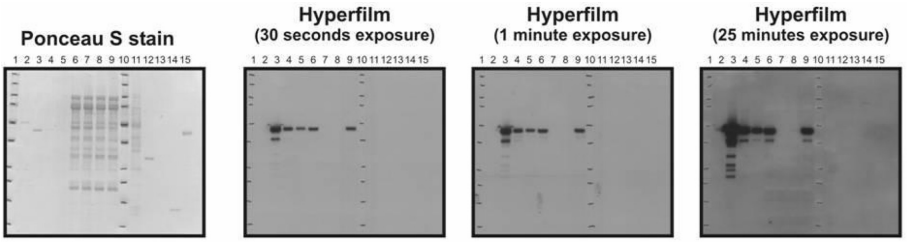

Figure 1. CP4 EPSPS protein detection by goat anti-CP4 EPSPS antibodies. Proteins were electrophoresed, immunoblotted, and then probed with anti-CP4 EPSPS antibodies to detect the CP4 ESPSPS proteins. Lane 1, molecular weight markers; Lane 2, rubisco (400 ng); Lane 3, *E. coli*-produced CP4 EPSPS (400 ng); Lane 4, *E. coli*-produced CP4 EPSPS (40 ng); Lane 5, plant-produced CP4 EPSPS soybean (40 ng); Lane 6, Roundup Ready soy protein extract 1 (10μg); Lane 7, Non-GM soy protein extract 1(10 μg); Lane 8, Non-GM soy protein extract 2 (10 μg); Lane 9, Roundup Ready soy extract 2 (10 μg); Lane 10, molecular weight markers; Lane 11, Rice protein extract (10 μg); Lane 12, *E. coli*-produced soy allergen P34 (Gly m Bd 30 k) (400 ng); Lane 13, plant-produced peanut allergen Ara h 2 (400ng); Lane 14, house dust mite allergen Der f 2 (400 ng); Lane 15, egg allergen ovalbumin Gal d 2 (400 ng).

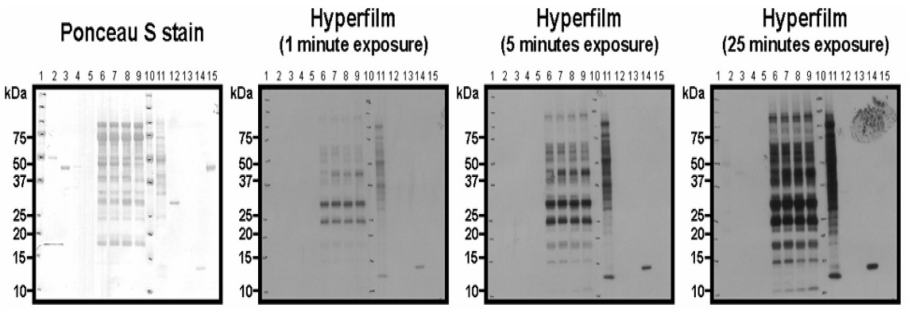

Figure 2. Serum IgE binding to purified proteins and plant extracts. Proteins were separated by SDS-PAGE, immunoblotted, and then probed with serum IgE from a European soy-allergic patient. Lane designations are the same as noted in the Fig. 1 legend.

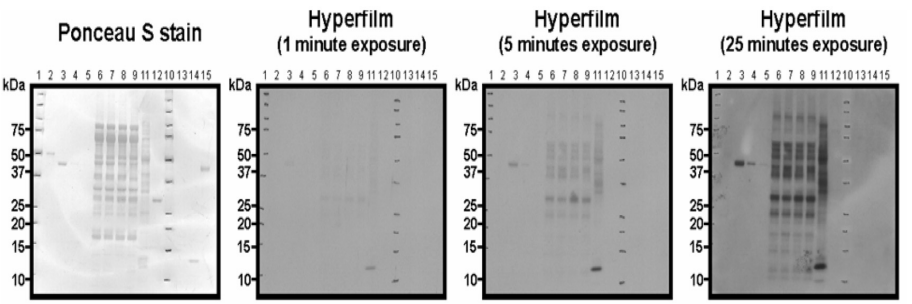

Figure 3. Apparent IgE binding to purified CP4 EPSPS protein. Proteins were separated by SDS-PAGE, immunoblotted, and then probed with serum IgE from European soy-allergic patient #8. Lane designations are the same as noted in the Fig. 1 legend.

ducibly bind to the CP4 EPSPS protein but did bind to many of the soy extract proteins. Furthermore, CP4 EPSPS protein had no effect on IgE binding to the soy extract proteins but the binding was markedly inhibited with non-transgenic and transgenic soy extract proteins, suggesting that the binding may be relatively nonspecific. In the case as shown in Fig. 4, inhibition with the rubisco protein also significantly reduced IgE to the soy extract proteins. Since IgE binding to the CP4 EPSPS protein was not reproducible and binding to soy extract could be inhibited by rubisco, it was concluded that there were no specific IgE to the CP4 EPSPS protein.

3.3 ELISA analysis

Preliminary tests were used to determine the optimum concentrations of protein for coating of microtiter plates, dilution of serum IgE and biotinylated anti-IgE, and optimum times for incubation of reagents to maximize specific signals while minimizing nonspecific binding. Thereafter, all

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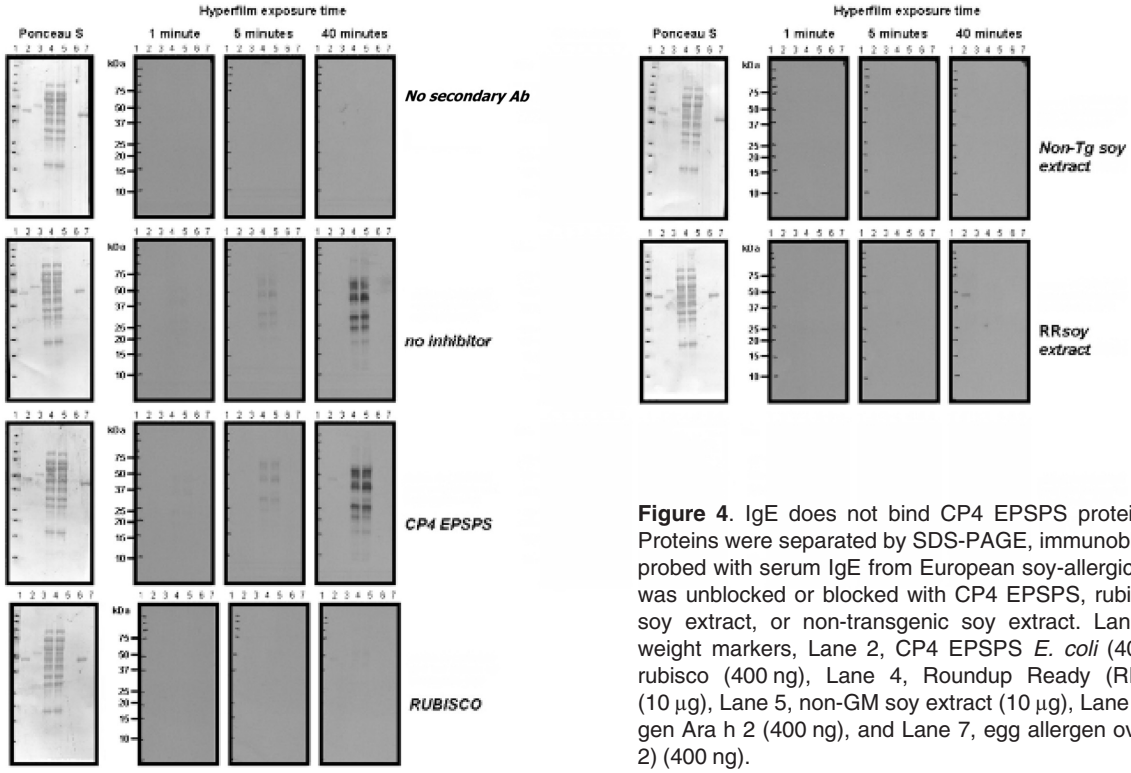

Figure 4. IgE does not bind CP4 EPSPS protein reproducibly. Proteins were separated by SDS-PAGE, immunoblotted, and then probed with serum IgE from European soy-allergic patient #8 that was unblocked or blocked with CP4 EPSPS, rubisco, transgenic soy extract, or non-transgenic soy extract. Lane 1, molecular weight markers, Lane 2, CP4 EPSPS *E. coli* (400 ng), Lane 3, rubisco (400 ng), Lane 4, Roundup Ready (RR) soy extract (10 μg), Lane 5, non-GM soy extract (10 μg), Lane 6, peanut allergen Ara h 2 (400 ng), and Lane 7, egg allergen ovalbumin (Gal d 2) (400 ng).

sera were tested according to the final protocol as specified in the Section 2. The values in Fig. 5 reflect the average of three independent wells without the background subtracted from the raw OD readings. All European patient serum IgE was used in ELISA assays with soy extract, rubisco, CP4 EPSPS or Der f 2 proteins as targets. Soy extract-binding values for the European soy-allergic patient population ranged from 0.44 to 0.82 with a population mean of 0.54. Mean IgE-binding values for this same population to rubisco, CP4 EPSPS and Der f 2 were 0.16, 0.05, and 0.36, respectively. Mean IgE-binding values for the mite allergic patients showed significant binding to Der f 2 (1.21) as expected with insignificant levels of IgE binding to rubisco (0.10) and CP4 EPSPS (0.052). Mean IgE-binding values of the mite allergic patients to soy extracts (0.44) were at the lower range of the values obtained for the soy-allergic patients.

Korean patient serum IgE was used in ELISA assays with soy extract, rubisco, CP4 EPSPS or ovalbumin proteins as targets. Just as was observed for the soy CAP values, ELISA results for the Korean patient population were higher than those reported for the European patients. Soy extract-binding values for the Korean soy-allergic patient population ranged from 0.47 to 2.87 with a population mean of 1.21. Mean IgE-binding values for this same population to rubisco, CP4 EPSPS and ovalbumin were 0.21, 0.06, and 1.31, respectively. Mean IgE-binding values for the atopic dermatitis patients that were secondarily diagnosed with egg allergy showed significant binding to ovalbumin (1.26) as expected; with insignificant binding to rubisco (0.08), CP4 EPSPS (0.06) and soy extract (0.53).

4 Discussion

The EPSPS family of enzymes is ubiquitous to plants and microorganisms. EPSPS proteins have been isolated from both sources, and their properties have been extensively studied [18–22]. The EPSPS protein is absent in mammals, fish, birds, reptiles, and insects [19]. The bacterial and plant enzymes are mono-functional with molecular weights of 44–48 kDa [20]. EPSPS proteins catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) [21]. Due to the specificity of EPSPS for its substrates, the only known catalytic product generated is EPSP, which is the penultimate product of the shikimic acid pathway. Shikimic acid is a substrate for the biosynthesis of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other aromatic molecules.

Roundup Ready soy contains the EPSPS gene derived from *Agrobacterium spp*. strain CP4 (*cp4 epsps*). The *cp4 epsps* coding sequence encodes a 47.6-kDa EPSPS protein consisting of a single polypeptide of 455 amino acids [2]. The CP4 EPSPS protein is structurally similar and function-

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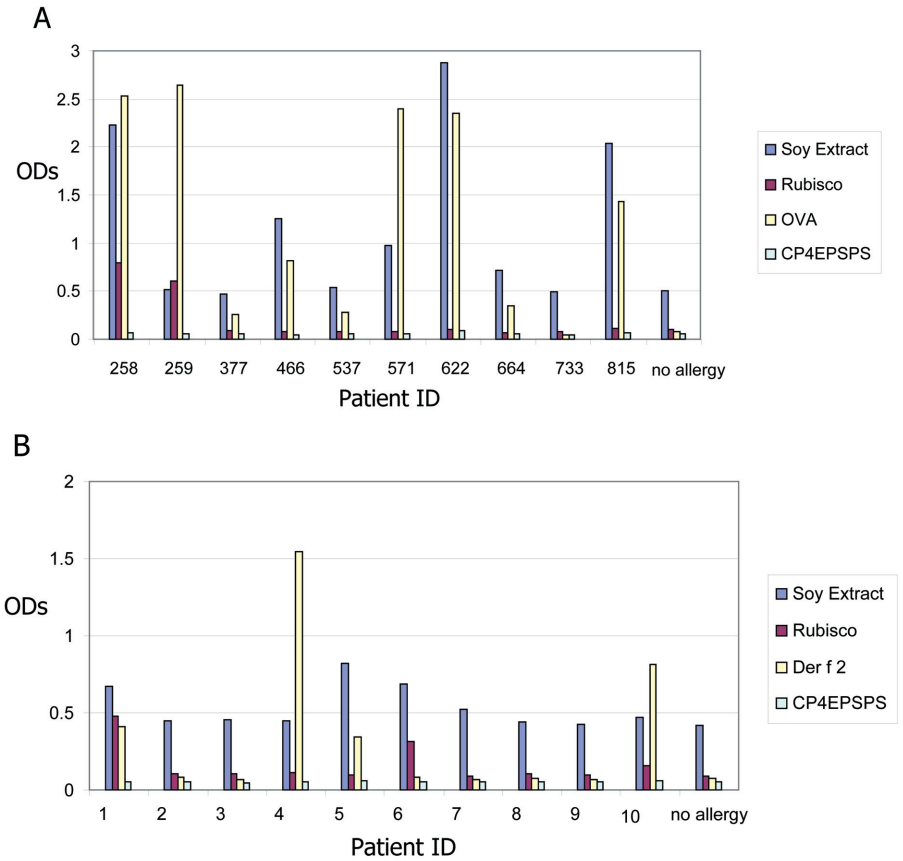

Figure 5. ELISA results from Korean and European soy-allergic patients. (A) Histogram showing IgE binding of Korean atopic dermatitis patients secondarily diagnosed with soy allergy to different purified proteins and extracts. (B) Histogram showing IgE binding of European soy-allergic patients to different purified proteins and extracts.

ally identical to endogenous plant EPSPS enzymes, but has a much-reduced affinity for glyphosate, the active ingredient in Roundup herbicides, relative to endogenous plant EPSPS [2]. In conventional plants, glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of shikimate-3-phosphate, thereby depriving plants of essential amino acids [22]. In Roundup Ready plants, which are tolerant to the Roundup family of agricultural herbicides, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate [2].

Harrison *et al*. [3] demonstrated the safety of the CP4 EPSPS protein according to the recommendations of the Codex Alimentarius Commission [6]. Briefly, they showed that the protein is from a non-allergenic source, the protein does not represent a relatively large portion of the total protein, the protein does not share structural similarities to known allergens or toxins based on the amino acid sequence, the protein is unstable to digestion in simulated gastric fluid, and the protein is not acutely toxic. Furthermore, others [23, 24] showed that the endogenous allergenicity of GM soy containing this gene is unchanged when compared with non-GM soy. These characteristics ensure that the CP4 EPSPS does not represent a known allergen and is unlikely to act as a cross-reactive allergen. They also demonstrate that it is unlikely the transfer of the gene altered the endogenous allergenicity of soy. The data obtained by IgE testing of extracts of GM and non-GM soy in this study confirm and underline these findings. Studies designed to determine whether the endogenous allergenicity of a GM crop has been modified when compared to its non-GM counterpart have repeatedly shown no change in IgE-binding capacity. Perhaps this is not surprising given that endogenous allergen expression would have to be significantly up-regulated as a result of the gene transfer event. Given the large size of most plant genomes and the very limited number of gene encoding allergens the trans-gene would have to be inserted proximal to an allergen gene in such a way as to up-regulate transcription. This seems like it would be an extremely rare event and perhaps we should reconsider whether this study brings any value to the allergy assessment process.

The studies mentioned above address two of the categories of potential allergenic risk to public health posed by genetically modified crops; transfer of a known allergen or likely cross-reactive protein and the potential increase in endogenous allergenicity of an already allergenic crop [4]. Recently, Batista *et al*. [25] attempted to test for evidence that CP4 EPSPS may have sensitized consumers by performing a post-market serum study to address the third cat-

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egory of allergenic risk to public health; expression of a novel protein that becomes an allergen. In their study serum IgE from a wide variety of allergic patients were tested to determine if they developed IgE to proteins newly introduced into food crops after having been exposed to them for a long period. The patients had positive histories of food allergy or inhalant allergy with the probability of these individuals having eaten GM soy containing the CP4 EPSPS protein near 100% based on the global distribution of GM soy containing CP4 EPSPS and ubiquitous use of soy protein in processed foods. Results from their study showed that none of the patients tested had detectable levels of IgE directed against the CP4 EPSPS protein and that there were no discernable differences between transgenic and non-transgenic extracts when used in skin prick tests [25].

In contrast, the present study tested a more focused study population that included soy-allergic patients from Europe and atopic dermatitis patients that were secondarily diagnosed with soy allergy from Korea as the test groups. Even though the patient populations tested in each of these studies were different there was no significant IgE binding to the CP4 EPSPS protein. In the IgE ELISA in which the proteins were tested under non-denaturing conditions, CP4 EPSPS had the lowest IgE-binding capacity of all antigens tested. Binding to CP4 EPSPS was even lower than to rubisco, a ubiquitous plant protein that has never been described as an allergen, though rubisco clearly bound IgE from some of our study participants by ELISA (Fig. 5). In IgE Western blotting experiments, sera from three subjects showed weak apparent binding to the biotech protein which could not, however, be reproduced in subsequent experiments. In attempts to confirm the specificity of the antibody binding, unspecific effects were observed with soy extracts, CP4 EPSPS, and rubisco. In part these effects may be due to low affinity binding to plant N-glycans (soybean extracts), but it is more likely that the high sensitivity of the enzyme immunologic detection of the Western blots did result in some unspecific binding only visible under the denaturing conditions of the Western blot assay, but not in the more physiological ELISA test. Therefore, we conclude that, 10 years after market introduction and consumption of Roundup Ready soy, our study revealed no evidence for allergenicity of the heterologous protein CP4 EPSPS.

5 References

[1] James, C., Executive Summary of Global Status of Commercialized Biotech/GM Crops: 2005. *ISAAA Briefs.* 2005, *No. 34*. ISAAA: Ithaca, NY, USA

[2] Padgette, S. R., Re, D. B., Barry, G. F., Eichholtz, D. E., *et al*., New weed control opportunities: Development of soybeans with a Roundup Ready gene. In: Duke, S. O. (Ed.), *Herbicide-resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects*. CRC Press, New York 1996, pp 53 – 84.

[3] Harrison, L. A., Bailey, M. R., Naylor, M. W., Ream, J. E., *et al*., The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. *J. Nutr*. 1996, *126,* 728 – 740.

[4] Goodman, R. E., Hefle, S. L., Taylor, S. L., van Ree, R., Assessing genetically modified crops to minimi9ze the risk of increased food allergy: A review. *Int. Arch. Allergy Immunol*. 2005, *137*, 153 – 166.

[5] Lehrer, S. B., Bannon, G. A., Risks of allergic reactions to biotech proteins in foods: Perception and reality. *Allergy* 2005, *60*, 559 – 564.

[6] Codex Alimentarius Commission. Proposed draft annex on the assessment of possible allergenicity of the draft guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants. *Joint FAO/WHO Food Standard Program,* 2003, Appendix IV; pp 57 – 60.

[7] Thomas, K., Bannon, G., Hefle, S., Herouet, C., *et al*., *In silico* methods for evaluating human allergenicity to novel proteins: Bioinformatics Workshop Meeting Report, 23 – 24 February 2005. *Toxicol. Sci*. 2005, *88*, 307 – 310.

[8] Thomas, K., Aalberse, M., Bannon, G. A., Bartels, M., *et al*., A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology Toxicol. Pharmacol*. 2004, *39*, 87 – 98.

[9] Sampson, H. A., Albergo, R., Comparison of results of skin test, RAST, and double blind, placebo-controlled food challenges in children with atopic dermatitis. *J. Allergy Clin. Imminol*. 1984, *74*, 26 – 33.

[10] Adolphson, C. R., Yunginger, J. W., Gleich, G. J., in: Rose, N. R., Friedman, H., (Eds.), *Manual of Clinical Immunology*, 2nd Edn., American Society for Microbiology, Washington, DC, 1980, pp 678 – 684.

[11] Burks, A. W., Brooks, J. R., Sampson, H. A., Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J. Allergy Clin. Immunol*. 1988, *81*, 1135 – 1142.

[12] Sampson, H. A., Scanion, S. M., Natural history of food hypersensitivity in children with atopic dermatitis. *J. Pediatrics* 1989, *115*, 23 – 27.

[13] Bock, S. A., Sampson, H. A., Atkins, F. M., *et al*., Double-blind-placebo-controlled food challenges (DBPCFC) as an office procedure. *J. Allergy Clin. Immunol*. 1988, *82*, 986 – 997.

[14] Sen, M. M., Kopper, R., Pons, L., Abraham, E. C., *et al.*, Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J. Immunol.* 2002, *169*, 882 – 887.

[15] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680 – 685.

[16] Mittag, D., Vieths, S., Vogel, L., Becker, W.-M., *et al*., Soybean allergy in birch pollen-allergic patients: clinical investigation and molecular characterization of allergens. *J. Allergy Clin. Immunol.* 2004, *113*, 148 – 154.

[17] Kleine-Tebbe, J., Wangorsch, A., Vogel, L., Crowell, D. N., *et al*., Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22. *J. Allergy Clin. Immunol* 2002, *110*, 797 – 804.

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[18] Klee, H. J., Muskopf, Y. M., Gasser, C. S., Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol. Gen. Genet.* 1987, *210,* 437 – 442.

[19] Schonbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V. *et al*., Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc. Natl. Acad. Sci. USA* 2001, *98,* 1376 – 1380.

[20] Steinrucken, H. C., Amrhein, N., 5-Enolpyruvylshikimate-3-phosphate synthase of *Klebsiella pneumoniae* 2. Inhibition by glyphosate [N-(phosphonomethyl)glycine]. *Eur. J. Biochem.* 1984, *143,* 351 – 357.

[21] Alibhai, M. F., Stallings, W. C., Closing down on glyphosate inhibition – with a new structure for drug discovery. *Proc. Natl. Acad. Sci. USA* 2001, *98,* 2944 – 2946.

[22] Kishore, G. M., Shah, D. M., Amino acid biosynthesis inhibitors as herbicides. *Annu. Rev. Biochem*. 1988, *57*, 627 – 663.

[23] Burks, A. W., Fuchs, R. L., Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *J. Allergy Clin. Immunol.* 1995, *96*, 1008 – 1010.

[24] Kim, S. H., Kim, H. M., Ye, Y. M., Kim, S. H., *et al*., Evaluating the allergic risk of genetically modified soybean. *Yonsei Med. J.* 2006, *47,* 505 – 512.

[25] Batista, R., Nunes, B., Carmo, M., Cardoso, C. *et al*., Lack of detectable allergenicity of transgenic maize and soya samples. *J. Allergy Clin. Immunol*. 2005, *116,* 403 – 410.

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