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Two-Dimensional Electrophoresis and Western-Blotting Analyses with anti Ara h 3 Basic Subunit IgG Evidence the Cross-Reacting Polypeptides of *Arachis hypogaea*, *Glycine max,* and *Lupinus albus* Seed Proteomes

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The allergenicity of seed storage proteins, the major components of edible legume seeds, may cause serious reactions in both children and adult population. Updated methodologies for evaluation of the activity of these proteins are needed. In this paper we used two-dimensional (2D) electrophoretic techniques to investigate the immuno-cross-reactivities of anti Ara h 3 basic subunit IgG to the seed proteomes of three legume species, namely, peanut, soybean, and lupin. The seed proteins, extracted with two different procedures, were separated by 2D electrophoresis, and the electrophoretic maps were analyzed by Western blot. In peanut proteome the antibodies strongly reacted with the 23 kDa polypeptides, corresponding to Ara h 3 basic isoforms, the antigen they were raised to, and three unidentified acidic polypeptides near 45 kDa. Remarkable cross-reactivities with lupin and soybean Ara h 3 homologous polypeptides and nonrelated proteins, namely, lupin conglutin γ and soybean Bg7S, were detected. Therefore, these proteins may be regarded as new putative allergens. The present findings show the potentiality of 2D electrophoresis in the identification of food allergens and open the way to the traceability of the new cross-reacting proteins in the food chain.

KEYWORDS: Arachis hypogeae; Glycine max; Lupinus albus; legume seeds; peanut allergens; IgG; IEF/SDS−PAGE; cross-reactivity

INTRODUCTION

The occurrence of allergies has increased during recent decades in many Western countries (*1*). Among the food-related allergies those caused by plant-derived foods, especially nuts and seeds, are the most important in adults (*2*). Due to the widespread occurrence of legume-based foods, it has become more and more necessary to face the allergenicity problem of legume seed protein components. In this respect the need for predictive tests, which may represent noninvasive preliminary steps in the analysis of food allergens, is acute. In this area the lack of integrated approaches and standardized techniques has often prevented reliable identification of allergens and comparison of results from different laboratories.

Two-dimensional electrophoretic techniques are becoming widely used for the "all-in-one" analysis of proteomes. The applications of this technique may vary dramatically according to the aim of the experimental approaches. An example of the use of 2D electrophoretic mapping for identification of plant food allergens is the work by K. Beyer et al. (*3*), who described the identification of a hazelnut-dominant allergen by this technique.

In the case of seed storage proteins, the major protein components of the edible seeds, analysis is greatly complicated by the polypeptide heterogeneity even in the same protein family and the variable extent of co- and posttranslational covalent modifications, including glycosylation. Also, the nomenclature of the most widely known storage proteins from legume seeds is complex and sometimes confusing due to the different classification criteria adopted. Legume seeds generally contain two types of major storage proteins that differ by size. The first group includes proteins with sedimentation coefficients around 11S. They are referred to as "legumin-like" or 11S globulins (*4*). Trivial names based on the botanical name of the plants from which they arise are also used, e.g., glycinin for soybean and arachin for peanut. The corresponding mature proteins consist of hexamers of two disulfide-linked subunits indicated as acidic subunits, with greater M_r, and basic subunits, with lower M_r. The second group of proteins has sedimentation coefficients around 7S. They are classified as "vicilin-like" or

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7S globulins, with trivial names such as conglycinin from soybean and conarachin from peanut. In addition to these proteins other minor components, but often more relevant from the viewpoint of the allergic responses, are present in the seeds, including a 2S class of smaller proteins also referred to as "sulfur-rich" proteins because of their remarkable content of sulfur-containing amino acids. In this class of protein Ara h 2, an immunodominant allergen of peanut, can be found (*5*).

In the present paper we report the global 2D electrophoretic analysis of the IgG cross-reactivities to a peanut allergen in three legume species: two widely spread ones, namely, peanut itself and soybean, and one representing a promising alternative food protein source, namely, lupin.

As far as peanut allergy is concerned, it affects 0.6−1% of the adult population and often results in fatal or severe immediate reactions (*6*, *7*). Several proteins have been identified as peanut allergens. They are named Ara h 1 to Ara h 8, with Ara h 1 and Ara h 3 corresponding to the 7S and 11S classes, respectively. Among these allergens Ara h 1 and Ara h 2 are usually considered the major allergens since they are recognized by 70−90% of sensitized subjects (*8*, *9*). Ara h 3 is considered a minor allergen, but it was recognized by most of allergic children who arrived at the Macedonio Melloni Hospital Emergency Department (Milano, Italy) with immediate allergic reactions after intake of whole peanut or peanut derivatives (*10*). This is the reason we focused this study on the immunological cross-reactivity of a specific Ara h 3 subunit.

As far as soybean is concerned, about 15 seed proteins have been shown to be allergenic molecules (*11*). Instead, very few reports on lupin allergens are available so far (*12*, *13*).

The present paper provides new experimental evidence of previously not recognized proteins as potential allergens from the three considered grain legumes.

MATERIALS AND METHODS

Materials. Raw peanut (*Arachis hypogaea* L. var. Israel) seeds were kindly supplied by Vincenzo Brandolini, University of Ferrara, Italy.

Nonroasted type I defatted soybean (*Glycine max* L.) flour was obtained from Sigma (Milan, Italy).

Dry mature seeds of white lupin (*Lupinus albus* L. var. Multitalia) were kindly provided by Massimo Fagnano, University of Naples, Italy.

Preparation of Protein Samples for 2D Electrophoretic Analyses. Raw seeds were manually dehusked, and the kernels were ground to a meal. The resulting flour was suspended in cold acetone to solubilize the lipid fraction and centrifuged at 10 000*g* for 30 min. This treatment was repeated three times.

For alkaline protein isolation the defatted flours were extracted with 25 mM Tris-HCl buffer pH 8.2 in a ratio of 1/30 (w/v) under stirring for 2 h at 4 °C. The pH was monitored regularly and, if needed, readjusted to the original value. The slurry was centrifuged at 10 000*g* for 30 min, and the pellet was extracted again following the same procedure but with a ratio of 1/5 (w/v). The two supernatants were combined prior to analysis.

For acidic protein isolation the flours were resuspended in 25 mM Na acetate buffer pH 4.6 in a ratio of 1/5 (w/v), and the same procedure described above was applied.

The extracted proteins were immediately analyzed or kept frozen at −80 °C until use.

2D Electrophoresis. Isoelectric focusing was performed on 7-cm pH 3−10 IPG strips (Amersham Biosciences, Milan, Italy); nonlinear pH gradients were used for alkaline protein samples and linear pH gradients with the acidic protein samples. The strips were rehydrated overnight in a solution consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM 1,4-dithiothreitol (DTT), and 2% IPG buffer pH 3−10 (Amersham Biosciences, Milan, Italy) containing 70 μg of the protein sample. Strips were focused at 6500 Vh, with a maximum of 3000 V, at 20 °C using the Multiphor II electrophoresis unit (Amersham Biosciences, Milan, Italy). Prior to the second dimension strips were incubated in equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol) with 65 mM DTT for 15 min and then with 243 mM iodoacetamide in the same buffer without DTT for 10 min. The separation was performed in 12% SDS−PAGE gels using a mini-PROTEAN III cell (Bio-Rad, Milan, Italy). The gels were Coomassie blue stained.

Antibody Preparation. The band corresponding to Ara h 3 basic subunit, $M_r \approx 23$ kDa, was excised with a scalpel from 1D Coomasssie blue stained gels. Various preparations of the band were made and divided into two aliquots: one was submitted to N-terminal amino acid sequencing by a Perkin-Elmer Applied Biosystems 492 pulse liquid-phase sequencer; the other was used for antibody production. The unique sequence obtained corresponded to Ara h 3 basic subunit (*10*). The antiserum to Ara h 3 basic subunit was then raised as described elsewhere (*10*). An aliquot of the serum was withdrawn before immunization and used as a negative control.

As far as anti-conglutin γ antibodies are concerned, they were purified from a total lupin protein antiserum by immunoaffinity chromatography with a non-glycosylated conglutin γ immobilized on the Sepharose 4B CNBr matrix according to standard procedures as described by Casey (*14*).

Western Blotting Analysis. 2D gels were transferred to PVDF membranes (Bio-Rad, Milan, Italy) by blotting according to Towbin et al. (*15*) on a Trans-blot Electrophoretic Transfer Cell (Bio-Rad, Milan, Italy).

The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution in 50 mM Tris-HCl, 150 mM NaCl, 5mM EDTA, 0.05% Triton-X. Membranes were then soaked in the same buffer containing rabbit anti Ara h 3 basic subunit polyclonal antiserum in a ratio of 1000:1 (v/v) or anti-conglutin γ in a ratio of 250:1 (v/v).

Antigen−IgG complexes were detected using 10 μL of mouse anti-rabbit IgG monoclonal antibodies labeled with alkaline phosphatase (Sigma, Milan, Italy). The developing solution contained 15% bromo-chloroindolyl phosphate (BCIP) and 30% nitro blue tetrazolium (NBT) (Sigma, Milan, Italy) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Amino Acid Sequence Alignments. Amino acid sequences retrieved from SwissProt and TrEMBL databases were aligned with either Dialign 2.2.1 (*16*) or ClustalW (*17*), both available on the Web at www.expasy.org.

RESULTS

Innovative analytical methods and novel applications of available techniques are required to face the food allergenicity problems in an integrated manner. One of these approaches, which has been used in this work as a first screening of immuno-cross-reacting polypeptides among related legume seed species, is the 2D electrophoretic polypeptide mapping in conjunction with Western-blotting procedures. In this study specific antibodies to Ara h 3 basic subunit raised by direct immunization with a polypeptide band excised from 1D gel were used, as described under Materials and Methods.

With the aim of including the vast majority of seed proteins in the 2D electrophoretic profiles, two protein extraction protocols were set up: one consisted of an alkaline extraction at pH 8.2 which solubilizes the storage proteins and other minor seed protein components; the other one consisted of an acidic extraction at pH 4.6 which solubilizes many other protein components with the exclusion of the storage proteins, insoluble at such pH (*18*, *19*). The striking differences in the electrophoretic patterns of the Coomassie blue stained 2D maps of each seed protein sample in the two mentioned extracting conditions can be seen in **Figures 1** and **2**, left panels, respectively.

To help the identification of as many spots as possible in the 2D gels, the 1D electrophoretic patterns of the seed protein extracts analyzed are compared with those obtained by 2D

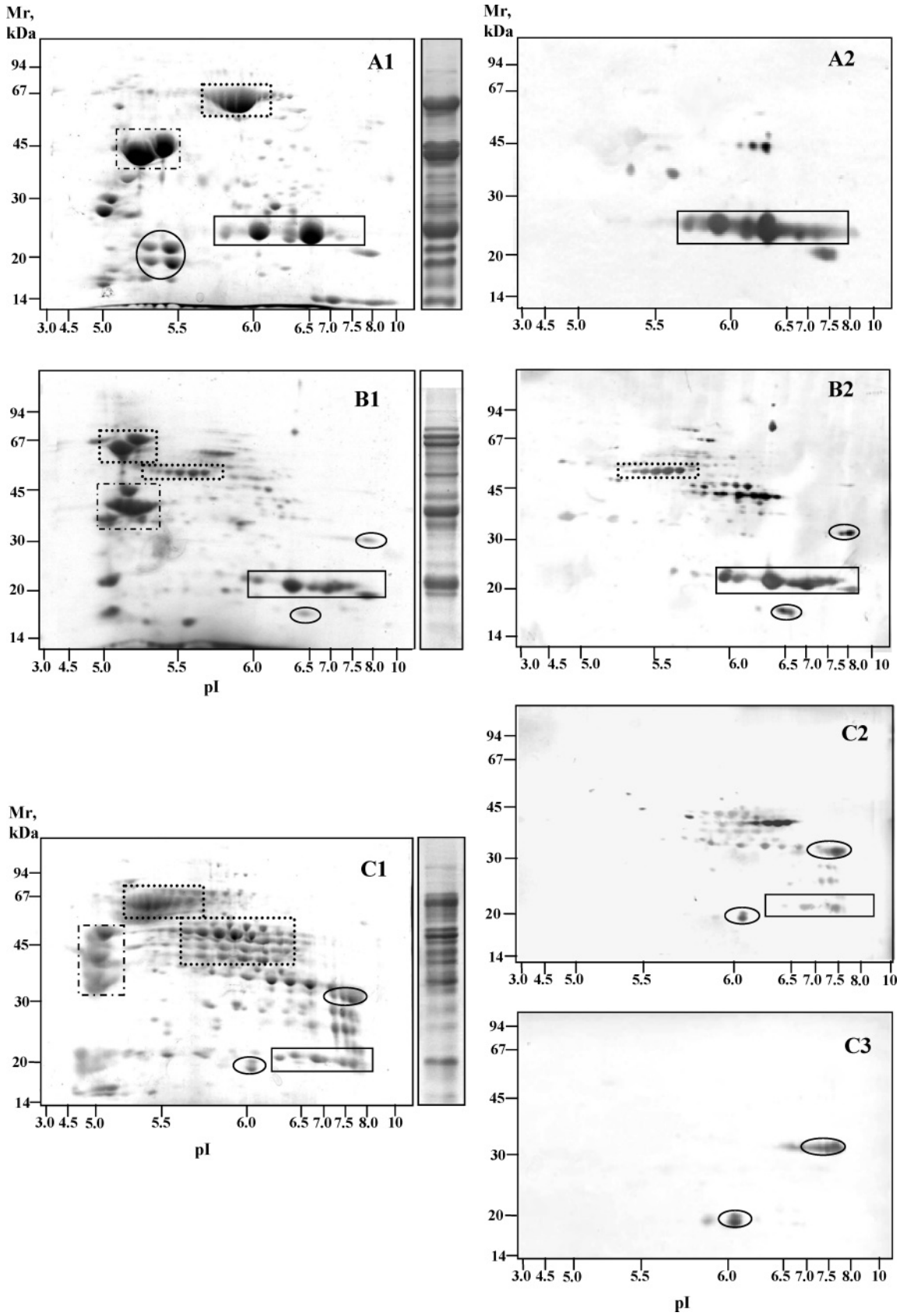

Figure 1. 2D electrophoretic maps of peanut (A), soybean (B), and lupin (C) alkaline protein extracts and Western-blotting analyses. Left panels (A1, B1, and C1) show the Coomassie blue stained 2D gels of the mentioned seed protein extracts as well as the 1D SDS–PAGE separation. Right panels are the corresponding Western-blotting analyses with anti Ara h 3 basic subunit (A2, B2, and C2) and anti-conglutin γ (C3) antibodies. Experimental details are given under Materials and Methods. Solid line rectangles enclose the basic subunits of the 11S globulins in the three species considered. Dash–dotted line rectangles enclose the acidic subunits of the 11S globulins in the three species considered. Dashed line rectangles enclose the subunits of the 7 S globulins in the three species considered. The solid circle in the peanut map encloses Ara h 2 polypeptides, according to ref (*36*). The solid ellipses in the soybean and lupin maps enclose the large and small subunits of conglutin γ-like proteins.

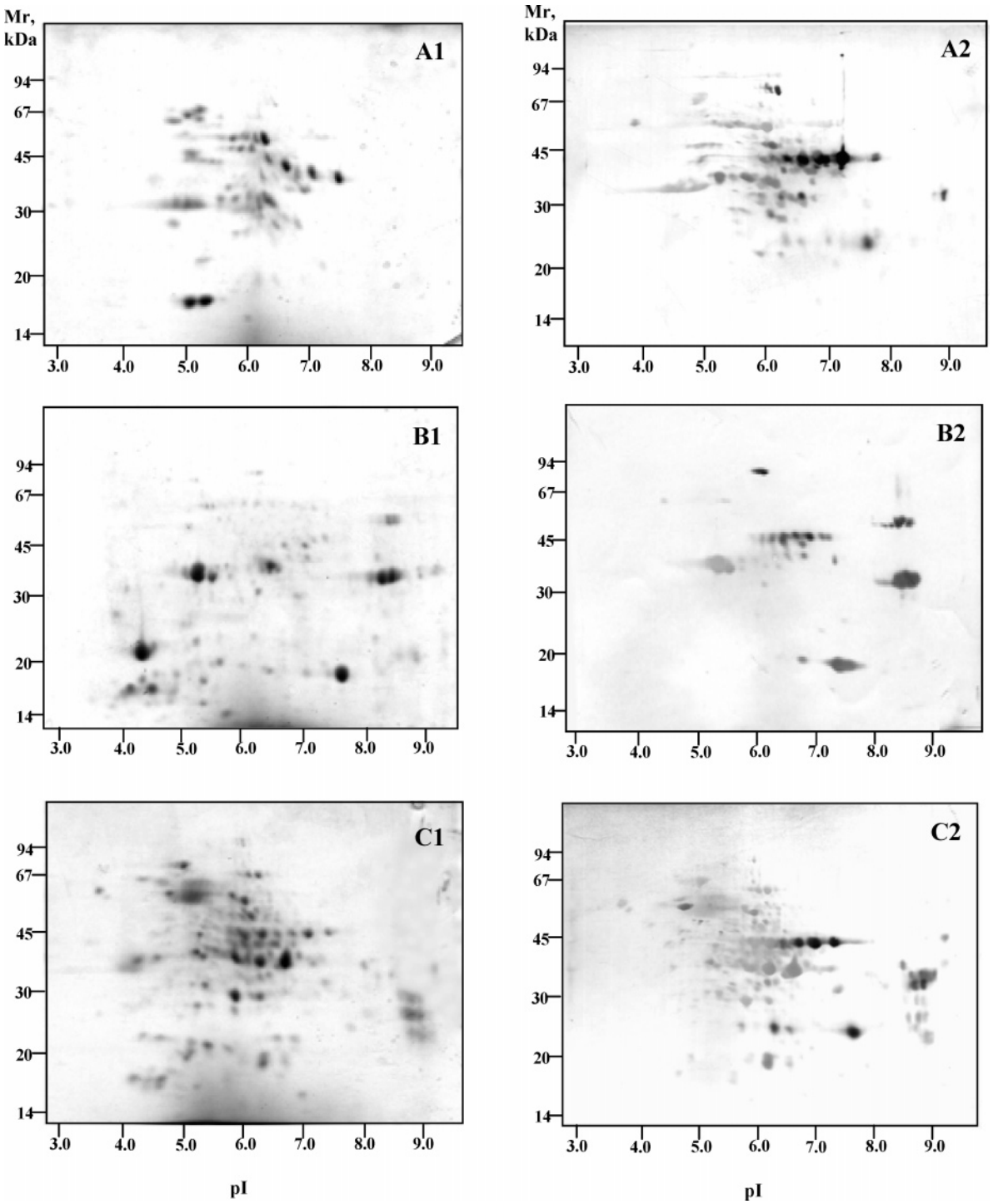

Figure 2. 2D electrophoretic maps of peanut (A), soybean (B), and lupin (C) acidic protein extracts and Western-blotting analyses. Left panels (A1, B1, and C1) show the Coomassie blue stained 2D gels of the mentioned seed protein extracts. Right panels are the corresponding Western-blotting analyses with anti Ara h 3 basic subunit antibodies (A2, B2, and C2). Experimental details are given under Materials and Methods.

techniques in **Figure** 1. The 1D patterns shown were in turn compared with the corresponding separations in previous papers (*20*−*23*). For the identification of lupin conglutin γ and the homologous soybean Bg7S, which proved to react with anti Ara h 3 IgGs, specific antibodies were used (see below).

The Western-blotting analyses of the 2D gels with IgGs to the basic subunits of Ara h 3 are shown in **Figures 1** and **2**, right panels. As expected, in the case of peanut protein alkaline extract, the antibodies strongly reacted with two major and few minor 23 kDa polypeptides (**Figure 1**, panel A2, solid rectangle) corresponding to Ara h 3 basic subunit isoforms (*24*), i.e., the polypeptides they were raised to. Three unidentified spots near 45 kDa with pI between 6.0 and 6.5 were also strongly detected by the antiserum. Since the M_r's of these polypeptides coincided with those of Ara h 3 acidic subunits, the reaction with the antiserum would have been misinterpreted in a 1D gel, thus preventing identification of these new reacting spots. No reaction was observed with Ara h 1, a 7S globulin, or with Ara h 2, a 2S protein, which are claimed to be the major peanut allergens (*25, 26*).

The Western-blotting analyses of soybean and lupin protein alkaline extracts showed cross-reactivity corresponding to the

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(1) GIEETICTASFKKNIGRNRSPDIYNPQAGSLKTANELQLNLLILRWLGLSAEYGNLYRNA   60
(2) GVEENICTLKLHENIARPSRADFYNPKAGRISTLN--SLTLPALRQFQLSAQYVVLYKNG   58
(3) GLEETICTARLLENIAKPSHTDLYNPSA(#)                                 28
    *:**.***  : :**.:   .*:***.** :.* *  .*.*  ** : ***:*  **:*.

(1) LFVPHYNTNAHSIIYALRGRAHVQVVDSNGDRVFDEELQEGHVLVVPQNFAVAGKSQSEN  120
(2) IYSPHWNLNANSVIYVTRGQGKVRVVNCQGNAVFDGELRRGQLLVVPQNFVVAEQAGEQG  118
    :: **:* **:*:**. **:.:*:**:.:*: *** **:.*::*******.** :: .:.


(1) FEYVAFKTDSRPSIANLAGENSFIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPSE  180
(2) FEYIVFKTHHNAVTSYLK---DVFRAIPSEVLAHSYNLRQSQVSELKY-----------  163
    ***:.***. ..  : *    ..:  :*.**:*:**.* :.*. :**

(1) QSLRAVA                                                        187
(2) -------
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Figure 3. Alignment of the 11S basic subunits amino acid sequences from peanut (1), soybean (2), and lupin (3). Swiss-Prot accession numbers are Q8LKN1 and P02858 for peanut and soybean sequences, respectively. (#) The amino acid sequence of lupin 11S basic subunit is incomplete with only 28 N-terminal amino acids determined (Duranti, unpublished results). (*****) Identical residues in all sequences. (**:**) Conserved substitutions in all sequences (**.**) Semiconserved substitutions in all sequences. Gaps (–) have been inserted to maximize the three alignments.

spots identified as the basic subunits of the respective 11S globulins (**Figure 1**, panels B2 and C2). This reaction was far more evident with soybean 11S basic subunits than with the lupin ones. Amino acid sequence alignments of the corresponding polypeptides are shown in **Figure 3**. The two complete amino acid sequences of the peanut and soybean 11S globulin basic subunits displayed 41% identity with an internal stretch of highly conserved consecutive 17 amino acid residues from Glu96 to Ala113 (numbers are referred to the peanut sequence). As far as the lupin 11S basic subunit is concerned, only 28 N-terminal amino acid residues are known (Duranti, Unpublished data), and this sequence appeared to be 53% identical to that of the peanut 11S basic subunit. Indeed, this region, which corresponds to the site of proteolytic cleavage of the 11S globulin pro-polypeptides, has been shown to be highly conserved among the proteins of this class (*27*). Nevertheless, the lower reactivity of lupin 11S basic subunits with respect to the soybean ones may indicate that the epitope(s) do(es) not lye in this highly conserved region.

Interestingly, four to six soybean polypeptides with M_r around 50 kDa and acidic pIs strongly cross-reacted with the antiserum anti Ara h 3 basic subunit. According to their position in the 2D map they could tentatively be identified as the soybean 7S globulin β subunits, although no reaction was observed with the highly homologous α and α' subunits of the same protein. This unexpected finding deserves further studies, especially if the IgG cross-reactivity of these spots could be confirmed by patient IgE analyses. As already remarked, a characteristic series of spots in the region of 45 kDa and pI from 6.0 to 6.5 strongly cross-reacted in the three seed extracts both in the alkaline (**Figure 1**) and acidic extraction conditions (**Figure 2**). Since the bands are barely visible in the corresponding Coomassie blue stained gels, especially in the alkaline extracts, they should contain strong epitopes. Identification of these bands is now being undertaken in our laboratories.

Two other relevant spots of soybean and lupin acidic and alkaline extracts cross-reacting with anti Ara h 3 basic subunit IgGs are located in the regions of 30 and 17 kDa and pIs 7.5−8.0 and 6.0−6.5, respectively. These spots have also been recognized by anti-conglutin γ IgGs on lupin (**Figure 1**, panel C3) and soybean (not shown) 2D blotted maps, and they were identified accordingly as the large and small subunits of lupin conglutin γ and the soybean highly homologous (*28*) protein, named Bg7S. Lupin conglutin γ and, by analogy, soybean Bg7S are soluble at either acidic and alkaline pH values (*29*); therefore, they appeared in both seed extracts (**Figures 1** and **2**). The cross-reactivity of these lupin and soybean proteins with anti Ara h 3 basic subunit IgGs is not straightforward, since these polypeptides belong to completely different protein families. Indeed, when the three sequences are globally aligned (accession numbers in SwissProt database Q9FSH9 for conglutin γ and P13917 for Bg7S) only 13% and 12% global identity of lupin and soybean proteins, respectively, to peanut 11S basic subunit were observed and also the local alignments revealed at best only three-residue identical stretches (not shown).

DISCUSSION

In this work 2D electrophoretic techniques have been used to screen three legume seed proteomes with the antibodies to a recently proved major peanut allergen (*10*), the basic subunits of the 11S globulin fraction. Due to the extremely greater concentrations of the globulin with respect to the albumin fractions in most legume seeds and in order to optimize spot detection and identification, two solubilization procedures were selected: the alkaline one aimed to solubilize the bulk of seed proteins with limited use of high salt concentrations, which would have hampered the IEF separation, and the acidic one, again in a low ionic strength medium, aimed to exclude the globulins and focus on the albumin fraction.

With this global approach the immuno-cross-reactivities among three related polypeptides from different legumes and with other less or nonrelated polypeptides by use of 2D electrophoretic maps were evidenced. Two strongly cross-reacting protein types deserved special interest for their mutual sequence homology and unrelatedness to the 11 S basic subunits; they were lupin conglutin γ and soybean Bg7S. As far as lupin conglutin γ is concerned, a major lupin polypeptide cross-reacting with IgE of peanut-sensitized patients displayed $M_r \approx$ 43 kDa (*13*). Indeed, this polypeptide could correspond to the nonreduced form of lupin conglutin γ, MW 45 608 Da from sequence data (*28*), or alternatively coincide with the series of spots already mentioned and also common to soybean protein extracts. Identification of lupin conglutin γ as a cross-reacting protein with anti Ara h 3 antibodies agrees with the recent identification of a conglutin γ-like protein as the major allergen of almonds (*30*). Such a potential allergen may be present in other nuts (*31*) and possibly in other edible seeds, although in peanut the presence of any conglutin γ-like protein has not been shown so far. As for soybean Bg7S, to our knowledge no claim

on its allergenicity has been made yet. For these two potentially new allergens further studies are needed in order to evidence IgEs reactivities and clinical effects and trace these proteins within the food chain. Both activities are currently being undertaken by our groups.

In the attempt to identify key sequence elements responsible for the cross-reactivities observed, in silico epitope analysis has been performed by aligning the amino acid sequences of the reacting polypeptides. The results showed that the cross-reactivities of the 11S globulin basic subunits may have a molecular basis on the sequence similarities among them, although identification of a clear epitope was not possible, also due to the incompleteness of lupin sequence. However, although the global alignments of lupin conglutin γ and soybean Bg7S with Ara h 3 basic subunit displayed very low to null identities, the antibodies to the peanut subunit significantly cross-reacted with both subunits of lupin and soybean proteins. On the other hand, 90% similarity in 10 amino acid residues between peanut 11S basic (residues 98−107) and soybean 11S acidic (residues 118−127) subunits has been found, but this did not result in any cross-reaction (**Figure 1**). These results confirm that epitope identification by using IgG cross-reactivity is not straightforward and would require mutagenesis studies once a putative reactive region has been selected. Nevertheless, it has been well documented that even a single amino acid substitution could give rise to an increase or complete disappearance of the immunological response (*32*).

Although it has been observed that immuno-cross-reactivity is not necessarily representative of clinical reactivity and also that IgE antibodies may fail to react with more than one member of each legume protein family (*33*), the present work emphasizes the potential role of the basic subunit of the 11S globulins in the immune response of sensitized individuals. Although the immuno-cross-reactivity between peanut Ara h 3 and soybean glycinin has already been described (*32*, *34*, *35*), no evidence for involvement of epitopes in the two 11S globulins basic subunits has been claimed, the human IgE reacting epitopes all residing in the acidic subunits according to these authors. Therefore, further studies are needed, and the first logical extension of this work will be testing the same proteomes with human IgEs arising from sensitized patients. This activity has already been undertaken by our groups, and partial results on the reactivity of the basic polypeptides of the 11S globulin with IgEs of peanut-sensitized patients (*10*) have confirmed the major findings of the present work.

2D electrophoretic mapping proved extremely powerful for the global detection of potential allergens in food proteins by allowing comparison of different proteomes, unmasking cross-reacting bands, and identifying reactive polypeptides either by simple comparison of their electrophoretic mobilities, as shown in this work, or through conventional proteomic approaches, including mass spectrometry and amino acid analysis of the reacting chains.

ABBREVIATIONS USED

Bg7S, basic 7S globulin; Ara h 3, *Arachis hypogaea* allergen 3.

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