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Neoallergens in Heated Soybean Hull

Key Words

Soybean allergens
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Storage
Heat

Abstract

Background: During the process of harvest, transport and storage, microbial and mold contamination can raise the temperature of soybeans to 75°C or higher. The purposes of this study were (1) to evaluate the allergenicity of fresh and stored soybean hulls and (2) to ascertain whether heat alters the allergenicity of stored soybean hulls. **Methods:** Allergen extracts were prepared from (1) stored soybean hulls, (2) fresh soybean hulls and (3) stored soybean hulls heated to 37°C (E1), 55°C (E2) and 80°C (E3) or kept at room temperature (E4) for 16 h. Individual serum from 68 soybean asthmatic (SA) subjects, 30 nonallergic subjects and two serum pools made from 4 SA sera and 4 sera from asthmatics not sensitive to soybean were studied. All sera and serum pools were assayed for content of specific IgE (radioallergosorbent test) and IgG4 (ELISA). The following additional studies were done for extracts E1–E4: (1) SDS-PAGE, (2) SDS-PAGE/Western blot for specific IgE and IgG4 using both serum pools, and (3) study of the effects of heat on inhibiting activity of the extracts prepared from stored soybean hulls using the pool of SA sera. **Results:** Test results demonstrated a reduced binding of specific IgE and IgG4 to fresh soybean hull extract compared to stored soybean hull extract, and an increased binding for heated extracts (E1–E3) compared to unheated ones (E4). Moreover, there was an increase in potency for IgE and IgG4 bindings for the heated (E1–E3) compared to unheated (E4) extract, as measured by the amount of protein to produce 50% inhibition. Several protein bands with a molecular weight (MW) higher than 20 kD were absent from the SDS-PAGE for E3 but were present in E1, E2 and E4, and a new protein band (MW 15.3 kD) appeared for E3 only. Two new protein bands, with MWs of 15.3 and 10 kD, which bind specific IgE, were present on Western blot and one of the 3 main soybean hull allergens, probably Gly m 2, disappeared in E3. IgG4 Western blot showed similar results, but only the 10 kD protein band was present. **Conclusion:** The results demonstrate that soybean hull allergenicity is affected by heat, and suggest that the heat generated during storage and transport of soybeans could generate 2 new allergen determinants or increases in epitope exposure as a result of conformational changes. The significance of these new IgE and IgG4 binding proteins has yet to be determined.

Introduction

The hull from soybean seeds was identified as the source of aeroallergens which causes soybean-induced asthma (SIA). Some of these proteinic aeroallergens were identified [1, 2] and three of them, Gly m 1A, Gly m 1B and Gly m 2, were purified and their N-terminal amino acid sequence reported [3–5]. Specific IgE and IgG4 to these three allergens was demonstrated in the sera of soybean asthmatic (SA) subjects [6, 7].

Stored grains are contaminated by insects, mites, bacteria and molds [8–10]. These contaminants must be considered in evaluating the etiology of SIA. Swanson et al. [11] found small amounts of soybean allergens in the leaves, stems and flowers of immature soybean plants, suggesting that the source of allergens is the soybean plant, and not contaminating microorganisms, mites or insects. While mites have not been found to be an etiologic allergen for SIA [12], four *Aspergillus* and two *Penicillium* species have been isolated from soybean hull [13] and, therefore, must be considered as contaminants possibly playing a role in the etiology of SIA.

Soybeans are difficult to store because they generate heat caused by the growth of storage fungi [8, 14]. Molds are allergenic, can produce mycotoxins, hydrolytic enzymes, and can raise the temperature of the grains through fermentation. Thus, either fungi allergens or thermally or chemically modified hull proteins might cause SIA. Neoallergens from heat or storage have been detected in apples, pecan nuts and shrimp [15–18]. The purpose of this study was to determine whether neoallergens might be derived from heated soybean hulls.

Materials and Methods

Human Sera

Sera from three groups of subjects from Barcelona, Spain, were studied: group I consisted of 68 sera from SA individuals attending an emergency room during one of the asthma outbreaks that occurred in Barcelona, group II of 30 sera from nonallergic individuals, and group III of 4 sera from asthmatic individuals not sensitive to soybeans. All subjects gave informed consent. Two serum pools were prepared, the first from 4 sera of group I and the second from 4 sera of group III.

Allergen Extracts

Extracts were prepared from stored hulls collected from silos in Barcelona, Spain, which were kept at 4°C until the extract was made, as well as from fresh hulls, not previously stored, obtained from soybeans picked from a field and peeled grain by grain. The stored hulls employed were found to be contaminated by molds in a previous study [13].

Extracts from stored hulls heated to 37°C (E1), 55°C (E2) and 80°C (E3) for 16 h were also prepared. An extract made from stored hulls maintained at room temperature for 16 h was used as a reference extract (E4). The amount of hulls obtained from fresh soybeans was not enough to perform heating experiments with fresh hulls during this phase of the study as previously described [7].

Specific IgE and IgG4 Determinations

Specific IgE and IgG4 levels to extracts prepared from fresh and stored hulls were determined in all individual sera of groups I and II, and to E1–E4 with the pool of SA sera following protocols previously described [7].

Effects of Heat on Inhibiting RAST and ELISA Activity of Hull Extracts

The allergen extracts E1–E4 were compared by RAST inhibition (for specific IgE binding) and by ELISA inhibition (for specific IgG4 binding). The procedures were performed essentially as those for specific IgE and IgG4 determinations, except that the coated solid phases with each of the 4 extracts were made to react with a mixture of 50 µl of the pooled SA sera diluted 1:4 along with 50 µl of the 4 allergen extracts in different concentrations. The relative potencies of allergen extracts were compared by the quantity of each extract needed to produce 50% inhibition.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of the Heated Hull Extracts (E1–E4)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 10–20% polyacrylamide Tris-tricine gradient gel (Ready Gels, Bio-Rad Laboratories, Richmond, Calif.) according to the discontinuous buffer system of Laemmli [19] following protocols previously described [7].

Western Blot Analysis

Transfer of the proteins to a 0.2-µm nitrocellulose (NC) membrane (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. [20]. After the transfer, the NC membranes were blocked overnight with blocking buffer. Assays of specific IgE and IgG4 were performed with the 2 pools of sera following protocols previously described [7]. Molecular weights (MWs) were determined by the method of Weber and Osborn [21].

Statistical Analysis

The statistical analysis was performed with the Statview program (Abacus Concepts, Berkeley, Calif.). To ascertain if the values were normally distributed, the Kolmogorov-Smirnov test was applied to the values found for each technique in group II. The means between groups I and II were compared with the Student t test for each IgE and IgG4 determination with fresh and stored hull extracts. A p value less than 0.05 was considered significant.

Table 1. Specific IgE and IgG4 to fresh and stored soybean hulls in groups I and II

Immunoglobulin	Group I		Group II	
	fresh	stored	fresh	stored
IgE				
Positive results	6 (8.8)*	57 (83.8)+++	1 (3.3)	1 (3.3)
Mean $\pm$ SD	0.106 $\pm$ 0.142*	13.6 $\pm$ 12.8+++	0.052 $\pm$ 0.046	0.072 $\pm$ 0.046
IgG4				
Positive results	2 (2.9)*	50 (66.1)++	1 (3.3)	3 (10.0)
Mean $\pm$ SD	0.028 $\pm$ 0.032*	0.300 $\pm$ 0.334++	0.019 $\pm$ 0.010	0.021 $\pm$ 0.016

Mean $\pm$ SD is expressed as % total counts bound for specific IgE and as OD units for specific IgG4. Figures in parentheses represent percentage. * $p < 0.0001$ vs. stored in groups I and II; ++ $p < 0.01$, +++ $p < 0.001$ vs. fresh and stored in group 2.

Results

Specific IgE and IgG4 to Fresh and Stored Hull Allergens

Results of the specific IgE and IgG4 assays of group II had a normal distribution, and the reference values were established as the mean plus two standard deviations. Results were considered positive if they were equal or higher to the reference values. Percentages of positive results were calculated in groups I and II. In group I, compared to group II, the mean values and the percentages of positive results for specific IgE and IgG4 to extract prepared from stored hulls were significantly higher than for extract prepared from fresh hull (table 1). The mean values and the percentages of positive results for specific IgE and IgG4 to extract prepared from stored hull were significantly higher in group I compared to group II (table 1).

Specific IgE and IgG4 to the Heated Stored Hull Extracts (E1–E4)

There was an increase in IgE and IgG4 binding, which started for E1 (37°C) and E2 (55°C), respectively, compared to E4 (fig. 1). The increase found for both IgE and IgG4 binding was significant for E3 (80°C) compared to E4 ($p < 0.0001$).

Effects of Heat on Inhibiting RAST and ELISA Activity of Hull Extracts

The amount of protein to produce 50% inhibition in the RAST inhibition study was approximately 0.014, 0.011, 0.0007 and 0.018 μ g for E1, E2, E3 and E4, respectively. In the ELISA inhibition study, the values for the same extracts were approximately 0.012, 0.010, 0.0007 and 0.016 μ g, respectively. These results indicate a small increase of potency for E1–E3, in comparison with E4, for specific IgE and IgG4 bindings beyond 37°C (E1). Figures 2a and b show

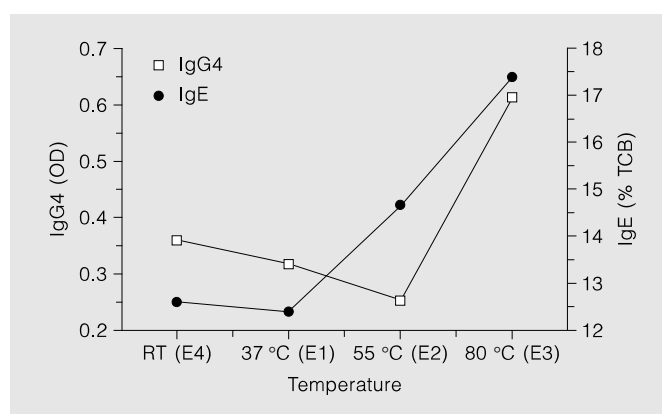


Fig. 1. Specific IgE and IgG4 to allergen extracts prepared from stored soybean hull heated at different temperatures (E1–E4). RT = Room temperature; TCB = total counts bound.

the inhibition slopes of both assays using E4 as a solid phase. As it can be seen, only small differences in the slopes of the inhibition curves were detected.

SDS-PAGE of the Extracts

The SDS-PAGE gel demonstrated for E3: (1) absence of protein bands with an MW higher than 20.5 kD present in E1, E2 and E4, (2) the disappearance of a band with a MW of approximately 8 kD, which probably is the allergen Gly m 2, present in E1, E2 and E4, and (3) the appearance of a new protein band with a MW of approximately 15.3 kD not detected in E1, E2 or E4 (fig. 3, lane 5).

SDS-PAGE/Western Blot

SDS-PAGE/Western blot for specific IgE demonstrated (1) an allergen of approximately 8 kD probably the allergen Gly m 2, which disappeared from E3, but was present in E1, E2 and E4, and (2) two bands, MWs 10 and 15.3 kD, which

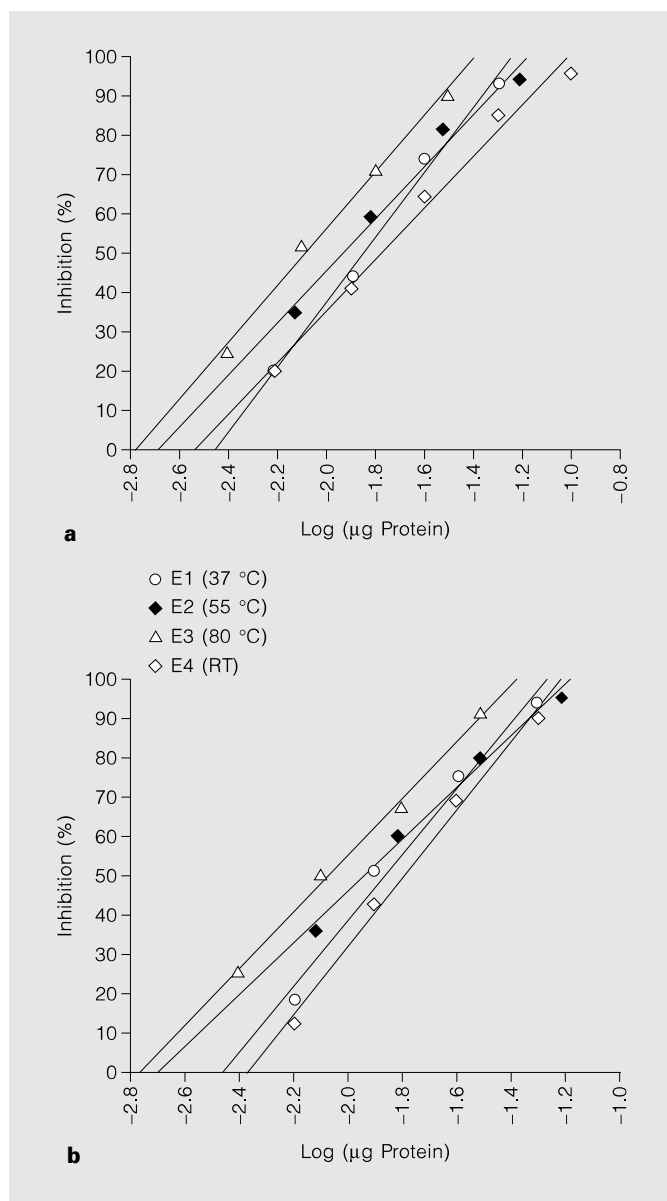


Fig. 2. RAST inhibition (**a**) and ELISA inhibition (**b**) assays using E4 as a solid phase. RT = Room temperature.

seem to be present in small amounts in E4, became more intense in E1–E3 (fig. 4, panel A). SDS-PAGE/Western blot for specific IgG4 demonstrated the disappearance of a 8-kD allergen, which is probably Gly m 2, and in addition, a new band, MW 15.3 kD, not detected in E1, E2 or E4, appeared in E3 (fig. 5, panel A).

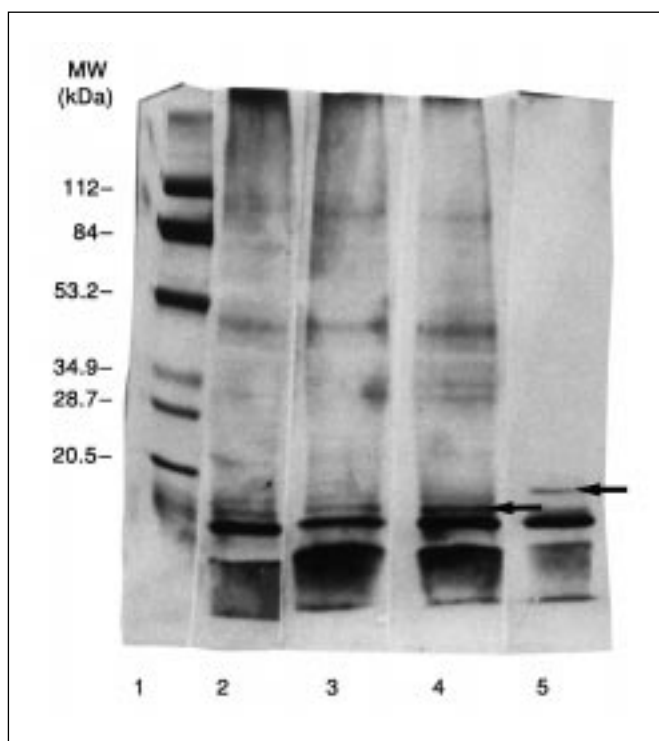


Fig. 3. SDS-PAGE of allergen extracts prepared from soybean hull heated at different temperatures (E1–E4). Lane 1 represents MW standards; lanes 2, 3, 4 and 5 represent E4, E1, E2 and E3, respectively. The arrow on lane 4 points to the 8-kD band, and the one on lane 5 to the 15.3-kD band.

Discussion

González et al. [3] identified an allergen from stored soybean hull, Gly m 1, responsible for the asthma outbreaks that took place in Cartagena, Spain. It contains two isoallergens, Gly m 1A and Gly m 1B, with MWs of 7.5 and 7 kD, respectively. Codina et al. [4] found a third allergen in stored soybean hull, Gly m 2, with a MW of 8 kD, using sera of patients with epidemic SA from Barcelona, Spain. It was demonstrated that the SA sera contained specific IgE and IgG4 which bound to the allergen Gly m 2, as well as to the two isoallergens, Gly m 1A and Gly m 1B. All three allergens were purified and sequenced [3, 5].

In this study the allergenicity of fresh and stored soybean hull was compared. The results of specific IgE and IgG4 determination to both extracts in a group of soybean-asthmatic sera and in a control group demonstrated that the allergenicity of hulls prior to their storage is minimal.

Heating experiments with stored hulls indicated an increase in allergenicity and potency of the heated extracts,

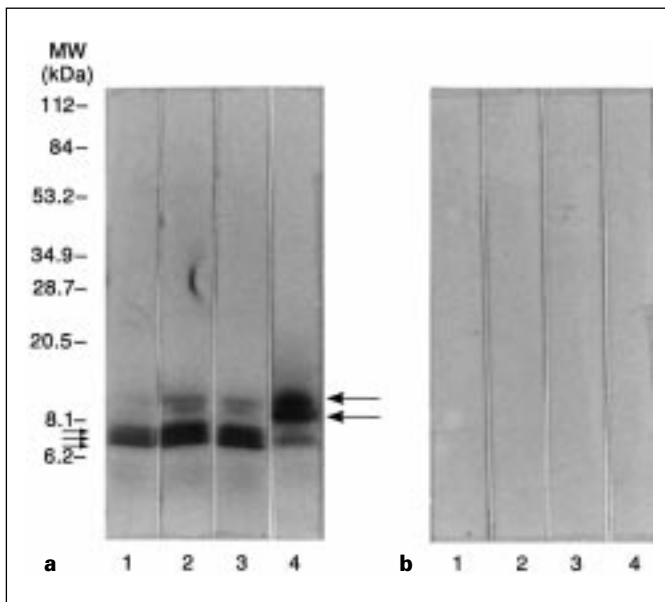


Fig. 4. SDS-PAGE/Western blot of the allergen extracts prepared from soybean hull heated at different temperatures (E1–E4), corresponding to the detection of specific IgE. **a** Incubated with the SA pool of sera. **b** Incubated with the non-SA pool of sera. Lanes 1, 2, 3 and 4 represent E4, E1, E2 and E3, respectively. The thin arrows on the left of the figure point to the allergens Gly m 2 (8 kD), Gly m 1A (7.5 kD) and Gly m 1B (7 kD). The thick arrows on the right of the figure point to the new IgE binding proteins (15.3 and 10 kD).

and changes in the allergenic composition were detected by SDS-PAGE/Western blot. The finding of new protein bands in extracts of stored hulls heated at increasing temperatures indicates that new allergen determinants might be created or that there is an increased exposure of epitopes upon heating. Self-heating of soybean is caused by the growth of storage fungi. Any growth of storage fungi is accompanied by respiration and heating, reaching temperatures of 75°C or higher, causing spontaneous combustion [8, 14], a potential hazard in silos where soybeans are stored. Storage fungi, especially *Aspergillus* and *Penicillium*, are the most xeno-phytic molds which can contaminate soybeans whose moisture content is as low as 14% and at temperatures of 8°C [8, 22]. In a previous work, two *Aspergillus* and four *Penicillium* species were isolated from hulls stored in the Barcelona silos [13], which were the same stored hulls as the ones employed in the present study. A possible role of molds in soybean-induced asthma was suggested. Molds are allergenic, but in addition they can generate heat and synthesize hydrolytic enzymes, which can also cause protein degradation.

Because heat denaturation changes protein configuration, heat treatment can modify allergenicity. However,

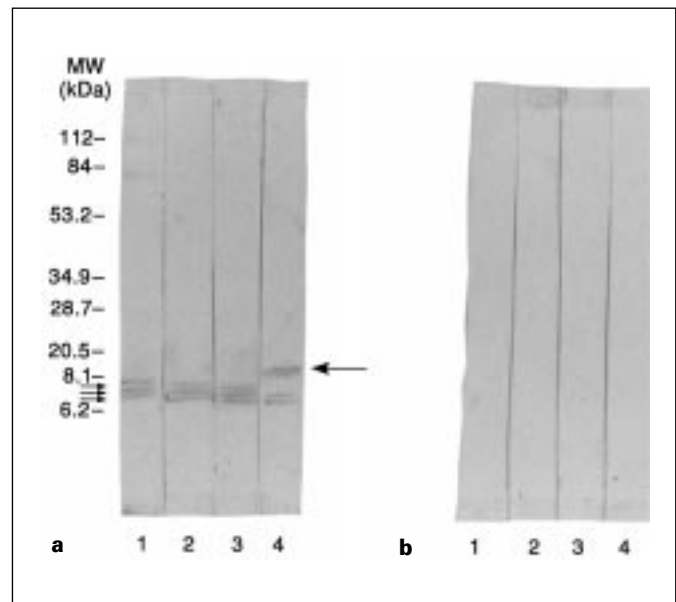


Fig. 5. SDS-PAGE/Western blot of the allergen extracts prepared from soybean hull heated at different temperatures (E1–E4), corresponding to the detection of specific IgG4. Panels and lanes are the same as in figure 3. The thick arrow on the right of the figure points to the new IgG4 binding protein (15.3 kD).

whether the allergenicity can be reduced or enhanced is not clear. When proteins are heated there is an increase in hydration, disulfide bond breakage and loss of tertiary and secondary structure [23].

Malanin et al. [17] presented the case of one patient who had no specific IgE to fresh pecans but had high titers to heated pecans or to pecans stored at 30°C for 1 week. They determined by SDS-PAGE/Western blot that the neoallergenic determinants were located on proteins with a MW of 15 kD. An increase in the allergenicity of soybean flour proteins when they are modified by interaction with oxidized lipids has also been demonstrated [24, 25]. Hsieh et al. [16] demonstrated an increased amount of the 18 kD allergen in apples during prolonged storage. They suggested that possible triggering factors for this phenomenon could be microbial infection, temperature, CO₂ or buildup. Rosen et al. [18] described a subject with negative skin prick tests to raw shrimp but positive tests to cooked shrimp. It appears that cooking shrimp produced new allergens not present in raw shrimp. Interestingly, Küstner of the classical Prausnitz-Küstner test was allergic to cooked but not raw fish [26].

The results of this study demonstrate that soybean hull allergenicity is affected by heat. Heat treatment increases

the potency of extracts made from stored hulls and induces formation of IgE and IgG4 binding proteins, which have about the same MW as many major allergens. Whether or not these new IgE and IgG4 binding proteins represent new allergen determinants or exposure of epitopes as a result of conformational changes is not known. The significance of these new proteins has yet to be determined and amino acid analysis should be performed.

Soybean dust discharged into the air in the harbor during soybean unloading activities is an uncommon very potent source of allergens and causes outbreaks of asthma. On the other hand, asthma from soybean dust has been reported only in isolated cases in soybean farmers or workers. The increase in allergenicity of soybean dust during transport has been attributed to mechanical reasons, such as abrading or milling in the hold of the ships during transport [11]. Based

on our findings, we suggest that soybean hulls have reduced allergenic properties by themselves and might be increased during transport and storage due to thermal modification caused by the growth of fungi. In addition, molds can release hydrolytic enzymes, which might contribute to the modification of soybean proteins, rendering them more allergenic. This mechanism could also explain the allergenicity of stored seeds other than soybeans. A possible measure to reduce allergenicity of soybeans could be to transport and store soybeans under controlled temperature and humidity conditions, below the limit that permits the growth of storage fungi.

In conclusion, this study demonstrates an increase in allergenicity when soybean hulls are thermally treated. The study of the effect of storage on the allergenicity of soybean hulls is an ongoing project at our institution.

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