

Molecular insight into IgE-mediated reactions to sesame (*Sesamum indicum* L.) seed proteins

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Background: Food allergy is becoming a major public health concern in recent times. Several sesame seed allergenic proteins have been identified. However, sensitization toward these proteins does not follow a common and unique pattern of clinical reactivity, as shown by the differential geographic recognition of single proteins.

Objective: To evaluate the sensitization profiles of 18 Italian individuals who experienced clinical symptoms after sesame seed consumption, including 4 anaphylactic reactions.

Methods: Using an in vitro approach, we adopted a 2-dimensional electrophoretic technique combined with immunoblotting analyses by using sera from 18 Italian sesame-allergic patients.

Results: We showed the prevalent and almost exclusive reactivity of the sesame 11S globulin. We shed light on the active role of the basic subunit of this globulin family. The limited accessibility of this polypeptide chain, unless the interchain disulphide bonds are cleaved, may be one of the reasons for its structural/functional stability and, thus, great potential for induction of IgE reactivity.

Conclusions: These results confirmed previous findings on the reactivity of the basic subunit of 11S globulin in various legume species. Moreover, this experimental approach proved to be useful for the noninvasive screening of specific reactivities in sensitized patients.

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INTRODUCTION

In Europe and the United States, food allergy affects approximately 1% to 2% of the general adult population and up to 5% to 7.5% of children.^{1–3} Symptoms may vary in severity from mild urticaria or localized oral effects to severe systemic reactions that can be fatal. Individuals with food allergy, mainly those who have experienced anaphylactic shock, require specific dietary restrictions.

Food sensitization is not synonymous with food allergy. The association between sensitization to a specific allergen in a food source and clinical symptoms is often uncertain. The relationship between dose and clinical reactions experienced by allergic individuals has not yet been accurately defined for every allergen. Cross-reactivity between food allergens is a well-known phenomenon but still deserving of study for its

definition within allergen families. The identification of an allergen is most difficult when food ingredients are numerous in an industrial food product.

An updated list of food protein allergens was recently published by Mari et al.⁴ The European Union adopted directives 2003/89/EC, 2006/142/EC, and 2007/68/EC, in which legume and sesame seeds must be mandatorily labelled among other allergenic food sources. The list of these allergenic sources includes some foods that are responsible for allergy whose severity and prevalence depend on the specific geographic area considered. Although frequent in Australia and Asia,⁵ some countries in North Africa, and Israel,⁶ allergy to sesame is rare or underestimated in Italy; the reasons for these differences can be different food habits or different sensitization patterns.

Sesame (*Sesamum indicum* L.) is used in the preparation of various foods: in Italy, the most common sesame food sources are bread and bakery products. From the molecular point of view, the allergens that have been reported to be associated with sensitization to sesame are (1) Ses i 1 and Ses i 2, two 2S albumins^{7,8}; (2) Ses i 3, a 7S globulin (vicilinlike protein)⁷; (3) Ses i 4 and Ses i 5, two oleosins⁹; (4) Ses i 6 and Ses i 7, two 11S globulins (leguminlike proteins)¹⁰; and (5) Ses i 8 profilin.¹¹

Starting from these previous findings, the aim of the present study was to evaluate whether the pattern of sensitization in Italian sesame-allergic individuals included the mentioned allergenic proteins and specific subunits thereof and to identify new IgE-reactive molecules by exploiting the

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feasibility of 2-dimensional (2-D) electrophoresis in defining patients' detailed individual sensitization profiles.

MATERIALS AND METHODS

Materials

Sesame seeds were purchased from Fratelli Ingegnoli S.p.A. (Milan, Italy). Chemicals were all reagent grade from Sigma-Aldrich Italia (Milan) and Fluka Italia (Milan).

Serum Features and Clinical Data

Enrollment of patients with sesame allergy was performed in collaboration with several clinical centers. Indeed, although sensitization to sesame (the low presence of circulating IgEs toward sesame extract) is frequent in Italy, the number of individuals with confirmed clinical symptoms is low. The protocol adopted in the present study was approved by ethical committees of the institutions involved in this study. Informed consent was obtained from the adult patients and from the children's parents.

Eighteen individuals (10 males and 8 females) were enrolled in the study; participant age ranged from 3 to 73 years (mean [SD] age, 26 [20.85] years). The serum samples used in the present study were from 18 individuals defined as being allergic to sesame seeds on the following basis: positive results to the skin prick test with a wheal diameter at least 3 mm wider than the negative control, or positive IgE detection using the sesame extract on the CAP test (Phadia, Uppsala, Sweden), or IgE detection using the Immuno Solid-phase Allergen Chip (VBC-Genomics, Vienna, Austria). All the

participants had reliable clinical reactions to sesame seeds, including hives, lip swelling, angioedema, urticaria, shortness of breath, wheezing, stridor, and abdominal pain. Four participants experienced anaphylaxis characterized by glottis edema, vomiting, throat swelling, and loss of consciousness (Table 1). Blood samples from healthy individuals or from patients with allergy to foods other than sesame seed were used as negative controls.

Purification of Sesame 11S Globulin

The 11S globulin was purified from sesame seeds as described by Orruno and Morgan.¹² Protein was prepared and immediately used for inhibition assays, as described later herein.

Preparation of Protein Samples for 2-D Electrophoretic Analyses

Sesame seeds were ground to a meal. The resulting flour was extracted with a solution consisting of 9M urea and 2% CHAPS in a ratio of 1:30 wt/vol under stirring at room temperature for 2 hours in the presence or absence of 65mM 1,4-dithiothreitol (DTT) to obtain disulfide-reduced and non-reduced protein samples, respectively. The slurry was centrifuged at 10,000g for 30 minutes, and the extracted proteins were immediately analyzed or kept frozen at -80°C until use.

2-D Electrophoresis

Isoelectric focusing was performed on 7-cm, pH 3 to 10, nonlinear immobilized pH gradient strips (Amersham Biosciences, Milan). The strips were rehydrated overnight in a

Table 1. Patients' Details

Patient No./Sex/Age, y	ISAC vs Ses i 1, ISU	CAP, kUI/mL	Skin prick test, mm	Clinical symptoms
1/M/29	ND	10.0 (Sesame)	10	Throat swelling, vomiting, shortness of breath
2/M/35	0	25.9 (Ses i 1)	ND	Urticaria, asthma
3/M/48	0	0.0 (Ses i 1)	ND	Urticaria, asthma
4/F/10	0	30.0 (Ses i 1)	ND	Asthma, gastrointestinal symptoms
5/F/5	0	45.9 (Ses i 1)	ND	Angioedema, asthma, gastrointestinal symptoms
6/M/10	0	1.6 (Ses i 1)	ND	Urticaria, asthma
7/F/67	0	6.0 (Ses i 1)	ND	Urticaria
8/M/50	44.3	ND	ND	Angioedema, urticaria, asthma, OAS
9/F/26	6.3	10.9 (Ses i 1)	ND	Angioedema
10/F/73	2.4	1.0 (Ses i 1)	ND	Urticaria
11/M/9	4.7	1.8 (Ses i 1)	ND	Urticaria
12/F/25	0.4	15.4 (Ses i 1)	ND	OAS
13/F/3	0	ND	ND	Urticaria
14/M/34	0	1.2 (Ses i 1)	ND	Angioedema
15/M/14	ND	3.8 (Sesame)	8	Anaphylaxis (glottis edema, vomiting, throat swelling, loss of consciousness)
16/M/16	ND	ND	7	Glottis edema, hives
17/F/9	ND	ND	19	Vomiting, cough, lip swelling, hives
18/M/4	ND	ND	7	Hives, urticaria, cough, wheezing, stridor, glottis edema, shortness of breath

Abbreviations: ISAC, Immuno Solid-phase Allergen Chip; ISU, IgE ISAC Standardized Units; ND, not done; OAS, oral allergic syndrome.

solution consisting of 9M urea, 2% CHAPS, 2% immobilized pH gradient buffer, pH 3 to 10 (Amersham Biosciences), containing 50 µg of the protein sample in the presence or absence of 65mM DTT. Strips were focused at 6,500 V/h, with a maximum of 3,000 V, at 20°C using the Multiphor II electrophoresis unit (Amersham Biosciences). Before the second dimension, strips were incubated in equilibration buffer (375mM Tris-hydrochloride, pH 8.8, 6M urea, 2% sodium dodecyl sulfate, and 20% glycerol) in the presence or absence of 65mM DTT. Then, strips were incubated with the equilibration buffer containing 243mM iodoacetamide for 10 minutes. The separation was performed in 8% to 16% linear gradient Tris-hydrochloride gels (Bio-Rad, Milan) using a mini-PROTEAN II cell. SeeBlue Plus2 prestained standard (Invitrogen, Milan) was used. The polyacrylamide 2-D gels were stained with SimplyBlue SafeStain (Invitrogen).

Antibody Preparation

Antibodies to lupin 11S basic subunit were raised in rabbits with a synthetic N-terminal 28-amino acid residue polypeptide (M.D., unpublished results, 2005) by using standard immunization procedures.¹³

Immunodetection With Animal IgGs From Lupin 11S Basic Subunit Immunized Rabbits

Two-dimensional gels were transferred to polyvinyl difluoride membranes (Bio-Rad) by means of blotting according to the method of Towbin et al¹⁴ on a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 3% gelatin for 2 hours and were washed 3 times with 0.25% gelatin solution in phosphate-buffered saline buffer (10mM sodium phosphate, pH 7.4, containing 150mM sodium chloride). Membranes were then soaked for 2 hours in phosphate-buffered saline buffer containing rabbit anti-lupin 11S globulin basic subunits in the ratio of 1,500:1 (vol/vol). The antibody-antigen complexes were revealed by using horseradish peroxidase conjugate with goat anti-rabbit antiserum 2,000:1 (vol/vol) (Bio-Rad) and hydrogen peroxide with 4-chloronaphthol as substrate.

Immunodetection With Human IgEs of Sesame-Allergic Participants and Inhibition Assay With Sesame 11S Globulin

Two-dimensional gels were transferred to polyvinyl difluoride membranes (Bio-Rad) and treated as described previously herein. Membranes were immersed in 10 mL of 0.25% gelatin solution containing 300 µL of allergic patient's serum. Antigen-IgE complexes were detected using 30 µL of goat anti-IgE polyclonal antibodies labeled with alkaline phosphatase (Sigma-Aldrich Italia). The developing solution contained 15% bromochloroindolyl phosphate and 30% nitro blue tetrazolium (Sigma-Aldrich Italia) in alkaline phosphatase buffer (100mM Tris-hydrochloride, pH 9.5, 100mM sodium chloride, and 5mM magnesium chloride). For inhibition immunoblots, human sera from patients 12 and 15 were preincubated with purified sesame 11S globulin at a final concentration of 70 µg/mL for 2 hours at room temperature

under gentle shaking and then were used as described previously herein.

Immunodetection With IgG4

After the blotting and blocking procedure as described previously herein, membranes were immersed in 10 mL of 0.25% gelatin solution containing 100 µL of allergic patient's serum. Four representative serum samples, namely, those of patients 4, 6, 7, and 18, were used for the IgG4 assay. The antibody-antigen complexes were revealed by using alkaline phosphatase conjugate with anti-IgG4 monoclonal antibody, 1,000:1 (vol/vol) (Zymed, San Francisco, California) and alkaline phosphatase substrate as described previously herein.

RESULTS

The identification of major sesame allergens has been considered by various authors.⁷⁻¹¹ However, at present, it is unclear whether the patterns of reactivity described are "universal" or whether various factors, including specific molecular features of the allergenic proteins or a kind of geographic differentiation, may affect the IgE recognition patterns. In this article, the sensitization profiles of 18 Italian individuals who experienced clinical symptoms after sesame seed consumption, including 4 anaphylactic reactions, are described.

Using the *in vitro* approach, we adopted a 2-D electrophoretic technique, which proved to be effective in the identification of specific allergenic proteins.^{7,15} The 2-D electrophoretic reference map under reducing conditions of sesame seed proteins stained with SimplyBlue SafeStain is shown in [Figure 1](#). The sesame seed whole protein pattern is characterized by 2 major spot groups around 35 and 20 kDa with acidic and basic isoelectric point values, respectively. It is known that the main protein component in sesame seeds belongs to the family of 11S globulins (Ses i 6 and Ses i 7), which represent 60% to 70% of the total proteins and consist of a group of acidic and basic polypeptide chains between 30 to 40 and 20 to 25 kDa, respectively.¹⁶ The acidic and basic subunits are linked by disulphide bridges, as occurs for most storage proteins of this class.¹⁷ Conversely, the 7S globulins (Ses i 3), another storage protein family, are much less represented in sesame seeds and consist of various chains, devoid of disulphide bridges, with molecular weight slightly higher than 45 kDa.⁷ On these bases, the 2 main spot groups detected in the map were assumed to correspond to the 11S globulin acidic and basic subunits, respectively. This assumption was confirmed by the use of antibodies to a conserved region of the 11S globulin basic subunits, which detected only a major 20- to 25-kDa spot and a few minor ones in the alkaline region of the map ([Fig 1B](#)). Other minor components were visible in the SimplyBlue SafeStain-stained gel, but their identification was not attempted in this work because of their lower or null IgE reactivity (see later herein).

In most IgE-revealed 2-D membranes, the predominant and, in some cases exclusive, reaction was with a basic and relatively low molecular weight spot ([Fig 2](#), panels 1 to 18). In all membranes, this polypeptide colocalized with the basic

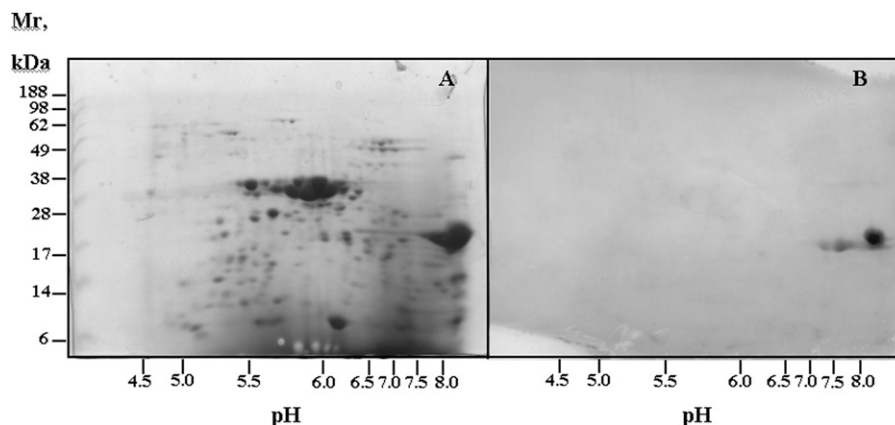


Figure 1. Two-dimensional electrophoretic reference map of sesame seed proteins under reducing conditions. A, Proteins (50 µg) were stained with SimplyBlue SafeStain. B, Blotted polyvinylidene difluoride 2-dimensional membrane revealed with anti-lupin 11S globulin basic subunit IgGs as primary antibody and peroxidase conjugate with goat anti-rabbit antiserum as secondary antibody, as detailed in the “Methods” section of the text. M_r indicates molecular weight.

11S globulin subunit of the map reported in Figure 1. However, the intensity of the reaction, with the same time exposure to secondary antibody and detection, differed significantly from one patient to another, thus suggesting either a variable specific IgE concentration or a different number of IgE-binding epitopes recognized by patients’ sera on the target molecule. Likewise, in all membranes, much less intense reactivity was visible with the 30- to 40-kDa acidic subunits of the 11S globulin family. In membranes 6 and 13, reactions with other spots, some of which aligned with the typical 7S globulin array of charge isoforms, were also detected. The membranes revealed with sera from healthy individuals did not show any significant staining (not shown). Pretreatment of some of the patients’ sera with purified 11S sesame globulin resulted in the complete inhibition of IgE binding to the 11S globulin spots, thus leaving membranes practically unstained (Fig 2, insets b), and confirming the specificity of the immunoresponses. To better define the antibody pattern in sesame-allergic patients, we evaluated the IgG4-mediated immunoreactivity against the sesame proteins separated in 2-D electrophoresis. No positive reaction was shown in the 4 patients tested (Fig 2, insets a). This is in line with the European Academy of Allergy and Clinical Immunology Task Force Report¹⁸ concluding that the presence of specific IgG4 should not be considered a hypersensitivity-inducing factor but rather an indicator for immunologic tolerance linked to the activity of regulatory T cells.

When the 2-D gel was run under nonreducing conditions and a total protein stain was used, the spots corresponding to the 11S globulin acidic and basic polypeptides disappeared, giving rise to a group of spots with a molecular weight of approximately 60 kDa and isoelectric point values intermediate between 6.0 and 8.0. These spots corresponded to the nonreduced 11S globulin protomer (Fig 3A, circled spots). In the same figure, some membranes run under nonreducing conditions and revealed with the most reactive sera are also shown. However, under these conditions, only very weak to

null reactions were visible in the map region corresponding to the whole 11S protomer. Reduced accessibility of some linear epitopes on the basic subunit likely prevented the recognition of this band, as discussed in the “Discussion” section.

DISCUSSION

In this study, a specific strong reaction of the sesame 11S globulin basic subunit in all 18 patients’ sera tested was recorded. However, this subunit becomes accessible to antibodies only on disulphide bond cleavage, as it is clear by comparing the corresponding profiles in Figures 2 and 3 where the gels are run under reducing and nonreducing conditions, respectively. This finding is consistent with the known structural feature that the basic subunit is buried in the native protein structure, as highlighted by the 3-D structure of crystalline soybean glycinin, the best characterized 11S globulin,¹⁹ and by a variety of other indirect findings.²⁰ For the same reason, the basic subunits are less accessible to endogenous and exogenous proteolytic enzymes than are the acidic subunits.²¹ This may explain the specific reactivity of the basic subunit, which, by transiting the gastrointestinal tract without major cleavage events, may trigger remarkable immunologic responses, as already shown for other legume seed 11S globulin basic subunits in previous studies.^{13,15}

In a previous study,⁷ 2-D electrophoresis was used to identify the main sesame seed allergenic proteins. However, all detected proteins belonged to protein families others than the 11S globulin, the main sesame seed protein revealed as a key allergen in the present work. Further evidence of major sesame allergens other than those detected in that and the present work is also available.⁸ However, in these cited studies, *in vitro* IgE reactivities were analyzed under conditions that prevented full exposure of acidic and basic 11S globulin polypeptide chains, thus likely preventing their recognition, as discussed previously herein. Only recently, 2 new sesame seed allergens belonging to 11S globulins were identified by using recombinant proteins and were classified

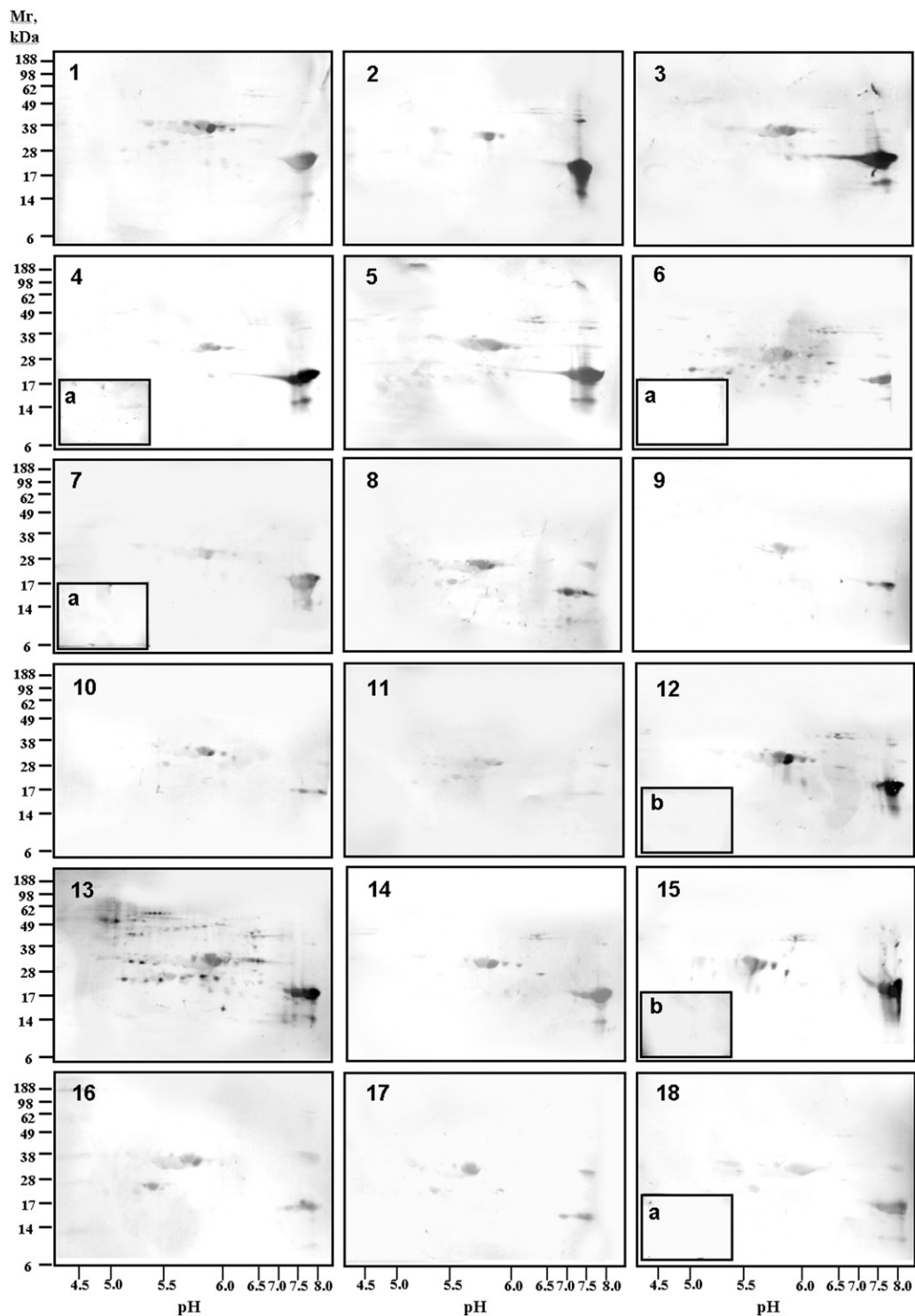


Figure 2. Two-dimensional blotted polyvinyl difluoride membranes of sesame seed proteins under the same conditions as in Figure 1 immunodetected with the sesame-sensitive patients' IgEs and revealed with phosphatase-conjugated goat anti-IgE polyclonal antibodies. Numbers in each panel refer to patients' sera. Insets "a": immunodetections with IgG4; insets "b": inhibition assays with sesame 11S globulin. M_r indicates molecular weight.

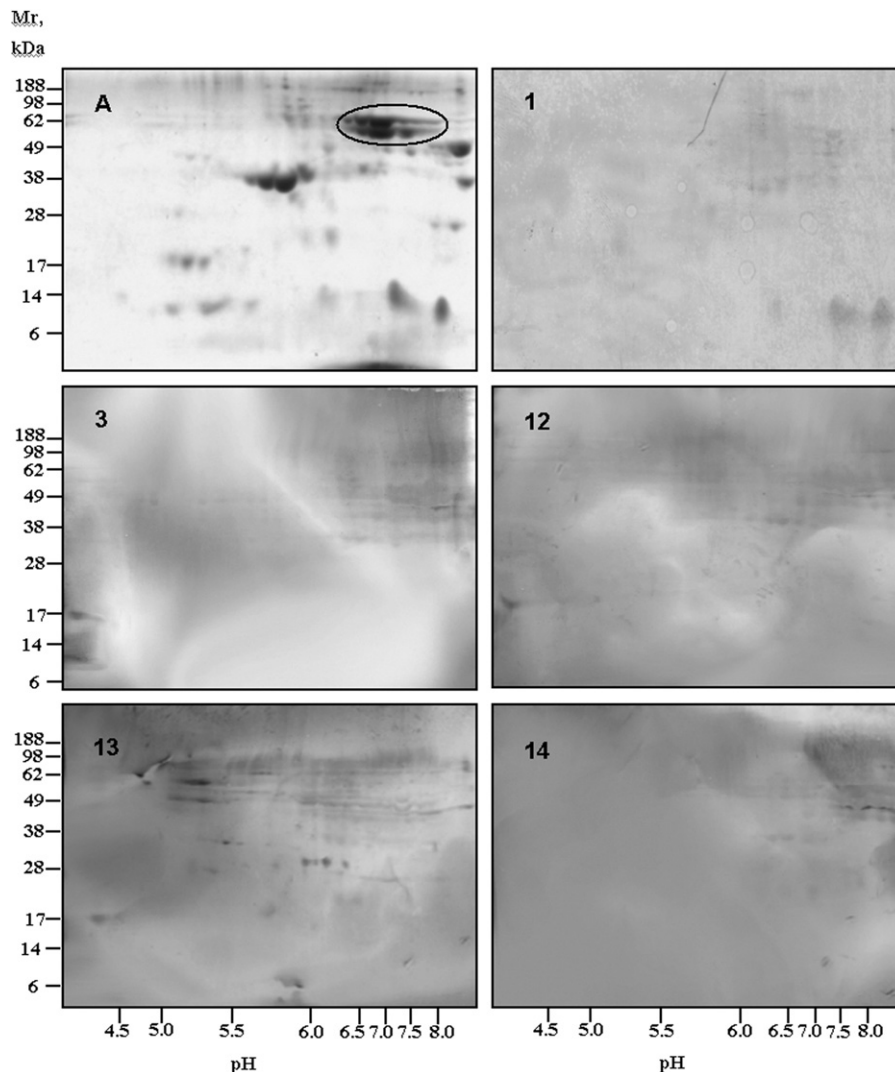


Figure 3. Two-dimensional electrophoretic maps of sesame seed proteins (50 μ g) under nonreducing conditions. A, SimplyBlue SafeStain–stained sesame seed proteins. The 11S globulin spots are circled on the map. The other panels, numbered according to patients' sera, show the blotted polyvinyl difluoride membranes immunodetected with the sesame-sensitive patients' IgEs and revealed with phosphatase-conjugated goat anti-IgE polyclonal antibodies. M_r indicates molecular weight.

as Ses i 6 and Ses i 7.¹⁰ In that study, however, only approximately half of the patients reacted with the recombinant proteins, whereas in the present case, all the patients' sera showed a positive reaction with the 2 11S globulin chains. It seems that the recombinant proteins are not proteolytically processed to give rise to the 2 separate chains in contrast to the natural protein, and, thus, the reactivity of the recombinant polypeptides may be comparably lower than that of the native 11S globulin–reduced subunits.

All the present findings support the conclusion that the reactivity of the sesame seed 11S globulin could frequently be underestimated and that the acidic and, mostly, basic subunits of this protein may actually represent the major sesame seed allergens, at least in the present cohort. Notably,

the basic subunits of 11S globulins had already been identified as potential major allergens in various legume species, including peanut, lupin, and soybean.^{13,15}

The results presented in this work confirm that the 2-D electrophoretic and immunoblotting approach is an excellent tool for the detection of reactive protein species in sensitized individuals. By applying this technique, specific reactivities that may be mixed up with comigrating bands, as occurs with 1-D approaches, or hindered by residual native conformation, as is the case in the present work, can be revealed.

In the present study, a specific strong reaction of the 11S globulin basic subunits in all 18 patients' sera tested has been recorded. These results also confirm previous findings^{13,15} on the reactivity of this relatively neglected polypeptide chain in

various legume species, such as peanut, soybean, and lupin, at least in our patient populations and under in vitro testing.

Moreover, this experimental approach proved to be useful for the noninvasive screening of specific reactivities in sensitized patients because it allows the identification of specific polypeptides that may act as allergens in a complex plant protein matrix, thus allowing us to get useful indications on the specific potential reactivity of each patient. According to this and previous studies, it could tentatively be suggested that in the Italian allergic population, the basic subunit of the globulin 11S plays a critical role in the sensitization of individuals experiencing clinical reactions, including anaphylactic shock, to sesame seeds.

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