

A Soybean G2 Glycinin Allergen

1. Identification and Characterization

Ricki M. Helm^a Gael Cockrell^a Cathie Connaughton^a Hugh A. Sampson^b
Gary A. Bannon^c Vadim Beilinson^d Donald Livingstone^d Niels C. Nielsen^d
A. Wesley Burks^a

^aDepartment of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Nutrition Center, Little Rock, Ark., ^bDivision of Pediatric Allergy and Immunology, Mt. Sinai Medical School, New York, N.Y., ^cDepartment of Pediatrics and Biochemistry and Molecular Biology, Arkansas Children's Hospital Research Institute, Little Rock, Ark., ^dUSDA-ARS and Agronomy and Biochemistry Departments, Purdue University, West Lafayette, Ind., USA

Key Words

Soybean · Glycinin · Food allergens · Epitopes

Abstract

Background: Multiple allergens have been documented in soybean extracts. IgE from individuals allergic to soybeans, but not to peanut, was shown by immunoblot analysis to bind to proteins with a molecular weight of approximately 21 kD. These findings suggested that unique proteins in soybeans might be responsible for soybean allergic reactivity. The objective of the present study was to identify unique proteins in soybean extracts that bind to specific IgE from soybean-sensitive individuals, and to characterize the allergen using physicochemical methods and IgE binding. **Methods:** Two-dimensional and preparative SDS-PAGE/IgE immunoblot analysis was used to identify a 22-kD soybean-specific allergen from crude soybean extracts. N-terminal sequence analysis was used to determine the identification of the protein binding IgE from soybean-sensitive individuals. **Results:** IgE immunoblot and amino acid sequence analysis identified the 22-kD protein as a member of the G2 glycinin soybean protein family. Further investigation revealed that the IgEs reacted with basic chains from each

member of the glycinin family of soybean storage proteins. **Conclusions:** Each of the subunits from glycinin, the storage protein that is the most prevalent component of soybean, are major allergens.

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Introduction

Allergenic reactions to food and food products are endemic in the industrialized world, and the number of incriminating foods identified as sources of allergens is increasing. Legumes such as peanuts and soybeans are being increasingly used as sources of protein for humans, a phenomenon that may in part be responsible for the rise in reported food allergies that has been observed [1]. The factors determining the allergenicity of soybean proteins and the wide variability in the degree of clinical responses among individuals are still not fully characterized. Reactions to soybean proteins may be either non-IgE mediated (e.g. enterocolitis) or IgE mediated, but they usually cease to be an important clinical factor after the first several years of life for those individuals who have either type of sensitivity [2, 3].

Identification of specific allergens in soybean extracts has been assessed based on their ability to bind IgE. This approach is complicated by the fact that both inhalant and ingestant allergies to soybeans have been reported. Thus, it is possible that different proteins of soybean cause food and respiratory allergies. Accordingly, proteins found as components of ingested foods would cause food allergies, whereas respiratory allergy would arise via inhalation of volatile dusts. Despite the significantly different patterns of proteins that have been described in the literature so far, both diseases could either occur together or separately in individuals presenting with different clinical symptoms. In studies in which the frequency of soybean aeroallergens was determined, at least 90% of the patients produced IgE that was specific to seed coat components with molecular weights of 7–8 kD [4, 5]. Only 13% of the patients had specific IgE that bound to components of seed coat-depleted soybean grains [4]. IgE of patients with soybean-induced asthma involved in the Barcelona asthma epidemic reacted weakly to two proteins with molecular weights of 42 and 21 kD [6]. Isoallergens identified as Gly m 1A (molecular weight 7.5 kD) and Gly m 1B (molecular weight 7 kD) recognized IgE from 95% of the patients with soybean-induced asthma [7]. A third soybean hull allergen, Gly m 2, with a molecular weight of 8 kD, has been reported to be partially responsible for soybean asthma outbreaks in Barcelona, Spain [8].

Frequency studies of IgE reactivity to different soybean proteins identified in food sources have reported values of between 1.4 and 65.2% among soybean-sensitive individuals [9–11]. Food allergy caused by the ingestion of soybean products has been documented in many reports about clinical symptoms, which range from mild gastrointestinal distress to suspected anaphylaxis. Shibasaki et al. [12] reported that the three classic globulin fractions that can be isolated from soybean (11S, 7S and 2S) all specifically bound IgE from young patients presenting with eczema after ingestion of soy. The 2S fraction contained a very allergenic protein with a molecular weight of about 21 kD that was identified as Kunitz soybean trypsin inhibitor [12]. Other ingested allergens include glycinin (11S proteins), β -conglycinin (7S proteins) and the protein Gly m Bd 30 kD [9, 10, 13–15].

Herian et al. [13] identified three different patterns of IgE-binding proteins in the sera of 7 patients that reacted to ingestion of soybean. Category 1 included a group of 4 patients with histories suggesting allergy to peanuts in addition to soybean, category 2 included a group of 3 subjects who had allergy to soybean but not peanuts, and category 3 comprised a single subject who did not fit either of

the other categories. In category 1, serum IgE bound to proteins with molecular weights of 50–60 kD, most likely subunits of β -conglycinin, while IgE from individuals in category 2 bound strongly to a protein(s) with a molecular weight of 20 kD. The identity of the latter protein was not certain, but was regarded neither as Kunitz soybean trypsin inhibitor nor a glycinin. In category 3, the serum IgE from a single individual did not recognize any peanut proteins. This subject had outgrown several legume sensitivities but had retained soybean sensitivity and a mild sensitivity to kidney beans.

The 11S and 7S seed storage proteins are widely distributed among both legumes and nonlegumes [16]. In children allergic to soy, both 11S and 7S bind IgE with similar binding activity [9]. Patients allergic to either 11S or 7S proteins from one legume species often have IgE antibodies that react with equivalent 11S or 7S proteins from other legumes [17–19]. Although immunochemical cross-reactions of this type are frequent, clinical responses to proteins in more than one legume are not common [18].

In an earlier study, immunoblot analysis was used to show that IgE from individuals allergic to soybeans, but not to peanut, bound strongly to proteins with a molecular weight of approximately 20 kD [13]. These findings suggested that unique proteins were present in soybeans that were responsible for soybean allergic reactivity. In another study [17], antigen affinity chromatography with peanut-specific IgE resulted in the removal of cross-reactive antibodies in soybean extracts. Using this approach, two unique allergenic fractions from soybean extracts were identified that had apparent molecular weights of 40 and 21 kD. In the present study, evidence is presented that shows that the basic chains from glycinin are major antigens.

Patients and Methods

Patients

Patients with atopic dermatitis and a positive immediate skin test greater than 2+ by conventional skin test responses to soybean extracts were confirmed as being sensitive to soybean protein by double-blind, placebo-controlled food challenges [9]. The known demographics of these patients are shown in table 1. Pooled and individual sera from 7 soybean-sensitive individuals were diluted 1/10 (v/v) in Tris-buffered saline (25 mM Tris-HCl pH 7.2, 150 mM NaCl with 0.05% Tween 20 (TBST)) for immunoblot analysis. Patients with atopic dermatitis, a >2+ positive immediate skin test and either a positive double-blind, placebo-controlled food challenge or a convincing history of peanut anaphylaxis were used as a serum source for peanut-specific IgE. Individuals with soybean sensitivity or peanut sensitivity neither reported nor exhibited clinical symptoms with the

Table 1. Demographic data of the soybean-sensitive individuals

Patient	Hist P/S	CAP-RAST	
		soybean	peanut
VL	-/+	91.3	Unk
IV	-/+	85.5	Unk
LD	-/+	>100	Unk
MP	-/+	80.3	Unk
S	NI/+	>100	>100
B	NI/+	>100	>100
K	NI/+	59.8	>100

Hist P/S = History for peanut and soybean; NI = never knowingly ingested; Unk = unknown.

alternate food source (i.e. soybean-sensitive individuals could eat peanuts and peanut-sensitive individuals could eat foods containing soybean products). Serum from a non-food atopic individual with a high IgE titer was used for nonspecific IgE binding to soybean and peanut proteins. The serum was diluted 1/5 (v/v) in TBST with blocking solution, as recommended by the manufacturer (Genosys Biotechnologies Inc., The Woodlands, Tex., USA), for mapping and identification of linear IgE-binding epitopes for the glycinin allergen. All of these studies, which were carried out at the University of Arkansas for Medical Sciences and Mt. Sinai School of Medicine, were approved by the Human Use Advisory Committees.

Allergen Identification by Two-Dimensional SDS-PAGE

Preparations of defatted soybean flakes were obtained from commercial sources (Dr. Jim Brooks, Ross Laboratories, Columbus, Ohio, USA). The defatted soybean flakes were ground to a fine powder in a mortar and pestle and extracted (1:20 wt/vol) in 20 mmol of sodium phosphate and 1 M NaCl, pH 7.2, overnight at 4°C.

For two-dimensional (2-D) SDS-PAGE analysis, a 5.0-ml aliquot of the soybean extract was dialyzed overnight against 1 liter of distilled water with three changes. The dialyzed material was lyophilized and resuspended in distilled water, and a 20-μg protein sample was analyzed by 2-D SDS-PAGE (i.e. isoelectric focusing in the first dimension, pH 3–10, followed by a 4–20% SDS-PAGE gradient gel in the second). Duplicate patterns, representing the protein profiles, were transferred to nitrocellulose membranes. One membrane was stained with Coomassie blue (0.1% Coomassie blue in 10% acetic acid, 50% methanol and 40% distilled water for 3 h with agitation) for protein identification and the other was prepared for IgE immunoblot analysis. Following protein transfer to the membrane, the remaining active sites were blocked using TBST in 1% BSA, and analyzed for IgE binding using serum from soybean-sensitive individuals. The serum from individuals with a clinical sensitivity to soybean was diluted 1/10 (v/v) with TBST in 1% BSA and incubated with the nitrocellulose membrane for at least 12 h at 4°C or for 2 h at room temperature. The membrane was subsequently washed and incubated with ¹²⁵I-radiolabeled anti-IgE (Sanofi Diagnostics, Chaska, Minn., USA) overnight at 4°C. After the membrane was washed and dried, it was exposed to X-Omat X-ray film at –80°C for 18–48 h. Immunoblots containing IgE-binding proteins identified by autora-

diography were reconciled against Coomassie-stained protein profiles to delineate those that reacted.

A model 491 Prep Cell (Bio-Rad, Richmond, Calif., USA) with a 12.5% preparative SDS-PAGE gel was used to separate the molecular components of a crude soybean extract using a modified Laemmli procedure [20]. A 6-cm gel was loaded with 25 mg of extract and continually electrophoresed at 40 mA of constant current at a flow rate of 60 ml/h until the tracking dye reached the bottom of the gel. At this point, a Bio-Rad fraction collector (Bio-Rad), was used to collect eluted material in 5-ml fractions. An aliquot (25 μl) from each 5-ml fraction was collected and analyzed using a Pharmacia 24-well 10% horizontal gel apparatus (Pharmacia Biotech Inc., Piscataway, N.J., USA). Replicate patterns were obtained. One gel was stained with Coomassie blue and the remaining gel was transferred electrophoretically to a nitrocellulose membrane [21]. The proteins that bound IgE were identified with ¹²⁵I-radiolabeled anti-IgE and visualized by X-ray autoradiography as described above. Individual aliquots representing 5-ml fractions that bound IgE were identified, and the 5-ml fractions were pooled and dialyzed against 100 mM ammonium bicarbonate (×4 × 4 liters) for 24 h and lyophilized using a Virtis 3L benchtop lyophilizer (The Virtis Company, Gardiner, N.Y., USA).

Coomassie-stained protein bands from both the 2-D and preparative SDS-PAGE analysis, which showed intense IgE binding in comparison to the autoradiograph, were sent to the W.M. Keck Foundation (Biotechnology Resource Laboratory, Yale University, New Haven, Conn., USA) for amino acid sequence analysis. Protein sequencing was carried out on Applied Biosystems instruments, which are equipped with on-line HPLCs for the identification of the resulting phenylthiohydantoin (Pth) amino acid derivatives. Before applying the sample, 25 pmol of a 16-residue internal sequencing standard peptide, which has the formula (norleucine-(succinyllysine)4)3-norleucine, is first spotted onto the sequencing filter. Since this internal sequencing standard is composed of non-naturally occurring amino acids, it does not interfere with sequencing the unknown protein. This internal standard provides the function of on-line monitoring of sequencer, which is critical to being able to keep the instruments operating at the peak performance, as is necessary to be able to routinely sequence at a level of <5 pmol. In addition, when a blocked eukaryotic protein is encountered, the presence of the sequence of the internal standard assures that the instrument was operating well and the failure to obtain a sequence was not the result of an instrument malfunction. In general, the 470A and 477 instruments are operated based on the manufacturer's recommendations, and 3 pmol of Pth standards are routinely used. In addition, the S4 solvent that transfers the Pth derivative to the HPLC contains 10 pmol of Pth-norvaline, which acts as an internal calibrant to independently monitor transfer to the HPLC. All sequences obtained in the Keck facility are immediately searched via the BLAST Network Service operated by the National Center for Biotechnology Information. This server accesses the Brookhaven, Swiss, PIR and GenBank databases and is updated daily. (Procedures carried out in the Protein Chemistry Section of the Yale/Keck Biotechnology Facility, New Haven, Conn., USA).

Binding of IgE to the Basic Chains of Glycinin

Each acidic and basic chain from glycinin was purified from the soybean breeding line CX635-1-1-1 as described by Moreira et al. [22]. Each of the purified polypeptides was lyophilized and then stored dry in a vacuum at room temperature. The proteins were dis-

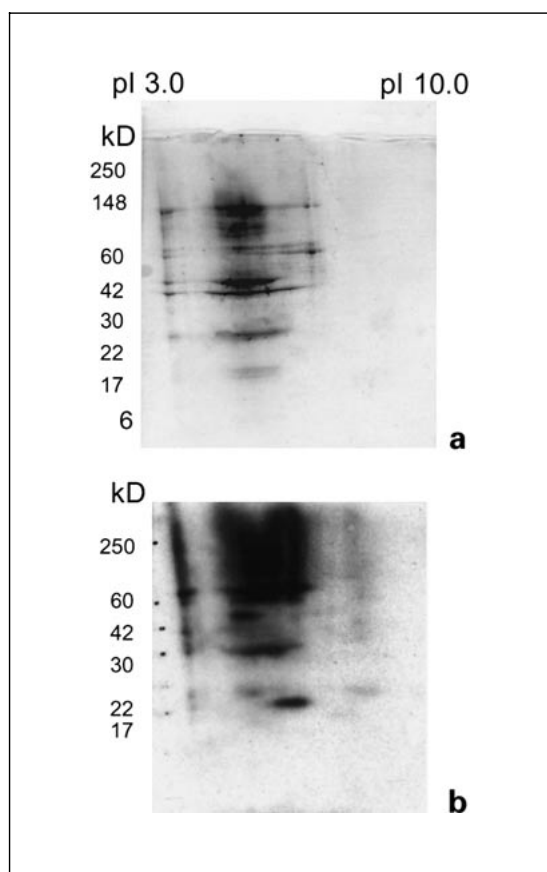


Fig. 1. 2-D SDS-PAGE analysis of a crude soybean extract. **a** Representative Coomassie-stained gel. **b** Autoradiograph of the transblotted proteins.

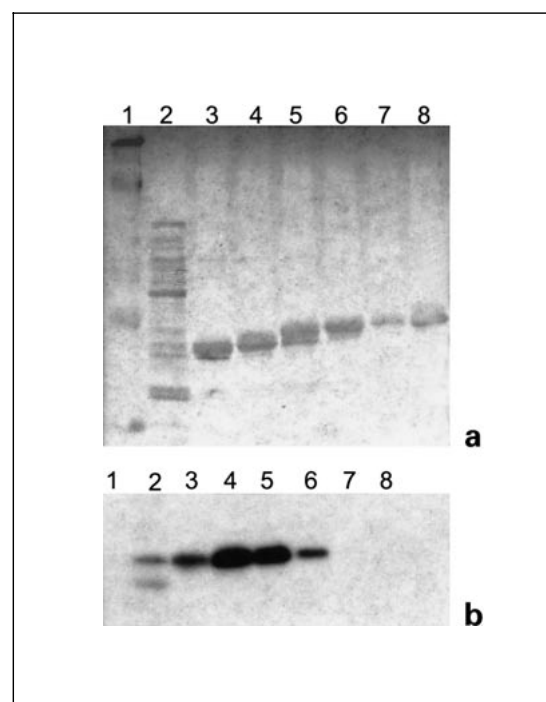


Fig. 2. Analysis of a soybean extract by preparative SDS-PAGE. Low-molecular-weight-range IgE-binding proteins were identified by soybean-sensitive serum enriched by preparative SDS-PAGE. **a** Coomassie blue-stained gel. **b** Autoradiograph of the transblotted proteins. Molecular weight markers were used as size standards. A similar blot was prepared and sent for N-terminal amino acid sequencing of the band identified in lane 4.

solved in extraction buffer that contained 4 M urea and separated by SDS-PAGE [21]. After electrophoresis, the proteins were transferred to PVDF membranes using the protocol recommended by the manufacturer (Bio-Rad Laboratories, Hercules, Calif., USA). The protein blots were probed [23] using sera from 6 patients allergic to soybean. To prepare the sera, 50 μ l from each patient were added to a final volume of 3 ml. Human antibodies bound to the membrane after incubation were identified using anti-human IgE labeled with alkaline phosphatase (BD PharMingen, Franklin Lakes, N.J., USA).

Results

Enrichment of IgE-Binding Soybean Proteins

2-D SDS-PAGE analysis was used to characterize IgE-binding proteins in crude soybean seed extracts using serum from soybean-sensitive individuals. A representative photograph of Coomassie blue-stained gel and the autoradiograph of an IgE immunoblot are shown in

figure 1. Based upon previous studies that identified unique soy-specific IgE-binding proteins [17], one with an approximate molecular weight range of 20–25 kD and a pI of ≈ 7.5 was selected for further characterization. To enrich the 20- to 22-kD proteins, crude soybean extracts were separated by preparative SDS-PAGE. Immunoblot analysis with pooled serum from soy-sensitive individuals revealed that IgE bound with high intensity to a protein band at approximately 20–22 kD (fig. 2). Serum IgE from the non-food atopic individual did not bind to any of the soybean proteins (data not shown).

The dialyzed, lyophilized and dH₂O-reconstituted fraction enriched in 22-kD proteins was subjected to 2-D SDS-PAGE/immunoblot analysis. As shown in figure 3, analysis by staining of blots with Coomassie blue and by autoradiographic analysis resulted in the detection of

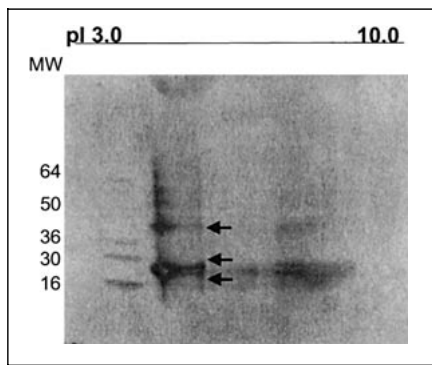


Fig. 3. 2-D SDS-PAGE analysis of fractions 24–36 from the preparative gel enrichment of a 22-kD soybean protein. The arrows point to the 22- and 45-kD bands that were N-terminal sequenced. MW = Molecular weight (in kD).

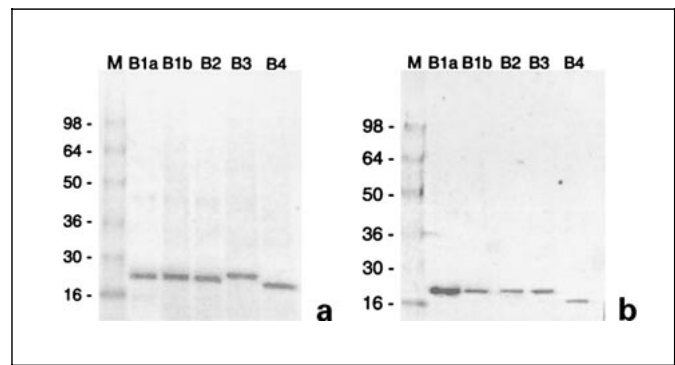


Fig. 4. SDS-PAGE/immunoblot analysis of the basic G2 chains of glycinin. **a** The results of a Coomassie blue-stained gel. **b** The results of immunoblot analysis with serum from soybean-sensitive individuals.

22-kD protein spots. The other proteins present at about 45 kD are aggregated forms of the 22-kD proteins.

Fractions enriched in the 22- and 45-kD bands were further purified by preparative SDS-PAGE and subjected to N-terminal amino acid sequence analysis. The N-terminal sequences of all three proteins were identified as mixed primary sequences of glycinin. The most prevalent primary sequence matched the basic glycinin polypeptide of G2 proglycinin as reported in the GenBank database (table 2). This result raised the question of whether the IgEs recognized only the G2 basic chain, or if they bound the basic chains from all glycinin subunits. To test the specificity of the reaction between the IgEs and glycinin, each basic chain was separated by SDS-PAGE, blotted to a PVDF membrane and analyzed. Figure 4 illustrates the results from such an experiment. Figure 4a shows a Coomassie blue-stained gel, while the corresponding blot is illustrated in figure 4b. It is apparent from the results that each basic chain from glycinin reacts with the IgEs to a similar extent. An analogous experiment was carried out using purified acidic chains. No binding to the acidic chains was detectable (data not shown). When the results from all of these experiments are considered, they convincingly demonstrate that the IgEs present in this serum sample bound specifically to the basic chains of glycinin.

Discussion

In the present study, proteins with a relative molecular weight of 20–22 kD were purified. They were selected based upon their ability to be recognized by serum IgE

from individuals who were shown to be clinically sensitive to soybean. Proteins of this size had previously been identified as potentially unique to soybean-sensitive individuals [17]. The N-terminal sequences of the proteins revealed that they were basic chains of a glycinin subunit from the 11S seed storage protein of *Glycine max*.

The 11S glycinin fraction accounts for the largest proportion (>50%) of the storage proteins found in most soybean varieties. When isolated from seeds in dilute salt solutions, this globulin is composed of six nonidentical subunits. Members of a small family of homologous nuclear genes encode the subunits. Both the genes and their products are extremely well characterized [16], and the critical properties of the five known glycinin subunits are summarized in table 3. Messenger RNAs encoding proglycinin precursors are translated on rough endoplasmic reticulum. After the cotranslational removal of signal sequences, the proglycinins assemble into trimers in the endoplasmic reticulum, and then are transported to protein storage vacuoles where they accumulate. Once the trimers arrive in storage vacuoles, an asparaginyl endopeptidase cleaves each of the subunits at a peptide bond between conserved asparagine and glycine residues. The cleavage results in the approximately 40-kD acidic and 20-kD basic chains that are characteristic of the subunits from mature glycinin. Cleavage of proglycinin trimers results in their assembly into hexamers, and these accumulate in storage vacuoles of seed cotyledons.

Although the proteins identified during the present investigation had a similar molecular weight, the N-terminal sequence clearly established them to be the basic chains of glycinin, not Kunitz soybean trypsin inhibitor.

Preparative gel SDS-PAGE 22-kD band (fig. 2): mix of two similar sequences in a 2.8:1 ratio: Primary (bottom) sequence and bold sequence both matched various soybean glycinin proteins.

Spot No. 1: 45-kD, 2-D SDS-PAGE (fig. 3): mix: primary (bottom) sequence matched various soybean glycinins.

Spot No. 1: 22-kD, 2-D SDS-PAGE (fig. 3): primary (bottom) sequence matched soybean glycinin A₂B1_a and G2 precursors. Asn, Ser, and Pro in cycles 18, 19 and 21 were determined by comparison to the known sequence.

Spot No. 3: 22-kD, 2-D SDS-PAGE (fig. 3): primary (bottom) sequence matched glycinin A2B1_a subunit precursor and G2 precursors from soybean.

Cys: Identified as an acrylamide Cys.

Table 3. Characteristics of the glycinin G2 subunits

Glycinin subunit	Database accession number	Calculated molecular weight	Calculated isoelectric point	Acidic chain		Basic chain	
				molecular weight	IEP	molecular weight	IEP
G1	CAA33215	53,624	5.78	33,161	5.37	20,481	7.99
G2	CAA33216	52,445	5.37	32,136	5.03	20,327	7.97
G3	AAB23211	52,311	5.22	31,718	5.09	20,611	5.69
G4	AAB23212	61,294	5.33	40,687	4.72	20,626	9.90
G5	FWSYG3	55,424	5.53	36,392	4.97	19,050	9.51

IEP = Isoelectric point.

As indicated earlier, the acidic and basic glycinin subunits are observed as prominent bands at approximately 40 and 20 kD, respectively, after SDS-PAGE. Djurtoft et al. [24] found IgE binding to all of the native acidic subunits, but little or none to the basic subunits. In experiments conducted by Zeece et al. [25], two closely spaced bands at approximately 40 kD were identified as acidic subunits of glycinin. The serum source these investigators used did not bind to proteins with molecular weights less than approximately 40 kD. Differences among serum sources are the most likely explanation for these differences.

It is not uncommon to find positive tests of IgE antibody to more than one legume protein, including peanut and soybean, although clinical observations have shown a low rate of coreactivity to peanut and soybeans. Eigenmann et al. [17] demonstrated that binding to whole peanut antigen was decreased by 73% after removal of soy cross-reacting antibodies by affinity chromatography. However, with peanut-absorbed sera from a single patient allergic to both soy and peanut, strong IgE antibody binding was observed to a 46-kD protein and a weaker reaction was apparent at 21 kD. These molecular weights are consistent with acidic and basic subunits of glycinins. The reduced binding, but not complete reduction of IgE antibody binding to peanut ELISA after absorption of cross-reacting antibodies to the respective antigen suggested that extensive cross-reactivity existed between soy and peanut allergens. This would not be unexpected considering the homologous nature of these two proteins. Bernhisel-Broadbent and Sampson [18] observed many in vitro cross-reactivity reactions to legumes, including peanut, soybean, green beans, peas and lima beans. Based on food challenge studies, they concluded that clinically important cross-reactivity to legumes in the children they studied was very rare.

In a study of severe food reactions in Sweden, Foucard and Malmheden Yman [26] suggested that soy allergy has probably been underestimated as a cause of anaphylaxis. In their study, four youngsters who were reported to have died from soy anaphylaxis with asthma were severely allergic to peanuts but had no previously known allergy to soy. They all had very high scores for IgE antibodies to peanut and moderate scores for soy. The estimated amount of soy intake in these fatal cases was established to be between 1 and 10 g. The authors regarded individuals at risk to be young people with asthma and peanut allergy so severe that they noted symptoms after indirect contact with other food sources.

As pointed out by Awazuhara et al. [11], the diversity of soybean products, the frequency of their consumption and differences in age and dietary behavior of subjects with soybean allergy all may contribute to differences in immunoblot identification of soybean allergens. In this report, the IgE binding data were derived from the serum of a patient who had a clinically documented soybean food sensitivity with a history of anaphylaxis following the ingestion of foods containing soybeans. It would be interesting to analyze serum from individuals having documented anaphylactic episodes to the ingestion of foods containing soybean products. What, however, does appear to be consistent is the report of a 20,000-dalton molecular weight protein in all these studies, including ours.

Acknowledgments

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