

Protein and allergen content of commercial skin test extracts for soybeans*

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Summary

The protein and allergen contents of four commercial soybean skin test extracts were tested by SDS-PAGE and immunoblotting using sera from soy-allergic adults. Polyacrylamide gels stained with Coomassie Blue showed an absence of several major soybean proteins, particularly those at higher molecular weights. The acidic subunits of glycinin and beta-conglycinin, major soybean storage proteins, appear to be absent or present in much reduced amounts. Immunoblots with soy-allergic sera indicate alteration, reduction, or loss of IgE-binding in the commercial extracts as compared to extracts of soy flour. In one soy-allergic patient, skin tests revealed a negative response to three of the commercial soybean extracts and a mild response to one extract. De-fatted soy flour obtained from two of the four extract manufacturers was extracted in the laboratory using a standard procedure for the isolation of soybean proteins. In one case, the extract still had an abnormal protein profile on gel electrophoresis while in the other case, the new extraction procedure gave significantly improved extraction of soy protein. Preparation methods appear to be partially responsible for the variable allergen content in commercial soybean skin test extracts.

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Introduction

Soybean allergy is one of the most common food allergies, especially among children. Epidemiological studies with children indicated that soybeans, peanuts, cow's milk, and eggs were the major allergenic foods [1–4]. The prevalence of soybean allergy in the adult population is unknown but it is thought to be increasing due to the expanding use of soybeans and soybean products in the American diet. Allergies tend to occur more frequently in populations where exposure to the allergen is greatest [5].

Very little research has been done to characterize soybean-allergic individuals and identify soybean allergens. In a recent study, it was shown that all soybean-allergic adults do not react to the same soy proteins [6].

Patients were separated into at least two distinct categories according to binding patterns of their IgE to soybean proteins and whether or not they were sensitive to peanuts as well as soybeans. Category I individuals were sensitive to both soybeans and peanuts and had IgE that bound several soy proteins of molecular weights ranging from about 50 000 to 70 000. Category II individuals were sensitive to soybeans but not peanuts and had IgE that bound a single soy protein of molecular weight about 20 000. IgE from Category II patients did not recognize any peanut proteins [6].

One method commonly used by clinicians to test individuals for soybean allergy is the skin prick test [7]. However, skin test results are not always consistent with oral challenge data [8] and skin test extracts can vary in quality [9]. The variability may be caused by different starting materials, storage conditions, methods of extraction, and batch-to-batch variability. This study investigated the protein and allergen content of several commercial soybean extracts using SDS-PAGE (sodium dodecyl

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sulphate polyacrylamide gel electrophoresis) and immunoblotting. Sera from both Category I and II individuals were used to test the allergenicity of the four commercial extracts.

Materials and methods

Soybeans products

Raw soybeans (Vinton variety) were generously supplied by Dr Patricia Murphy, Department of Food Technology, Iowa State University, Ames, Iowa, U.S.A. Soy flour was purchased from a local grocery store in Madison, Wisconsin, U.S.A.

Commercial soy extracts

Soybean skin test extracts were purchased from four suppliers (Allergy Labs of Ohio, Columbus, Ohio, U.S.A.; Center Laboratories, Port Washington, New York, U.S.A.; Greer Laboratories, Inc., Lenoir, North Carolina, U.S.A.; Hollister-Stier, Spokane, Washington, U.S.A.). All extracts were 1:10 w/v aqueous preparations (as defined by the manufacturer) preserved with 0.4% phenol. The methods of production of these extracts are unknown and are the proprietary information of the companies. All extracts were purchased immediately

prior to these experiments and were stored appropriately. The studies were completed before the expiration of the shelf-life of the extracts.

Extraction of soy flours

De-fatted soybean flours used by Greer Laboratories, Inc. and Hollister-Stier for extract preparation were generously supplied by these firms. In addition, the raw Vinton soybeans and commercial soy flour were defatted according to the method of Nordlee *et al.* [10]. All defatted flours were extracted with 0.1 M ammonium bicarbonate, pH 8.0, a procedure reported previously for the extraction of peanut proteins [10]. The extracts of the raw Vinton soybeans and the commercial soy flour were used for comparative purposes in electrophoretic experiments (Fig. 1). Since the Vinton soybean extract was used as a reference throughout the experiments, aliquots were frozen (-20°C) until use. No changes in protein concentration or Coomassie blue staining pattern were observed.

Soybean-allergic sera

Sera were obtained from seven atopic patients with clinical symptoms of soybean allergy. Diagnosis was confirmed in each case by a convincing oral history, skin

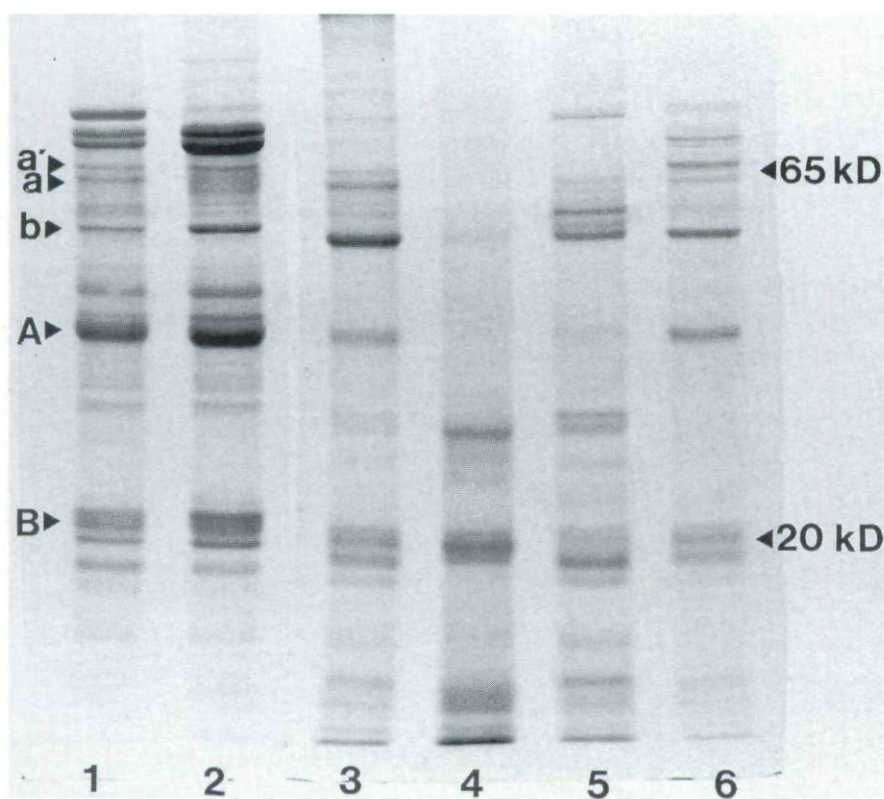


Fig. 1. Coomassie Blue R stained 12% SDS-PAGE gel of two soybean extracts prepared in the laboratory (1, raw soybean, Vinton variety, 2, soy flour) and four commercial soybean skin test extracts (3, Hollister-Stier, 4, Greer, 5, Center Labs, 6, ALO). About 60 μg of protein from each extract was applied per lane. All samples were reduced with 2-mercaptoethanol. Tentative identification of some bands is based on mobilities and comparison to previous work [12]. B-basic subunits of glycinin, A-acidic subunits of glycinin, and beta conglycinin subunits (a, a', and b).

Table 1. Characteristics of soybean-allergic subjects

Patient (category)	Sex	Age	Known or suspected legume sensitivities	RAST against raw soybeans (binding $\times$ normal)	Skin test results	Symptoms
1 (I)	F	23	Peanuts, soybeans, peas, lima beans	68 $\times$	3+	Laryngeal oedema, bronchospasm, hives, itching, abdominal pain
2 (I)	F	35	Soybeans, kidney beans, peanuts, garbanzo beans, green peas	11 $\times$	2+	Hives, swelling, difficulty breathing, nausea, diarrhoea, lip oedema
3 (II)	F	67	Soybeans	25 $\times$	2+	Laryngeal oedema, bronchospasm, wheezing, swelling, itching
4 (I)	M	36	Peanuts, soybeans	2.3 $\times$	2+	Mild respiratory distress, hives, itching, oral tingling
5 (II)	M	54	Soybeans, kidney beans, lentils	3 $\times$	2+	Hives, localized swelling, bronchoconstriction, metallic taste
6 (I)	F	25	Peanuts, soybeans, green peas	5 $\times$	2+	Respiratory difficulty, bronchoconstriction, wheezing
7 (?)	F	42	Soybeans, kidney beans; lost allergy to peanuts, lima beans	66 $\times$	3+	Hives, asthma, watery eyes, wheezing, rhinitis

prick testing, and positive RAST. Details about each patient are described in Table 1. Four patients fit the criteria for Category I soybean allergy, two patients fit the criteria for Category II soybean allergy, and the seventh patient did not fit into either of the categories [6]. Control non-atopic sera consisted of a pool of six individuals with no history of allergy.

SDS-PAGE and immunoblotting

SDS-PAGE gels and immunoblots were prepared as reported previously [6].

Skin testing

A commercial soy extract from Greer Laboratories was diluted to a 1:20 w/v concentration with a sterile solution of 50% glycerin and 0.4% phenol. The extract was applied by the puncture technique and the weal size recorded after about 10–15 min. Positive controls for the skin tests contained 1 mg/ml histamine in 50% glycerin plus 0.4%

phenol. One patient did not give a positive skin test to the Greer extract; this patient was tested with an extract of soy flour prepared as described above.

RAST (RadioAllergoSorbent Test)

RAST values for individual patients were determined by a method similar to that of Adolphson *et al.* [11]. Soybean solid-phase was prepared by coupling raw soybean extract to cyanogen bromide-activated microcrystalline cellulose particles. The soybean extract used for preparation of the RAST solid-phase was an extract of raw Vinton soybeans prepared as described above. Microcrystalline cellulose (0.5 mg) with coupled soybean proteins (1 mg cellulose/ml), 0.05 ml serum from soy-allergic subjects or controls, and 0.2 ml RAST diluent [11] were incubated in 12 $\times$ 75 mm test tubes at 25°C for 20–24 hr with constant rotation. After incubation, the solid-phase was washed with RAST wash solution [11] and 0.1 ml (approximately 200 000 d.p.m.) ^{125} I radiolabelled rabbit anti-human IgE and 0.65 ml FCS diluent [11] was added to each tube. After 20–24 hr of incubation at 25°C, the solid-phase was again washed with RAST wash solution, and the amount of radioactivity in the tubes containing

the solid-phase was determined with a gamma scintillation counter. Results are expressed as per cent binding and are calculated as [counts bound to allergic serum minus counts bound to normal serum] divided by [total counts added minus counts bound to normal serum] $\times 100$. The results were also expressed as a ratio of the counts bound by allergic sera divided by counts bound by control non-atopic sera.

Results

Subjects

Table 1 lists the sex, age, known legume sensitivities, RAST values, skin test results, category of soybean allergy, and symptoms of the seven subjects whose sera were used in this study.

Gel electrophoresis

Figure 1 shows a 12% SDS-PAGE gel of raw soybean extract (Vinton variety), soybean flour extract, and the four commercial skin test extracts stained with Coomassie Blue R. Some protein bands have been tentatively identified on the basis of electrophoretic mobility and comparison to previous work [12]. Very noticeable is a marked reduction of several soybean proteins in the commercial extracts. Beta-conglycinin and the acidic subunits of glycinin are dramatically reduced or even absent in the commercial extracts.

A second 12% SDS-PAGE gel stained with Coomassie Blue R run with extracts of Hollister-Stier and Greer soy flours prepared according to the procedure of Nordlee *et al.* [10] is shown in Fig. 2. Although the Hollister-Stier extract now gives an improved protein pattern more like that of raw soybean or soybean flour extracts, the Greer preparation still lacked the beta-conglycinin subunits.

Immunoblots

Figures 3, 4, and 5 illustrate three distinctly different types of IgE binding responses to the four commercial soybean extracts. Figure 3 illustrates the IgE-binding pattern of Patient number 1, a Category I individual. Serum from Patient 1 binds to proteins in all four commercial soy extracts. However, the IgE-binding pattern is different than that usually obtained with extracts of raw soybeans, soy flours and other soy products [6]. The major bands appear at lower molecular weights than the reactive bands previously reported. It is not known whether these lower molecular weight proteins are related to the IgE-binding proteins identified in past studies.

The IgE binding of the Allergy Labs of Ohio (ALO) extract is most consistent with results previously obtained



Fig. 2. Coomassie Blue R stained 12% SDS-PAGE gel of 0.1 M ammonium bicarbonate, pH 8.0, extracts prepared according to the procedure of Nordlee *et al.* [10] using soybean flour obtained from Hollister-Stier (1) and Greer Laboratories (2).

with extracts of raw soybeans, soy flours and some soy products. Sera from other Category I patients (Patients numbers 2, 4, and 6) gave similar but not identical IgE binding to that observed with Patient 1 (blots not shown). All patients reacted to proteins in all of the commercial soybean extracts, although Patients 2 and 4 showed generally weak responses. Serum from Patient 2 also gave binding to a few additional minor bands.

Serum IgE from Patient 3, a Category II patient, reacts only to one extract (ALO) with only a single reactive band of low molecular weight about 14 000 visible (Fig. 4).

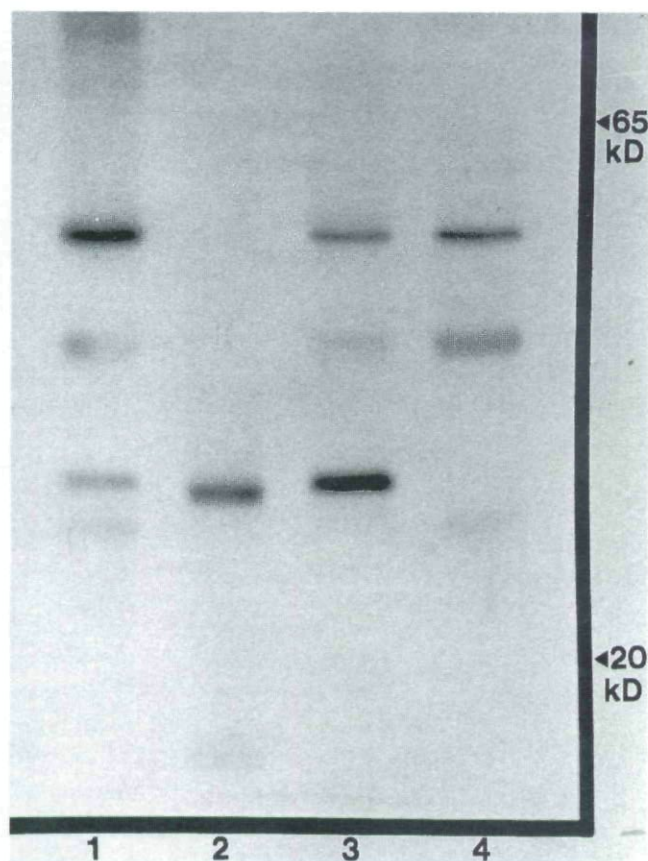


Fig. 3. Immunoblot of serum from a Category I patient (Patient 1). Extracts tested (1–4) are Hollister-Stier, Greer, Center Labs, and ALO.

Serum from Patient 5, also from Category II, shows a similar lack of IgE binding to the extracts (blots not shown). Skin tests of Patient 3 with the same extracts confirmed a negative response for three of the extracts (Greer, Hollister-Stier, Center Laboratories) and a mild positive response for the ALO extract.

The IgE-binding pattern of Patient 7 serum is shown in Fig. 5. This patient is not included in either Category I or II because of a distinctive IgE binding pattern to a variety of soybean proteins [6]. This patient's case history is also unique in that several legume sensitivities were outgrown including one to peanuts, but a strong sensitivity to soybeans and a mild sensitivity to kidney beans were retained. Serum IgE from Patient 7 binds strongly to one or more soybean proteins of molecular weight about 14 000 [6]. The four commercial soy extracts contain this protein with three of the extracts giving strong IgE binding with the serum of Patient 7. The Greer extract gives only weak binding.

Immunoblots showing IgE binding by representative soybean-allergic individuals to freshly prepared extracts

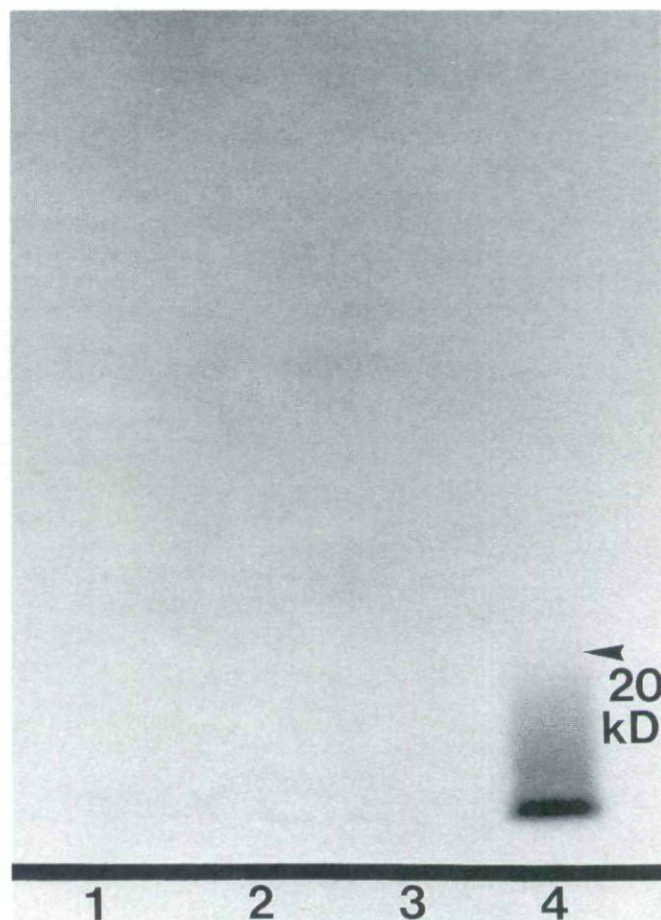


Fig. 4. Immunoblot of serum from a Category II patient (Patient 3). Extracts tested (1–4) are the same as those in Fig. 2.

of Greer flour and Hollister-Stier flour using 0.1 M ammonium bicarbonate pH 8.0 are displayed in Fig. 6. This approach attempted to determine whether commercial extraction methods are the cause of reduced protein content or altered IgE binding. When tested with Category II serum (Patients 3 and 5), the ammonium bicarbonate extract of Hollister-Stier flour contained the protein (MW 20 000) that characteristically binds IgE of Category II soy-allergic individuals [6], but the Greer flour extract still did not contain this IgE-binding protein (Fig. 6). IgE binding by the serum of Patient 7 was improved for the ammonium bicarbonate-extracted Greer flour. However the Greer extract still gave the weaker response of the two extracts (Fig. 6).

When comparing IgE binding by sera of Category I soybean-allergic patients, some differences were noted between the ammonium bicarbonate extracts of the Greer and Hollister-Stier soy flours (Fig. 6). In particular, several of the IgE-binding proteins were much more evident in the Hollister-Stier extract than in the Greer

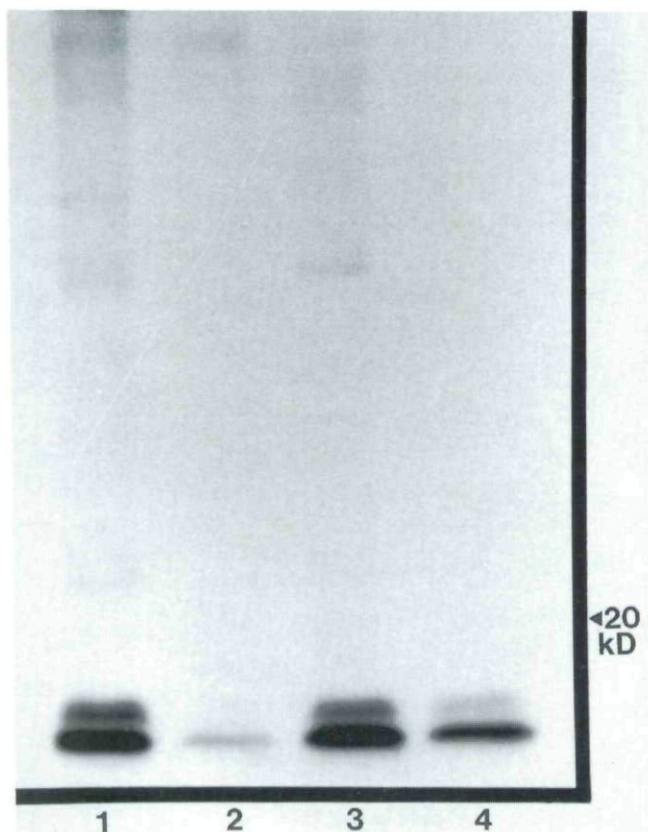


Fig. 5. Immunoblot of serum from Patient 7. Extracts tested (1–4) are the same as those in Fig. 2.

extract. The number of IgE-binding proteins in the ammonium bicarbonate extract of the Greer soy flour was greater than the number present in the commercial extract from the same manufacturer (Fig. 3). However, there was no difference in the number of IgE binding proteins between the two Hollister-Stier extracts.

Immunoblots with normal control serum did not show visible binding to any of the extracts.

Discussion

Extracts used by clinicians for skin testing are often criticized as being of poor or inconsistent quality [9]. Reasons include the use of variable raw materials, improper extract storage and handling, lack of information about the quantity and stability of specific allergens, the use of inappropriate extraction methods, and the lack of standardization of most extract materials [13]. Since one of the most commonly used methods for identifying allergic individuals is the skin test, it is important to have extracts with high and consistent allergenic activity. Poor extracts can result in false negative and sometimes even false positive results in the skin test. Obviously, false negative skin tests can be misleading to the clinician and potentially dangerous to the patient.

Extracts of soybeans and soybean products contain several IgE-binding proteins [6]. Based on the IgE-binding patterns of their sera and the absence or presence of co-existing peanut sensitivity, soy-allergic patients

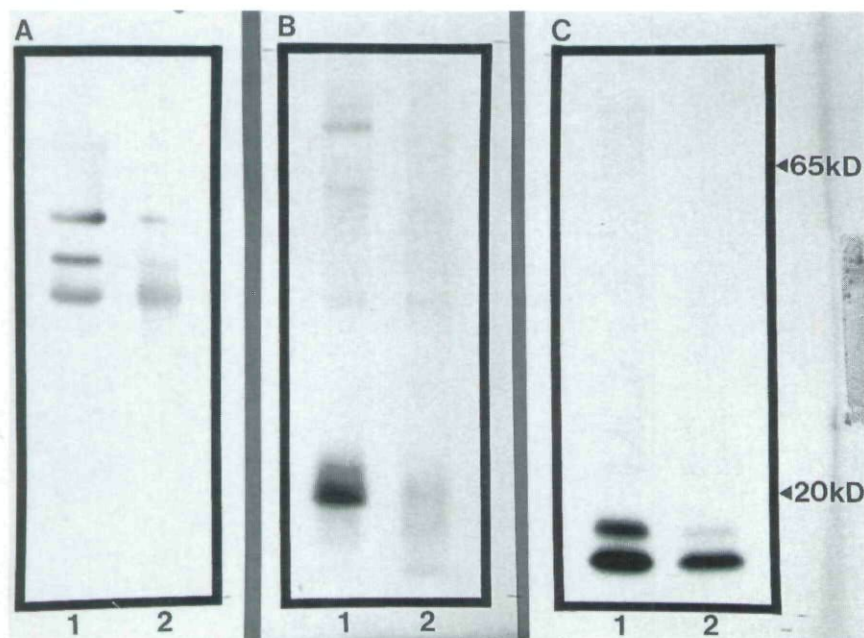


Fig. 6. Immunoblots of sera from Category I patient 1 (blot A), Category II patient 3 (blot B), and patient 7 (blot C). Extracts tested were prepared by the ammonium bicarbonate extraction procedure of Nordlee *et al.* [10] using soybean flour obtained from Hollister-Stier (1) and Greer Laboratories (2).

were divided into two major categories. Sera from Category I individuals bound several soy proteins ranging in molecular weight from about 50 000 to 70 000. Sera from Category II individuals bound a single soy protein of molecular weight about 20 000.

In this study, four commercial skin test extracts were tested for protein content by SDS-PAGE and for IgE binding using the immunoblotting technique and sera from seven soybean-allergic individuals. SDS-PAGE of the four commercial extracts showed a marked reduction or even a loss of several soybean proteins. Higher molecular weight proteins (40 000 to 70 000) seemed particularly affected. Beta-conglycinin and the acidic subunits of glycinin are nearly absent. This could indicate poor extraction or processing steps that destroy or fail to solubilize these proteins.

IgE from sera of Category I individuals gave binding with all four commercial extracts. However, the protein bands which showed binding were of different molecular weights than those previously reported for extracts of raw soybeans and various soy-based food products [6]. In particular, the IgE-binding proteins of higher molecular weight were absent. It is not known whether the smaller molecular weight proteins are related to the proteins identified in past studies. Of the four extracts tested, Greer gave the weakest IgE binding with Category I serum while the ALO extract gave a binding pattern most similar to that obtained in earlier studies [6].

Sera from Category II soybean-allergic individuals showed virtually no IgE binding to any of the commercial extracts. Lack of allergenic activity in the extracts was confirmed with negative skin tests on Patient 3, a Category II patient.

Serum from Patient 7 binds strongly to a soybean protein of molecular weight about 14 000 [6]. Three of the four commercial soy extracts contain large amounts of this protein, giving strong IgE binding with this patient's serum. Patient 7 has a unique clinical history. This patient has outgrown several legume sensitivities including one to peanuts, but has retained a strong sensitivity to soybeans and a mild sensitivity to kidney beans. It is unusual to outgrow peanut allergy as sensitivity to peanuts is more likely to persist than sensitivity to other foods [14]. The unique history likely correlates with her unique IgE binding pattern.

The poor reactivity of the commercial extracts to Category II serum (and somewhat to Category I serum) was surprising considering the rather consistent IgE-binding patterns of extracts of raw soybeans and a variety of soy-based food products [6]. Unfortunately, very little information is available on the commercial methods used for preparing these extracts. One possible explanation for lack of reactivity could be a problem with the methods

used to prepare the extracts. Defatted soy flours obtained from Greer Labs and Hollister-Stier and extracted with a standard ammonium bicarbonate extraction procedure used in previous studies on soybean and peanut allergens [6,10] indicated that the extraction procedure was not solely responsible for the problems.

SDS-PAGE of the ammonium bicarbonate extract of the Hollister-Stier soy flour showed a nearly identical protein profile to that observed with typical soy flour. Beta-conglycinin and the acidic subunits of glycinin that were previously absent are now visible. An immunoblot with serum of a Category II patient (Patient 3) revealed that the 20 000 molecular weight allergen is now present (Fig. 6). Apparently, the extraction procedure used by Hollister-Stier is not extracting this protein. However, the soy flour used by Hollister-Stier appears to be adequate as a starting material.

SDS-PAGE of the ammonium bicarbonate extract of the Greer soy flour continued to show an absence of beta-conglycinin. However, acidic subunits of glycinin did appear. An immunoblot with the serum from a Category II patient (Patient 3) showed no binding to this extract (Fig. 6). These results suggest that the method used to prepare the Greer flour irreversibly denatured proteins thereby preventing their extractability with the ammonium bicarbonate procedure.

Variability in the allergen content of soybean skin test extracts appear to result in part from the methods used to prepare the extracts, selection of starting materials, and from methods used to prepare those materials. Samples included in this study were randomly selected, but only one lot was obtained from each manufacturer. Therefore, other factors, which were not evaluated, such as age of the extracts, age of raw materials, and batch-to-batch variation may also affect reliability. Clearly, the diagnosis of soybean allergy could be improved by the availability of improved soybean extracts for skin testing and the implementation of standardization procedures to assess the quality and potency of these extracts. Standardization of skin test extracts has proven to be quite valuable in the case of pollen extracts [15]. For example, a thorough evaluation of commercial rye pollen extracts determined that considerable variability existed and led to the development of a standardized extract [15]. Similar evaluations and decisions on the standardization of food extracts would likely improve the quality and consistency of such extracts including soy extracts. This preliminary investigation will hopefully stimulate such efforts.

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