

Purification and characterization of major inhalant allergens from soybean hulls

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Summary

Proteins responsible for respiratory allergy to soybean have been purified from an extract of soybean hulls. The purification procedure combined size exclusion and reverse-phase HPLC. Two pure glycoproteins (S1 and S2) exhibiting IgE-binding ability, as demonstrated by immunoblotting and ELISA techniques, were obtained. Both proteins displayed low molecular weight values on SDS-PAGE (S1, 7.0 kD; S2 7.5 kD). Protein S1 showed charge microheterogeneity, rendering two bands at pH 6.1–6.2 on IEF, whereas S2 showed a single band at pH 6.8. Amino acid composition analyses revealed a strong homology between S1 and S2 and, as a characteristic feature, a high percentage of hydrophobic residues, mainly leucine and isoleucine. Concerning the allergenic activity, both proteins were recognized by the specific IgE from 95% of patients who suffered asthma attacks during the asthma outbreaks of 1987 and 1988 in Cartagena (Spain), caused by soybean dust. Besides, proteins S1 and S2 were able to, separately, inhibit up to 75% the binding of specific IgE to the whole extract. Moreover, purified proteins totally crossreacted, even though protein S2 seemed to be slightly more active in all the immunochemical techniques employed. Results presented allow us to conclude that both proteins are isoallergens and to name them as *Gly m* IA (protein S2) and *Gly m* IB (protein S1), according to the IUIS-allergen nomenclature system.

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Introduction

In addition to the well-established fact of food allergy to soya (*Glycine max*) [1,2], the presence of inhalant allergens in different types of soybean dust and flour has been described as a cause of allergic manifestations, such as rhinitis [3] and occupational asthma [4,5].

During the past few years, several asthma outbreaks occurred in Barcelona and Cartagena (Spain), and an unequivocal relationship between these outbreaks and soybean unloading at the harbour, in days with concurrent favourable weather conditions for soybean dust spreading over the towns, could be established [6,7].

In a previous work from our laboratory [8], a low

molecular mass (about 8 kD) allergen, localized in soybean hulls, was identified as the main protein responsible for the asthma epidemics of 1987 and 1988 in Cartagena. This allergen, to which 90% of patients who suffered asthma attacks on epidemic days had specific IgE, was also demonstrated to be different from the proteins identified as causing food allergy to soya.

Other authors have identified a low molecular mass glycopeptide (< 14 kD) as the main soybean-dust allergen involved in the Barcelona asthma epidemics [9], which probably coincides with that described by us. They also investigated the localization of the allergen in all parts of the plant, and they found that the hulls and pods were the richest sources [10].

In this paper, we describe the purification, and further biochemical and immunological characterization, of soybean hull components responsible for respiratory allergies.

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Materials and methods

Source material and preparation of the extract

Soybean samples were obtained from those materials unloaded at the seaport of Cartagena during the epidemic days in October 1987. These legumes had been imported from the U.S.A.

The extract was prepared from soybean hulls, obtained by peeling one by one all the beans used in this work, as previously described [8]. Briefly, the soybean hulls were ground with a coffee grinder and extracted with 0.9% NaCl at a 5% (w/v) ratio, by stirring for 1 hr at room temperature. The extract was separated from the solid material by centrifugation at 22000 *g* for 20 min, and dialysed against distilled water for 24 hr. Afterwards, the extract was centrifuged again as before, filtered through a 0.22 μ m pore size membrane, aliquoted and freeze-dried. The lyophilized extract was stored at 4°C until use. This soybean hull extract will be hereafter denominated SE.

Human sera

Serum samples used throughout this work were obtained from 20 individuals who were admitted to the emergency room during the asthma outbreaks that occurred in Cartagena (Spain) in October 1987 and April 1988, and who gave voluntary consent. Eight-five per cent of patients had positive skin prick test to SE and none of them complained about having food allergy to soybean. For some experiments, a serum pool, made by mixing equal volume aliquots from the individual sera, was used.

Control serum samples were obtained from asthmatic patients who had also called for medical attention in the same hospitals, but on days different from those of the asthma outbreaks. All controls were negative when prick-tested with soybean extract. Furthermore, there were no significant differences between patient and control groups when prick-tested with the 18 most common allergens of the geographic area where they lived.

All the serum samples were stored at -80°C.

Electrophoretical procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed with a 8–25% acrylamide gradient in a high-molarity Tris buffer system [11]. The following polypeptides were used as molecular weight markers: phosphorylase b (92.5 kD), ovalbumin (45 kD), carbonic anhydrase (31.5 kD), soybean trypsin inhibitor (21.5 kD), myoglobin (16.9 kD), and myoglobin fragment I (8.2 kD).

Analytical isoelectric focusing (IEF) of proteins was performed on a FMC Isogel (Rockland, Maine, U.S.A.)

agarose plate, pH gradient from 3 to 10, according to the manufacturer's instructions. Isogel pI markers were run on the same gel.

Protein detection on the gels was done by Coomassie Blue staining.

Immunoblotting

Electrotransfer of polypeptides separated by SDS-PAGE onto nitrocellulose membrane was carried out according to Towbin *et al.* [12]. Transfer of proteins from IEF agarose plates to nitrocellulose membrane was performed by press blot for 5 min. Further immunodetection of IgE-binding proteins was accomplished by sequential incubation of the membranes with patients serum pool (diluted 1:3 with PBS) and [¹²⁵I]-HE2 MoAb, a mouse anti-human IgE monoclonal antibody previously produced in our laboratory [13]. Finally, nitrocellulose membranes were washed, dried, and autoradiographed with intensifying screens [14] for 48 hr at -70°C.

HPLC equipment

All the chromatographic procedures were carried out on HPLC equipment from Waters (Milford, Massachusetts, U.S.A.) comprising a Model 680 gradient programmer, two 510 pumps, a U6K injector, and a 490 multiwavelength u.v./visible detector.

Amino acid analysis

Samples were hydrolysed at 110°C for 24 hr with constant boiling HCl in the presence of phenol and 2-mercaptoethanol (0.05%). The hydrolysates were analysed by derivatization with dabsyl chloride [15] and separation of dabsyl-derivatives of amino acids with a C18 Ultrasphere column (Beckman, San Ramon, California, U.S.A.). Analyses were performed in triplicate, no corrections were made for destruction during hydrolysis or incomplete hydrolysis. Tryptophan was not determined.

Purification of soybean allergens

The lyophilized extract from soybean hulls was dissolved in 100 mM Na₂SO₄-20 mM NaH₂PO₄ buffer, pH 6.8, and chromatographed in a size exclusion HPLC column (Beckman Spherosgel TSKG 3000 SW, 30 × 2.1 cm, 13 μ m particle size, 25 nm pore size), using the same solvent as the mobile phase at a constant flow rate of 1 ml/min.

Collected fractions were analysed by SDS-PAGE and immunoblotting, and the allergenic activity measured by a competition ELISA, as described below.

Then the fraction with the highest IgE binding capacity

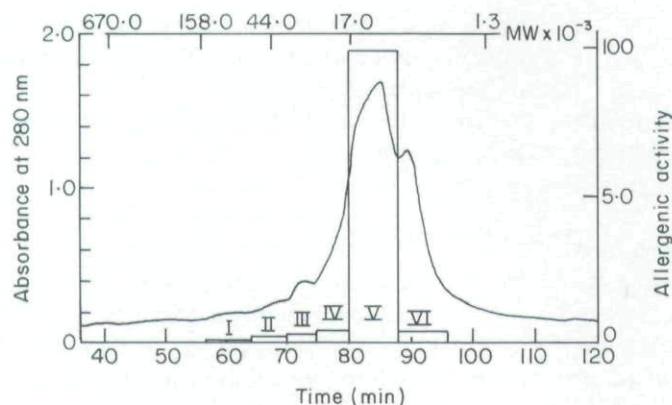


Fig. 1. Elution profile of SE on size exclusion HPLC monitored at 280 nm. The lyophilized extract (10 mg) was dissolved in 0.1 M Na_2SO_4 , 0.02 M NaH_2PO_4 , pH 6.8, and applied to a Spherogel TSK G 3000 SW column equilibrated and eluted with the same solvent. The retention times of the standard proteins are indicated at the top of the figure. The bar graph depicts the allergenic activity of each fraction evaluated by a competition ELISA as described in Materials and Methods.

was applied onto a short alkyl chain-bonded silica column (Vydac C4, 25×1.0 cm, $10 \mu\text{m}$ particle size, 30 nm pore size) and eluted with a 10–50% gradient of acetonitrile in 0.1% trifluoroacetic acid at a constant flow rate of 1 ml/min. Collected fractions were analysed using the same techniques as above. Fractions containing pure IgE-binding proteins (soybean allergens S1 and S2) were dialysed against distilled water, using dialysis tubings with molecular weight cut off 3500, and lyophilized.

Protein determination

Protein concentration of the extract and fractions from size exclusion HPLC was estimated by the method of Lowry *et al.* [16] using bovine serum albumin as a standard. Concentration of purified S1 and S2 allergens samples was measured by the difference between spectrophotometric absorptions at 215 and 225 nm [17]. This technique showed a good correlation with amino acid analysis.

Glycoprotein detection

Glycoproteins were detected by an enzyme immunoassay, based on periodate oxidation of sugars followed by derivatization of the aldehyde groups formed with digoxigenin-hydrazide, and further immunodetection with anti-digoxigenin antibodies alkaline phosphatase conjugated, using a DIG Glycan Detection kit supplied by Boehringer Mannheim GmbH Biochemica (Mannheim, Germany). Samples were blotted onto a nitrocellulose membrane (dot-blot) which was subsequently processed following the manufacturer's instructions. Transferrin, as a positive control, and recombinant *Escherichia coli* creatinase, as a negative control, were included in this experiment.

Immunoassays

Serum-specific IgE for SE and for purified S1 and S2 proteins was evaluated by ELISA experiments as follows.

Either SE (at a $100 \mu\text{g/ml}$ protein concentration in distilled water) or purified proteins (at $5 \mu\text{g/ml}$) were adsorbed to plastic microtitre wells (Immulon II, Removawells, Dynatech, Alexandria, Virginia, U.S.A.) by incubating $100 \mu\text{l}$ per well of these solutions at 37°C for 1 hr. Unbound protein was then washed with PBS containing 0.1% Tween 20. Unoccupied well sites were blocked with $100 \mu\text{l}$ of PBS containing 1% bovine serum albumin for 1 hr at room temperature.

The adsorbed antigens were made to react with $50 \mu\text{l}$ of individual human sera at 4°C overnight and, after washing, with $50 \mu\text{l}$ of a stock solution of anti-human IgE HE-2 MoAb, conjugated with peroxidase, for 1 hr at room temperature. The plates were washed five times, as previously, and $50 \mu\text{l}$ of a solution of 1,2-phenylenediamine dihydrochloride (OPD) were added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped with $50 \mu\text{l}$ of 8 N H_2SO_4 , and the absorbance measured at 492 and 630 nm in an automatic microplate reader.

Competition ELISA experiments were performed essentially as above, except that the allergosorbent was made to react with a mixture of $50 \mu\text{l}$ of patients serum pool (diluted 1:3 with PBS) and $50 \mu\text{l}$ of serial dilutions of the samples to be tested.

To evaluate the activity of samples, an arbitrary value of 1 was given to the amount of the protein used as inhibitor (in μg) needed to inhibit up to 50% the binding of specific IgE to SE.

Results

Purification of inhalant soybean allergens

Purification of soybean allergens from SE was achieved in two chromatographic steps. The first purification process consisted in size exclusion HPLC. A sample of SE was chromatographed on a Spherogel column, rendering the elution profile shown in Fig. 1. The presence of IgE-

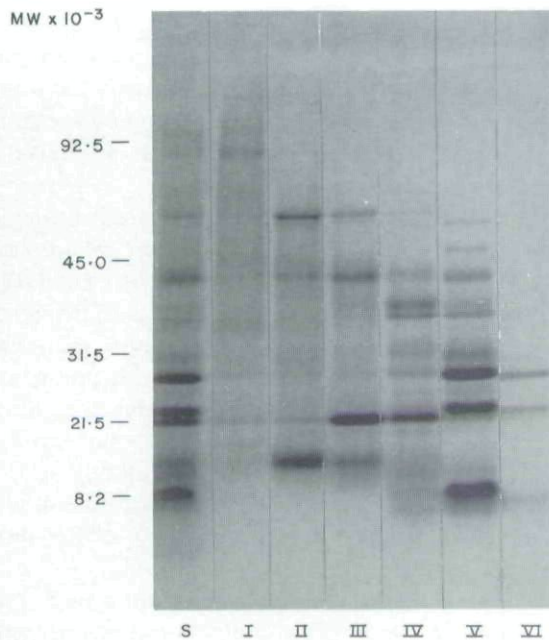


Fig. 2. SDS-PAGE under non-reducing conditions of fractions I–VI from size exclusion HPLC (Fig. 1). Lane S shows the electrophoretic pattern of SE.

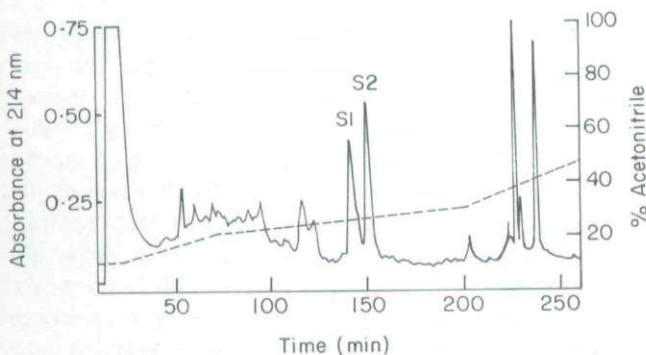


Fig. 3. Reverse-phase HPLC of fraction V from size exclusion chromatography (Fig. 1). Lyophilized fraction V (8 mg) was dissolved in 0.1 TFA and injected onto a Vydac C4 column. Elution was performed as described in Materials and Methods. The dashed line depicts the acetonitrile gradient shape.

binding activity was evaluated in all elution fractions, and those fractions which showed similar activity were pooled. Fraction V possessed the highest IgE-binding capacity (Fig. 1), and also it had the highest percentage of the protein eluted from the column. The estimated protein yield for pool V amounted to 36% of the total protein applied to the column.

Analysis of polypeptide composition of pools I–VI by SDS-PAGE (Fig. 2), revealed that pool V contained a group of proteins, with molecular mass about 8 kD, that

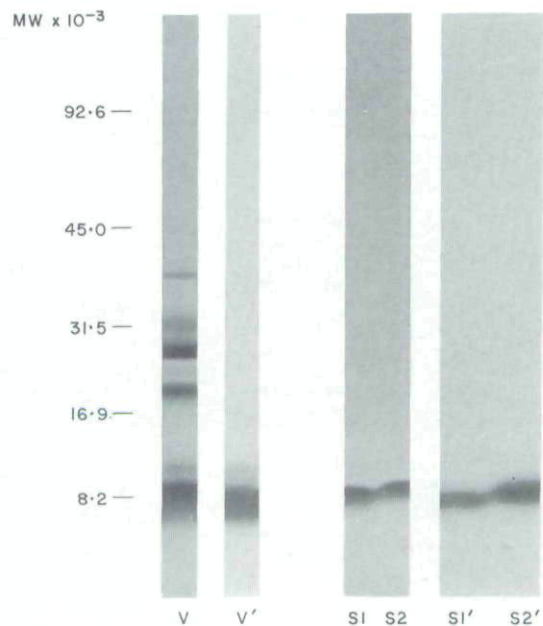


Fig. 4. SDS-PAGE patterns of fraction V, used as the starting material for reverse-phase chromatography, and proteins S1 and S2 eluted from the Vydac C4 column (Fig. 3). Lanes V', S1', and S2' show the corresponding immunodetection of the same samples with patients serum pool and ^{125}I -anti-human IgE, carried out as described in Materials and Methods.

had been identified as the main soybean hull components responsible for respiratory allergies in a previous work from our lab [8].

Consequently, pool V was selected as the starting material for the subsequent purification step. Figure 3 shows the chromatographic profile obtained when pool V was chromatographed on a reverse-phase Vydac C4 column. Eluted fractions were analysed as above, and IgE-binding activity was only detected in two peaks eluted in the central region of the chromatogram (S1 and S2 in Fig. 3), at an acetonitrile concentration of approximately 25%.

SDS-PAGE analysis showed that two proteins with low molecular weights eluted in these peaks. Fractions containing pure proteins were separately pooled, and their analysis by SDS-PAGE, together with a sample of pool V applied to the reverse-phase column, are shown in Fig. 4. This figure also includes the immunoblotting pattern with a patients serum pool, that confirms the preservation of IgE-binding capacity of proteins S1 and S2 upon the purification process. Besides, radiostained bands produced in autoradiograms S1' and S2' coincided with the strong broad band observed in lane V', that is, that produced by the main inhalant allergens from soybean hulls.

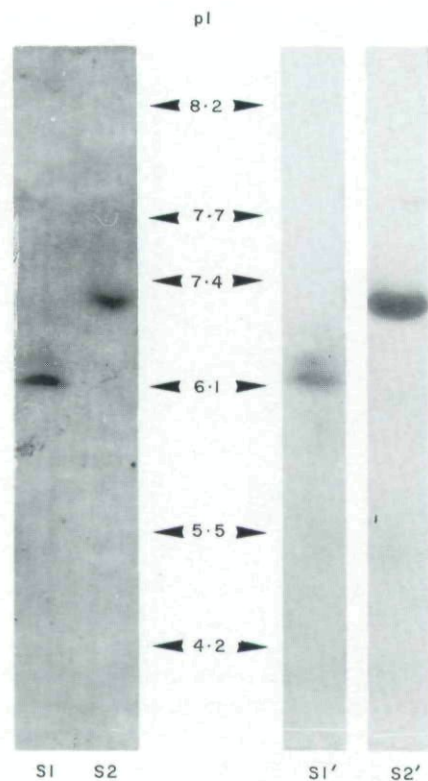


Fig. 5. Analytical IEF of reverse-phase HPLC purified S1 and S2 soybean hull allergens. On the left, Coomassie Blue staining of proteins (lanes S1, S2); on the right, immunodetection with patients serum pool and [^{125} I]-anti-human IgE (lanes S1', S2').

Protein yield for allergens S1 and S2 in reverse-phase chromatography was approximately 10%, giving an overall yield of 3.5% for each protein in the complete purification procedure, since amounts of about 350 μ g of both proteins S1 and S2 were obtained from 10 mg of SE.

Biochemical characterization of soybean allergens S1 and S2

Apparent molecular weight values for proteins S1 and S2 were estimated from SDS-PAGE under non-reducing conditions (Fig. 4). Protein S1 showed a molecular weight (7.0 kD) slightly lower than that of protein S2 (7.5 kD).

The presence of carbohydrate prosthetic groups, linked to the polypeptide backbones of both proteins, was evidenced by the positive staining obtained when allergens S1 and S2 were submitted to a glycoprotein detection procedure as described in Materials and Methods.

Analytical isoelectric focusing patterns of S1 and S2, and their corresponding immunoblot patterns with a patients serum pool, are presented in Fig. 5. Protein S2 displayed a neat band with pI 6.8; however, protein S1, although homogeneous when analysed by the preceding

techniques, showed a certain degree of charge heterogeneity, displaying a major band with pI 6.1 and a minor one with pI 6.2. Apart from that, it is worth mentioning that protein S1 rendered, upon immunodetection, a radiostained pattern weaker than that produced by protein S2 (Fig. 5, lanes S1' and S2').

Amino acid analysis of soybean hull allergens demonstrated a high homology (almost identity) between them (Table 1). In fact, allergen S2 only differed from allergen S1 in having one alanine, one isoleucine, and two leucine residues more. For both proteins, the most remarkable results are the high content of hydrophobic amino acids (leucine and isoleucine account for about one third of total residues), and the almost absence of aromatic residues (only 1% tyrosine). On the other hand, the occurrence of polymorphism at certain positions might be an explanation for the minute amount of phenylalanine found.

Molecular weight estimations from amino acid composition (omitting the possible contribution of tryptophan content) were 7185 D for the polypeptide backbone of allergen S1, and 7596 D for polypeptide S2.

Allergenic significance of proteins S1 and S2

In order to show the allergenic significance of proteins S1 and S2, their ability to bind specific human IgE was studied with serum samples from 20 patients who suffered asthma attacks during the epidemic days, by ELISA technique. The results, listed in Table 2, indicated that 19 out of the 20 sera tested (95%) had specific IgE to the purified allergens, and the only case in which no IgE-binding to proteins S1 and S2 was detected (serum number 11) also displayed a negative result with the whole extract. On the other hand, the absorbance values were higher for S2 than for S1 for 12 out of the 19 reactive sera.

In addition, the allergen activity of both proteins was evaluated by means of a competition ELISA technique. Results, shown in Table 3, demonstrated that either protein S1 or S2 could inhibit to a high extent the binding of specific IgE to SE (S1: 76%; S2: 72%), then suggesting that these proteins bear major allergenic determinants of soybean extract.

Furthermore, the IgE-binding capacity of protein S2 was found to be higher than that of protein S1, since lower concentrations of protein S2 were needed to inhibit up to 50% the binding of specific IgE to the allergosorbent phases.

Discussion

In this work, soybean proteins responsible for respiratory allergies have been purified and characterized. Isolation

Table 1. Amino acid composition of soybean allergens S1 and S2.

Amino acid	Allergen S1		Allergen S2	
	Per cent composition	Residues per molecule (<i>n</i>)	Per cent composition	Residues per molecule (<i>n</i>)
Asx	12.1	8	11.4	8
Glx	4.6	3	3.6	3
Ser	7.4	5	7.0	5
Thr	5.3	4	5.1	4
Arg	7.2	5	6.9	5
Gly	13.1	9	12.7	9
Ala	6.2	4	7.1	5
Pro	6.9	5	6.7	5
Val	2.8	2	2.7	2
Met	—	—	—	—
Ile	10.4	7	11.5	8
Leu	18.8	13	20.4	15
Phe	0.4	—	0.2	—
Cys	2.4	2	2.8	2
Lys	1.2	1	0.9	1
His	—	—	—	—
Tyr	1.2	1	1.0	1
Total	100.0	69	100.0	73

Asx represents the sum of aspartic acid and asparagine percentages, and Glx that of glutamic acid and glutamine. Figures in the first column for each protein represent the number of residues per 100 residues. The number of residues per molecule, estimated by adjusting percentage results to the closest integer, are given in the second columns.

of these proteins was approached by taking advantage of data on their physico-chemical characteristics previously reported by us [8]. Thus, these proteins had been defined as low molecular weight polypeptides and, consequently, size exclusion chromatography was attempted as the first fractionation step, so as to separate the allergen molecules from higher molecular weight structures present in SE (Fig. 1). This process rendered a fraction containing most IgE-binding capacity of SE and, as expected, it consisted of low molecular weight proteins (Fig. 2, lane V), that was subsequently chromatographed on reverse-phase HPLC column. In this second chromatographic step, two proteins exhibiting IgE-binding ability, were separated in two close well-resolved peaks (S1 and S2, Fig. 3). Both proteins displayed very similar molecular weight values when analysed on SDS-PAGE (Fig. 4). Because of this, these allergens had previously been thought to be only one allergen [8], since the SDS-PAGE system employed failed to resolve them when they were applied together to the gel as components of a complex mixture (e.g. lane S in Fig. 1, lane V in Fig. 4), producing a single broad

radiostained band in immunoblotting patterns (lane V', Fig. 4).

On the other hand, isoelectric points of proteins S1 and S2 are rather dissimilar: 6.1 and 6.8, respectively. This difference in electric charge does not seem to be originated from divergences in amino acid compositions, since they differed only in four neutral amino acid residues, except that the acid/amide ratios in Glx and Asx were very different for S1 and S2. Then, the results obtained strongly suggest that post-translational modifications of the proteins might be responsible for the difference in isoelectric points. In fact, allergens S1 and S2 have been demonstrated to be glycoproteins and, thus, different carbohydrate moieties linked to the polypeptide chains might account for the different pIs observed and, also, it would explain the charge microheterogeneity of protein S1.

Concerning the high similarity between amino acid compositions, it seems to suggest that both allergens could be essentially the same protein but differently post-translational processed. Obviously, the complete

Table 2. Binding of specific human IgE to SE and purified proteins S1 and S2

Patients' sera	SE	S1	S2
1	0.625	0.543	0.930
2	0.785	1.401	1.067
3	2.258	1.669	1.994
4	1.023	1.828	1.739
5	0.431	0.979	0.824
6	0.725	1.187	1.298
7	2.050	1.518	2.036
8	0.472	0.152	0.438
9	2.136	2.051	1.974
10	0.568	0.648	0.755
11	0.022	0.036	0.033
12	1.352	1.593	1.500
13	0.191	0.190	0.056
14	1.945	1.821	1.826
15	0.203	0.142	0.196
16	2.188	2.027	2.027
17	0.581	0.226	0.278
18	0.937	1.072	1.390
19	0.343	0.450	0.604
20	1.366	1.177	1.419
NC	0.025	0.028	0.024

Figures represent values of absorbance obtained in an ELISA screening. Allergosorbent wells were prepared with either SE or pure proteins S1 and S2, and sequentially incubated with individual sera, from patients who suffered asthma attacks on epidemic days, and peroxidase-conjugated anti-human IgE HE2 MoAb, as described in Materials and Methods. A negative serum pool (NC) was used to determine the non-specific binding.

sequences of both allergens will be needed so as to clarify this point.

Soybean allergens S1 and S2 behaved experimentally as hydrophilic proteins; thus, they were easily extracted from soybean hulls in aqueous saline solutions, and they eluted from the reverse-phase HPLC column at a rather low concentration of acetonitrile. Besides, they must be easily released from soybean hull dust, and permeate the mucosal membranes of the respiratory tract, to produce asthmatic reactions in patients. But, in contrast with this behaviour, a relevant feature of the amino acid compositions of proteins S1 and S2 is the high percentage of hydrophobic amino acids, mainly leucine and isoleucine which contribute about 30% of total residues. These facts might be suggesting that the proteins should be formed by a hydrophobic core, with most leucine and isoleucine residues internalized in the protein, and polar amino acid

Table 3. Cross-inhibition of specific IgE-binding among SE, allergen S1 and allergen S2

Allergosorbent	Inhibitor		
	SE	S1	S2
SE	12.3 / 98	1.8 / 76	1.4 / 72
S1	29 / 85	1.2 / 98	0.5 / 92
S2	37 / 87	1.9 / 89	0.8 / 93

The cross-inhibition of the binding of specific IgE, from a soybean-sensitive patients serum pool, was evaluated by a competition ELISA, as described in Materials and Methods. The upper value in each entry means the amount of protein (in micrograms per well) needed to reach 50% inhibition. The lower values represent the maximum inhibition reached (%).

residues and carbohydrates exposed, then increasing the hydrophilicity of proteins surfaces.

As far as the allergenic activity of proteins S1 and S2 is concerned, data presented in this work clearly evidence that both proteins are the most clinically relevant inhalant allergens from soybean hulls. At least 95% of patients who suffered asthma attacks during epidemic days, due to the unloading of soybean at the harbour, in Cartagena (Spain), had specific IgE to the purified proteins, and even, in some cases, the *in vitro* response was higher for S1 and S2 than for the whole extract (Table 2).

Besides, allergens S1 and S2, independently, accounted for a high percentage (about 75%) of total allergenic activity (Table 3), demonstrating that they are the main allergens from soybean hull, and consequently, that other allergens present in the extract must have a little allergenic importance when compared to that of S1 and S2, as it had been previously anticipated from immunoblotting results [8].

On the other hand, S1 and S2 cross-reacted in IgE-binding inhibition experiments (Table 3), completely inhibiting the binding of specific IgE to each other. Then, it seems clear that both proteins bear the same allergenic epitopes, and this fact supports the idea of the extremely high homology between them.

Nevertheless, a slight difference between S1 and S2 was observed for the amount of protein needed to inhibit up to 50% the binding of specific IgE to each other or to SE (Table 3). In each case, about twofold S1 to S2 amounts were necessary to reach similar inhibition levels. In addition, immunoblotting experiments after SDS-PAGE

(Fig. 4) and, especially, IEF (Fig. 5) also revealed a stronger radiostaining for protein S2. All these observations reflect different affinities of the allergenic epitopes on each molecule for IgE, that might arise from protein conformation differences caused by either carbohydrate prosthetic groups or the little divergence of amino acid composition discussed above.

The physico-chemical features of S1 and S2 allergens seem to indicate a possible identity with some of the soybean-dust aeroallergens that caused asthma outbreaks in Barcelona, Spain, recently identified and partially characterized by other authors [9]. They described a group of glycopeptides with molecular weight < 14.4 kD as the main IgE-binding components in soybean hull and dust extracts. In addition, they found four predominant bands on immunoblot after IEF, with pIs of 6.55, 5.85, 5.20 and 4.55. The two former might coincide with S2 and S1, respectively, taking into consideration that the different electrophoretic conditions employed by us could account for the divergence in the pIs values observed.

In conclusion, two proteins from soybean hulls, responsible for respiratory allergies, have been purified using an efficient chromatographic procedure, that rendered a good recovery of active pure proteins. Physico-chemical and immunochemical properties of the purified allergens have been established and, according to the IUIS-allergen nomenclature system [18] and taking into account the close immunological relationship between them, they must be hereafter denominated as isoallergens *Gly m* IA (protein S2, molecular mass 7.5 kD, pI 6.8) and *Gly m* IB (protein S1, molecular mass 7.0 kD, pI 6.1–6.2).

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