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One- and Two-Dimensional Electrophoretic Identification of IgE-Binding Polypeptides of *Lupinus albus* and Other Legume Seeds

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The prevalence of food allergies in the world population requires integrated approaches to identify new potential allergens, especially those of plant origin. The aim of this work was the allergen in vitro analysis of *Lupinus albus* seed proteome, a promising food protein source, and the assessment of IgE cross-reactivities with other more diffused legume species. A combination of one- and two-dimensional gel electrophoresis and immunoblotting analyses with specific IgGs for band identification and lupin-sensitized patients' circulating IgEs for allergenicity studies has been used. Two lupin proteins, namely, conglutin γ and 11S globulin basic subunits, strongly reacted with all patients' sera. Also, cross-reactivities with the homologous polypeptides of other legume species were observed. Otherwise, no reaction at all was detected with a 2S-type lupin protein. This global electrophoretic approach has allowed the identification of a new potential lupin allergen and confirmed the cross-reactivity among the legume 11S globulin basic subunits.

KEYWORDS: Food allergy; *Lupinus albus*; conglutin γ; 11S basic subunits; legume seed proteins

INTRODUCTION

Legume seeds are commonly considered to be a rich source of valuable proteins for food and feed. For this and other relevant reasons, ways of expanding the use of these proteins and concomitantly reducing the intake of animal proteins are increasingly pursued, especially in the developed countries. Legume seeds may also represent a source of nutraceutical proteins: recent studies in animal models have shown that soybean 7S globulin α' subunit and lupin conglutin γ are, respectively, powerful hypocholesterolemic and hypoglycemic agents (*1*, *2*). On the other hand, some typical constraints of legume seed proteins, including the antinutritional and allergenic activities of some of them, limit their wider use as food raw materials or ingredients. The need for actions aimed at identifying deleterious components is therefore acute.

The prevalence of food allergy in the general population declines with age, from ∼8% in children to <1% in adults (*3*). Most plant food allergens belong to a few protein families and superfamilies, such as the cupin superfamily, including 11S and 7S seed storage proteins, the prolamin superfamily, including 2S albumins and hydrolase inhibitors, and other less related families of structural, metabolic, and defense plant proteins (*4*).

Among the legume seeds, lupin is an interesting one, owing to both its high protein content (*5*), comparable to that of soybean, and the lowest presence of antinutritional compounds (*6*). Various ongoing research projects, not just in Europe, witness the recently renewed interest in this seed and its protein component. Lupin seed contains two major types of storage proteins. The first group, that is, conglutins α, includes proteins with sedimentation coefficients of ∼11−12S. They are referred to as 11S or "legumin-like" globulins and consist of hexamers of two disulfide-linked heterogeneous subunits indicated as acidic, M_r ranging from 54 to 47 kDa, and basic subunits, M_r of ∼20 kDa, respectively. The second group of proteins, that is, conglutins β, has sedimentation coefficients of ∼7S. They are classified as 7S or "vicilin-like" globulins, and their trimeric structure consists of several polypeptides of 20−80 kDa, with no disulfide bridges. In addition to these proteins, other conspicuous components are the conglutin γ, a basic tetrameric protein with sedimentation coefficient 7S, consisting of disulfide-linked 30 and 17 kDa polypeptides, and the conglutin δ, a

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monomeric protein with a sedimentation coefficient of 2S made up of disulfide-linked polypeptide chains of 9 and 4 kDa (*5*).

A relevant issue, when considering lupin seeds as an alternative protein source, concerns the evaluation of its allergenic potential. Although some papers have already dealt with this issue (*7*, *8*), the molecular identity of lupin allergenic polypeptides has not been determined so far.

In previous works (*9*−*11*), two-dimensional (2D) electrophoresis combined with immunoblotting analyses was used as a powerful tool for the global analysis of allergens in plant proteomes. In particular, a dominant allergenic activity of the peanut 11S globulin basic subunit (*10*) and the cross-reactivities of IgG specific to this polypeptide chain with other legume seeds (*9*) have been described.

The aim of this work was the assessment of the potentially allergenic polypeptides in *Lupinus albus* seeds by using one-dimensional (1D) electrophoresis and immunoblotting analyses with lupin-sensitized patients' sera. Also, comparison of the reactivities to other legume seed extracts was carried out. Moreover, the use of IEF/SDS-PAGE electrophoresis (2D electrophoresis) allowed the unambiguous identification of the novel putative allergens in lupin seed proteome.

MATERIALS AND METHODS

Materials. Dry mature seeds of white lupin (*Lupinus albus* L. var. Multitalia) were kindly provided by Massimo Fagnano, University of Naples, Italy. Peanut (*Arachis hypogea* L. var. Israel) seeds were kindly supplied by Vincenzo Brandolini, University of Ferrara, Italy. Soybean (*Glycine max*), pea (*Pisum sativum*), and common bean (*Phaseolus vulgaris*) seeds were of commercial origin.

Chemicals were all of reagent grade from Sigma-Aldrich and Fluka.

Patients' Sera. The sera of five children suffering from lupin allergy and experiencing rhino-conjunctivitis episodes and severe asthma (*7*) were collected at the Meyer Hospital, University of Florence, with informed consent from the children's parents. As a negative control, sera from two milk-allergic children and four nonallergic subjects were used.

One-Dimensional Electrophoresis. The defatted flours were suspended in the 0.5× sample buffer at a final concentration of 10 mg/mL. The sample buffer contained 0.25 M Tris-HCl, pH 6.8, 7.5% glycerol, 2% SDS, and 5% β-mercaptoethanol. After heating at 100 °C for 5 min, the slurries were centrifuged at 10000*g* for 15 min, and 10 μL of each supernatant was loaded onto the gel. The SDS-PAGE was performed in NuPAGE Novex Bis-Tris 10% gels using an XCell SureLock Mini-Cell (Invitrogen, Milan, Italy). NuPAGE MES SDS running buffer and SeeBlue Plus2 prestained standard (Invitrogen) were used. The gels were stained with SimplyBlue SafeStain (Invitrogen).

Preparation of Protein Samples for 2D Electrophoretic Analyses. Seeds were manually dehulled and the kernels ground to a meal. The resulting flour was suspended in cold acetone to solubilize the lipid fraction and centrifuged at 10000*g* for 30 min. This treatment was repeated three times. The defatted flours were extracted with a solution consisting of 7 M urea, 2 M thiourea, 2% CHAPS, and 65 mM 1,4-dithiothreitol (DTT) in the ratio 1:30 w/v under stirring for 2 h. The slurry was centrifuged at 10000*g* for 30 min, and the extracted proteins were immediately analyzed or kept frozen at −80 °C until use.

Two-Dimensional Electrophoresis. Isoelectric focusing was performed on 7 cm pH 3−10 nonlinear IPG strips (Amersham Biosciences, Milan, Italy). The strips were rehydrated overnight in a solution consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer pH 3−10 (Amersham Biosciences) 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). Prior to the second dimension, strips were incubated in equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 65 mM DTT for 15 min. Then strips were incubated with the equilibration buffer containing 243 mM iodoacetamide for 10 min. The separation was performed in 12% SDS-PAGE gels using a mini-PROTEAN III cell (Bio-Rad, Milan, Italy). The polyacrylamide 2D gels were Coomassie Blue stained.

Immunoblotting. Replicas of both 1D and 2D gels were transferred to PVDF membranes (Millipore, Milan, Italy) by blotting according to the method of Towbin et al. (*12*) on a Trans-blot electrophoretic transfer cell (Bio-Rad).

IgG Preparation. Antibodies to conglutin γ and lupin 11S basic subunits were raised in rabbits with the purified protein (*13*) and a synthetic N-terminal 28 amino acid residue polypeptide (Duranti, unpublished data), respectively, by using standard immunization procedures (*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 further purified by immunoaffinity chromatography with a nonglycosylated conglutin γ immobilized on the Sepharose 4B CNBr matrix, according to the procedures described by Casey (*14*).

IgG Analysis. The membranes were blocked with 3% gelatin for 2 h and washed three times with 0.25% gelatin solution in PBS buffer (10 mM NaP_i, pH 7.4, containing 150 mM NaCl). Membranes were then soaked for 2 h in PBS buffer containing rabbit anti-lupin 11S globulin basic subunits in the ratio of 2000/1 (v/v) or anti-conglutin γ in the ratio of 1000/1 (v/v). The bands were revealed by using horseradish peroxidase conjugate with goat anti-rabbit antiserum at 2000/1 (v/v) (Bio-Rad) and hydrogen peroxide with 4-chloronaphthol as substrate.

IgE Analysis. The membranes were blocked with 1% gelatin for 2 h and washed three times with 0.25% gelatin solution in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, and 0.05% Triton-X to prevent nonspecific adsorption of the immunological reagents. Membranes were then immersed in 10 mL of 0.25% gelatin solution containing 300 μL of allergic children's sera. Antigen−IgE complexes were detected using 20 μL of goat anti-IgE polyclonal antibodies labeled with alkaline phosphatase (Sigma, Milan, Italy). The developing solution contained 15% bromochloroindolyl phosphate (BCIP) and 30% nitro blue tetrazolium (NBT) (Sigma) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

RESULTS

The first step of this work used five lupin-sensitized children's sera for immunoblotting analyses of lupin and other legume protein extracts 1D electrophoretic patterns. To include in our analyses as many polypeptide bands as possible, both dedicated gels and improved staining procedures were used (see details under Materials and Methods). The gel of **Figure 1A** shows the typically complex electrophoretic profiles of legume seed proteins, which comprise the subunits of the bulk storage proteins, mainly the 7S and 11S globulins. To help identify lupin conglutins δ and γ, which represent minor seed protein constituents, purified samples of these two proteins were added (**Figure 1A**,**B**, lanes 1 and 7).

The five patients' IgE-treated membranes, of which a representative one is reported in **Figure 1B**, showed identical patterns and allowed the main reacting polypeptides in all seed extracts to be identified. Strongly reactive bands around 20 kDa were visible in soybean, peanut, lupin, and pea extracts. Other bands around 17 and 30 kDa were also detectable in lupin and soybean extracts. According to the electrophoretic mobilities of these bands and their recognition by specific antibodies (**Figure 1C**,**D**), they were identified as the lupin and soybean conglutins γ and the 11S globulin basic subunits of soybean, peanut, lupin, and pea, respectively. No reaction was observed in panel **D** with the common bean extract, probably due to the very low amount of the 11S globulin in this legume seed (*15*). Purified conglutin γ confirmed strongly positive IgE response (**Figure 1**, lane 7). Conversely, conglutin δ, a 2S-protein-type of lupin seed, did not react at all with any of the patients' sera

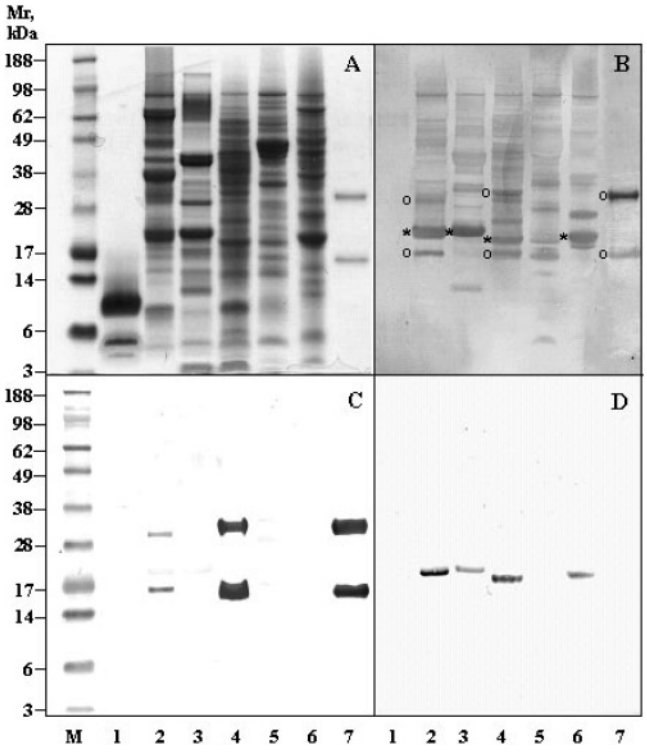

Figure 1. One-dimensional SDS-PAGE and immunoblotting analysis of selected legume seed protein extracts: (**A**) SimplyBlue SafeStain colored gel of the protein extracts, run under reducing conditions; (**B**) corresponding membrane reacted with lupin-sensitized patient circulating IgEs; (**C**, **D**) immunoblotting analyses with anti-conglutin γ and anti-11S globulin basic subunit IgGs, respectively. Experimental details are given under Materials and Methods. Sample lanes are the following: M, prestained marker proteins; 1, lupin conglutin δ; 2, soybean; 3, peanut; 4, lupin; 5, common bean; 6, pea; 7, lupin conglutin γ; ○, conglutin γ and conglutin γ-like large and small subunits; ∗, 11S globulin basic subunits.

either in the purified form or in the lupin protein extract (**Figure 1B**, lanes 1 and 4, respectively). No positive response was observed with sera from milk-allergic children or nonallergic subjects (not shown).

To confirm the attributions of lupin IgE-binding polypeptide and to unmask potentially hidden reactive polypeptides (*9*), the high-resolution power of 2D IEF/SDS-PAGE electrophoresis has been used. The Coomassie blue stained 2D map is reported in **Figure 2A**. Identification of the main groups of spots in the gel was carried out both by comparison with 2D maps of purified reference lupin protein fractions (not shown) and, for relevant proteins, by specific antibodies (**Figure 2C**,**D**). The groups of polypeptides were labeled accordingly in **Figure 2A**.

When the replicas of the gel of **Figure 2A** were blotted to PVDF membranes and reacted with the patients' sera, the pattern of IgE-binding polypeptides was revealed (**Figure 2B** shows one of the five membranes). Several spots were recognized, although at different intensities, not necessarily corresponding to the relative amounts of the polypeptides in the 2D map. This is the case of the low-reacting polypeptides in the center of the membrane, which belong to the quantitatively conspicuous 7S globulin family. In lupin, these polypeptides appear so numerous and indistinct due to the extensive endogenous post-translational cleavage of the corresponding precursors (*16*); consequently, they were hardly distinguishable in the 1D gel of **Figure 1B**. Among the reacting spots, some have especially drawn our interest due to their clear and sharp detection. To unambiguously identify these strongly reactive spots, specific antibodies to conglutin γ and 11S globulin basic subunits were used (**Figure 2**, panels **C** and **D**, respectively). On this basis, two spots were assigned as the 30 and 17 kDa conglutin γ subunits and the other spots as the heterogeneous basic subunits of lupin 11S globulin, M_r 20 kDa. The high-resolution power of the 2D gels revealed that the basic subunit isoforms all reacted with the circulating IgEs, suggesting the presence on them of a common epitopic region. On the other hand, some major spots visible in the Coomassie blue stained gel, such as those corresponding to the 11S globulin acidic subunits, were not visible at all, indicating that no IgE binding to them had occurred. These findings fully confirmed the results shown in **Figure 1**.

DISCUSSION

The combined use of 1D and 2D electrophoresis has allowed us to obtain a twofold result: identification of the polypeptides reacting with lupin-sensitized patients' circulating IgEs among lupin seed proteins and comparison of the observed reactivities with those revealed in other legume seed protein extracts.

A new lupin potential major allergen has been identified as the protein named conglutin γ. This identification is supported by a number of previous findings. Some years ago, Moneret-Vautrain et al. (*17*) showed 1D electrophoretic patterns of lupin protein extracts reacted with IgE from peanut-sensitized patients and described a number of reacting polypeptides with different M_r values. Among these polypeptides, one had M_r around 43 kDa, which might coincide with the unreduced conglutin γ. Indeed, we have also recently observed cross-reactivity of anti-peanut IgE with conglutin γ (*9*). A result similar to that of Moneret-Vautrin et al. (*17*) was obtained directly with the IgE of a lupin-sensitized child by Novembre et al. (*7*). More recently, Poltronieri et al. (*18*) described a polypeptide similar to conglutin γ as one of the dominant allergens of almond seeds. In soybean seed extracts, two similar polypeptides corresponding to the conglutin γ homologous protein, named Bg7S, also significantly reacted with the patient's IgEs (**Figure 1B**). Interestingly, the conglutin γ polypeptides were also found to cross-react with IgG specific to the basic subunit of Ara h 3, a well-known peanut allergen of the 11S globulin family (*9*), although no sequence similarity between the two protein types was found (accession numbers of Swiss-Prot/TrEMBL database Q9FSH9 and Q8LKN1 for conglutin γ and Ara h 3, respectively). All of these findings support the conclusion that conglutin γ, first described in *L. albus* seed (*5*) but seemingly present in many other seeds and nuts, may be a major allergenic protein.

Noteworthy is that although the conglutin γ large subunit is glycosylated, the small one is not (*19*). Therefore, the involvement of saccharide moieties in the IgE reactivity can be ruled out. This also held true with the 11S globulin basic subunits, where the natural lack of covalently linked carbohydrate moieties did not prevent the reaction from taking place.

Another conclusion that can be drawn from our findings is that also the basic subunits of the 11S globulins may represent an allergen. Indeed, these bands, regardless the antiserum used, are always clearly visible in lupin, as well as in all other legume seeds containing such polypeptides (**Figure 1B**). The cross-reactivities of the 11S basic subunits of the lupin, peanut, soybean, and pea globulins are not surprising due to their high sequence homology, especially in the region proximal to the proteolytic processing site, which is very much conserved among these proteins (*20*). Our present data confirm a previous finding (*10*) and an observation by Koppelman et al. (*21*) suggesting that the basic subunits of the 11S globulins are the dominant allergens in the individuals screened in Europe instead of the acidic subunits (*22*).

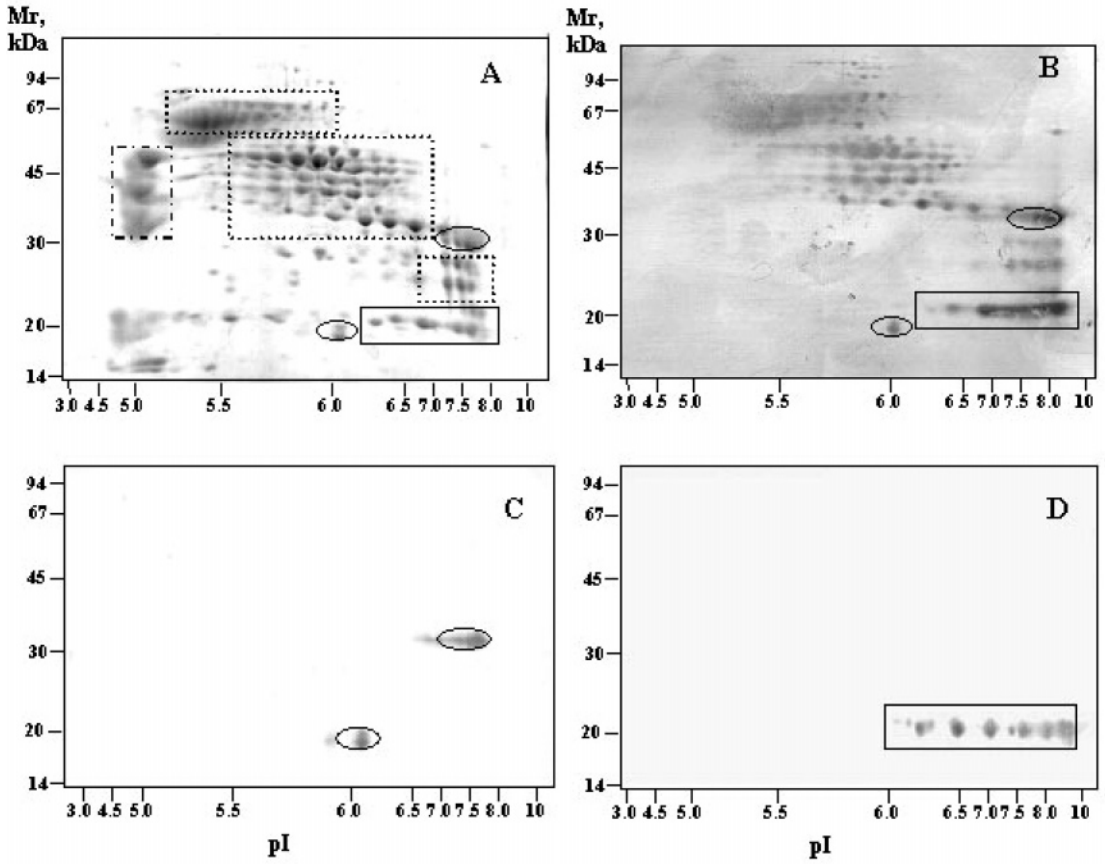

Figure 2. Two-dimensional electrophoretic maps of lupin protein extracts (**A**) and immunoblotting analyses with lupin-sensitized patient IgEs (**B**), anti-conglutin γ (**C**), and anti-11S globulin basic subunits (**D**) IgGs. Panel **A** shows the Coomassie blue stained 2D gel of the mentioned seed protein extract. Experimental details are given under Materials and Methods. Solid line rectangles enclose the basic subunits of the 11S globulins. Dashed–dotted line rectangles enclose the acidic subunits of the 11S globulins in panel **A**. Dashed line rectangles outline the polypeptides of the 7S globulins. The solid ellipses enclose the large and small subunits of conglutin γ.

In our study conglutin δ, the 2S protein, which is claimed to be a powerful allergen in the other seeds, did not react at all with any of the patients' sera. It is not clear at the moment if this is due to the peculiar nature of *L. albus* 2S protein or the reactivity of the sera used. However, we have recently shown that also the peanut 2S protein, Ara h 2, is not involved in peanut allergy, at least in our patients' population (*10*), contrary to previous studies (*21*).

Although these in vitro studies are extremely useful for the identification of potential allergens and cross-reactivities, other approaches, including the use of food challenges, are needed to confirm the actual clinical significance of these data. Indeed, although it is possible to find IgEs against other legumes in patients showing symptoms to only one legume, clinical manifestations of these cross-reactions are relatively uncommon (*23*, *24*). Nevertheless, the possibility of crossed peanut−lupin allergy cannot be ruled out, as also suggested by the great percentage of positive reactions to lupin in peanut-sensitized children (*17*). Dairy habits as well as pollen exposure may strongly influence the epidemiology of legume including lupin allergy. Therefore, due to the relatively minor use of lupin seeds and their isolated proteins as food, further studies, more oriented to epidemiological aspects, are needed to evaluate the actual prevalence of lupin allergy. In this respect, it might be worth mentioning that only 1 health center of 10 contacted has been able to supply the patients'sera used in this work.

In conclusion, combined 1D and 2D electrophoresis and immunoblotting analysis have revealed and allowed us to identify two dominant IgE-reacting polypeptides of lupin seeds: one corresponding to *L. albus* conglutin γ and the other corresponding to the basic subunits of lupin 11S globulins. In addition, patients' sera were used to assess in vitro cross-reactivities with the proteins of other legume seeds, confirming the potential allergenicity of these mentioned polypeptides when present in the seed extracts. This work also confirmed the efficacy of the 2D electrophoretic maps for the identification of allergens and the assessment of potential individual sensitivities to food allergens.

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