

# Characterization of IgE Binding to Lupin, Peanut and Almond with Sera from Lupin-Allergic Patients

Lise Holden<sup>a</sup> Gaynour B.G. Sletten<sup>a</sup> Helene Lindvik<sup>b</sup> Christiane K. Fæste<sup>a</sup>  
Maaïke M.B.W. Dooper<sup>a</sup>

<sup>a</sup>National Veterinary Institute and <sup>b</sup>Voksentoppen, Rikshospitalet-Radiumhospitalet Medical Center, Oslo, Norway

## Key Words

Allergen · Allergenicity · Almond · IgE · Inhibition ELISA ·  
Lupin · Peanut

## Abstract

**Background:** The increasing number of applications of sweet lupins in food is paralleled by an increase in immunoglobulin E (IgE)-mediated allergic reactions to lupin proteins. In particular, lupin allergy seems to appear in patients with an existing peanut allergy. In the present study, IgE-binding studies towards fractionated lupin seed proteins, and peanut and almond proteins were performed using sera from patients with confirmed lupin allergy. **Methods:** Immunoblotting and indirect ELISA were performed to investigate IgE binding to protein extracts. ELISA inhibition experiments were performed to investigate the presence of cross-reactive allergens in the protein extracts. **Results:** Immunoblotting and ELISA experiments demonstrated IgE binding to all lupin conglutins ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) as well as to peanut and almond proteins, with a unique IgE-binding profile for each patient. High IgE binding to  $\alpha$ -conglutin was observed and IgE from the majority of patients similarly recognized two proteins within the  $\alpha$ -conglutin-containing fraction, 40 and 43 kDa in size. Inhibition ELISA experiments showed that preincubation of sera with lupin conglutins, peanut and almond resulted in decreased IgE binding to lupin flour. **Conclusions:** Overall, these results indicate that  $\alpha$ -,  $\beta$ -,  $\gamma$ - and

$\delta$ -conglutins are candidate allergens in lupin and suggest a particularly strong allergenicity of  $\alpha$ -conglutins. Furthermore, the results indicate the presence of cross-reactive allergens in lupin, peanut and almond.

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## Introduction

There is growing interest in industrial exploitation of alternative vegetable protein sources to soy, which may be genetically modified. Sweet lupin seeds (such as *Lupinus angustifolius* and *Lupinus albus*) are similar to soy in protein and amino acid content [1] and in this respect, lupin has been called ‘the new soy’. However, in the past 10 years, an increasing number of immunoglobulin E (IgE)-mediated allergic reactions to lupin has been reported [2–9], which parallels the increasing number of food applications of this legume. The most common clinical pattern of lupin allergy is the triggering of an allergic reaction via ingestion of lupin in peanut-allergic individuals [2–4, 6, 9], although triggering via ingestion, inhalation and occupational exposure in individuals without peanut allergy has also been reported [5, 7, 8]. The allergic reactions range from mild local reactions to systemic anaphylaxis.

An important issue, when considering lupin seeds as an alternative protein source, is the evaluation of their al-

**Table 1.** Clinical characteristics of lupin allergic patients

| Pa-tient No. | Age years | Sex | Total IgE kU/l | Symptoms following food challenge with lupin <sup>a</sup>                             | Specific IgE, kU/l |        |        | Other food allergies    |
|--------------|-----------|-----|----------------|---------------------------------------------------------------------------------------|--------------------|--------|--------|-------------------------|
|              |           |     |                |                                                                                       | lupin              | peanut | almond |                         |
| 1            | 12        | M   | 326            | itching in throat, discomfort on swallowing, swelling and urticarial rash around eyes | 6.0                | >100   | 7.5    | peanut                  |
| 2            | 16        | F   | 3,710          | itching in throat, stomach pain, nausea, listless, drowsy                             | 74.9               | >100   | 6.5    | peanut                  |
| 3            | 11        | M   | 409            | facial swelling, respiratory symptoms                                                 | 4.2                | >100   | 2.5    | peanut, fenugreek       |
| 4            | 17        | F   | 2,450          | facial and body itching, labial swelling                                              | 2.0                | 5.5    | 10.5   | peanut, hazelnut        |
| 5            | 7         | M   | 263            | rhinitis, asthma, urticaria                                                           | 3.4                | 62.2   | 1.2    | peanut                  |
| 6            | 7         | M   | 2,430          | asthma                                                                                | 21.2               | >100   | <0.35  | peanut, egg, milk, nuts |

<sup>a</sup> Patient 4 underwent double-blind, placebo-controlled food challenge, the remaining patients underwent open oral food challenge.

lergenic potential and the characterization of potential allergenic proteins. The majority of proteins in lupin seeds are comprised of  $\alpha$ - and  $\beta$ -conglutins, and to a lesser extent  $\gamma$ - and  $\delta$ -conglutins [10–12].  $\alpha$ -Conglutin belongs to the 11S globulin family of hexameric proteins, whereas  $\beta$ -conglutin and  $\gamma$ -conglutin belong to the 7S globulin family of trimeric proteins [13]. Both the 11S and the 7S globulins are members of the cupin superfamily.  $\delta$ -Conglutin belongs to the 2S albumin family and is a member of the prolamins superfamily [13]. Overall, the subunit structure of these conglutins appears to be complex and differences in the subunits in different *Lupinus* cultivars and species have been described [11, 12]. Although previous studies have been performed in which the allergenicity of lupin proteins was investigated [3, 9, 14, 15], no lupin allergens have yet been identified.

In earlier studies performed in this laboratory, anti-lupin polyclonal and monoclonal antibodies have been generated, characterized and used in the development of enzyme-linked immunosorbent assay (ELISA) methods for the detection of lupin in foods [16–18]. In the comprehensive specificity testing of the antibodies, the monoclonal antibody was found to cross-react with almond, which was a somewhat unexpected finding because these plants are phylogenetically distant.

In the present work, the allergenicity of the lupin conglutins was investigated in sera from confirmed lupin-allergic patients using immunoblotting and ELISA. The use of inhibition ELISA further allowed the identification of novel putative allergens in lupin. Finally, the effect of processing on allergenicity and IgE cross-reactivity to peanut and almond was examined in these patients.

## Patients and Methods

### Patients

Six patients with positive skin prick test (SPT) and specific serum IgE to lupin flour ( $\geq 2$  kU/l, ImmunoCAP 100; Phadia, Uppsala, Sweden) were recruited at Voksentoppen, Department of Pediatrics, Rikshospitalet-Radiumhospitalet Medical Center, Oslo, Norway. All patients reacted positively to an open oral or double-blind, placebo-controlled food challenge with lupin-containing pancakes or muffins. In the following, the patients are referred to as P1–P6, and their clinical characteristics and specific serum IgE values for lupin, peanut and almond are given in table 1. All patients had a coexisting peanut allergy. These diagnoses were based on anamnesis, positive SPTs and high IgE levels to peanut. In addition, 1 patient (P5) reacted positively to peanut in an open oral food challenge. SPTs with almond were performed in 4 patients. P2 and P6 were positive, while P3 and P5 were negative. Two atopic patients without lupin and peanut allergy and two non-atopics were used as negative controls. Blood was obtained 2 weeks after challenge. Informed consent was obtained from all patients before they were included in the study, which was approved by the Regional Committee for Medical Research Ethics, Southern Norway.

### Materials

Native proteins from *L. angustifolius* seeds, in the form of lupin flour, were kindly provided by Soja Austria (Lupipan; Vienna, Austria). Processed proteins from *L. albus* seeds, in the form of a tofu-like product (Lopino; Lupina, Visbek, Germany) were purchased from a local store in Germany. This product consists of heat-treated proteins; that is, seeds were soaked and blended, and the filtrate obtained was boiled and pressed. Dry, mature *L. angustifolius* seeds were kindly provided by Frank and Miedendorp Food Products (Twello, The Netherlands). Native and roasted peanuts (*Arachis hypogaea*, variety *Jumbo runners*) were a gift from a Norwegian manufacturer. Native almond (*Prunus dulcis*) was obtained from a local store. Human serum albumin (Calbiochem, La Jolla, Calif., USA) and  $\beta$ -lactoglobulin (Sigma-Aldrich, Steinheim, Germany) were used as negative control proteins.

### Protein Extractions and Lupin Protein Fractions

All protein extracts, if not otherwise stated, were prepared as described previously [17]. In brief, homogenized samples (2 g) were extracted with 10 ml of 0.1 M Tris-0.5 M glycine, pH 8.7, overnight at 45°C, and centrifuged at 39,200 g for 25 min at 4°C. Protein extracts were stored at -20°C. Procedures for the isolation of globulins and the generation of protein fractions containing  $\alpha$ - or  $\beta$ -conglutins (indicated as  $\alpha$ - or  $\beta$ -conglutins in the text) have been described previously [16]. For the isolation of  $\gamma$ -conglutin, *L. angustifolius* seeds were soaked in deionized H<sub>2</sub>O for 16 h and heated at 60°C for 4 h. Precipitated  $\gamma$ -conglutin was obtained by centrifugation and dissolved in 50 mM Tris buffer, pH 7.5.  $\delta$ -Conglutin was principally isolated as described elsewhere [19]. In short, defatted lupin flour was extracted in deionized H<sub>2</sub>O, pH 8.0–8.5, and centrifuged, and the proteins in the supernatant were precipitated by lowering the pH to 4.5. After centrifugation, the pellet (containing  $\alpha$ -,  $\beta$ - and  $\delta$ -conglutins) was resuspended in a 0.1 M calcium carbonate buffer, pH 4.0, containing 0.4 M sodium chloride and 40% ethanol.  $\alpha$ - and  $\beta$ -conglutins were removed by centrifugation and  $\delta$ -conglutins, present in the supernatant, were obtained by cold precipitation. Total protein concentrations were determined according to the Lowry method (DC protein assay; Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (BSA) as a standard.

### Electrophoresis and Immunoblotting

Protein extracts (5–10  $\mu$ g) were separated by SDS-PAGE under reducing conditions using 4–12% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, Calif., USA). Gels were either stained with SimplyBlue Safestain (Invitrogen) or the separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 0.05% Triton X-100 and 3% BSA for 1 h, following incubation with patient serum (diluted 1:20) overnight at 4°C. For IgE detection, membranes were first incubated with rabbit anti-human IgE (1:6,000, DakoCytomation, Glostrup, Denmark) and then with goat anti-rabbit IgG horseradish peroxidase conjugate (1:5,000, DakoCytomation), each for 1 h. IgE binding was finally revealed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Zymed, San Francisco, Calif., USA). All washing and incubation steps were performed at room temperature with gentle shaking, if not otherwise stated.

### Serum IgE Indirect ELISA

High-binding 96-well microplates (Corning, Corning, N.Y., USA) were coated overnight at 4°C with 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich), containing 4  $\mu$ g/ml protein extract (100  $\mu$ l/well). Plates were washed with phosphate-buffered saline (PBS), pH 7.6, containing 0.05% Tween-20 (PBS-T) and blocked with 1% BSA in PBS-T (200  $\mu$ l/well). Test and control sera, diluted with 1% BSA in PBS-T (1:20, 100  $\mu$ l/well), were incubated for 1 h at room temperature under gentle shaking. For IgE detection, wells were incubated with rabbit anti-human IgE (1:15,000, 100  $\mu$ l/well, DakoCytomation) followed by incubation with goat anti-rabbit IgG horseradish peroxidase conjugate (1:10,000, 100  $\mu$ l/well, Zymed). Binding was finally revealed with K-Blue TMB substrate solution (75  $\mu$ l/well, Neogen, Lexington, Ky., USA), the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> (50  $\mu$ l/well) and optical density (OD) was read at 450 nm.

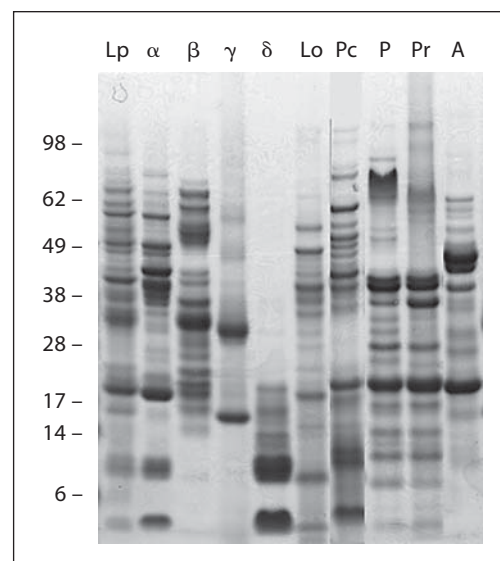


**Fig. 1.** SDS-PAGE of protein extracts used in the present study. Lp = Lupin flour (Lupinan);  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  =  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -conglutin; Lo = highly processed lupin proteins (Lopino); Pc = moderately processed lupin proteins (pancake); P = raw peanut; Pr = roasted peanut; A = almond. Protein sizes (in kDa) are indicated on the left.

### Inhibition ELISA

Only protein extracts giving an OD 450 nm reading  $\geq 0.2$  in the indirect ELISA were used in the inhibition assay. Inhibition experiments were performed by coating 96-well microplates with 4  $\mu$ g/ml lupin flour extract and blocking as described above. Diluted sera were preincubated with protein extracts at a final concentration of 0.2, 1, 5, 25, 125 and 625  $\mu$ g/ml for 2 h at room temperature under gentle shaking. Subsequently, the inhibitor mixtures (including sera with no inhibitor as zero-inhibition control) were transferred to coated and blocked wells (100  $\mu$ l/well) and incubated for 1 h. The assay was completed as described above. Percent inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 100 - (\text{OD}_{450} \text{ of serum with inhibitor} / \text{OD}_{450} \text{ of serum without inhibitor}) \times 100.$$

The concentration required for 50% inhibition of IgE binding (IC<sub>50</sub>) was also calculated.

## Results

### Composition of Protein Extracts

In order to examine the protein composition of extracts from lupin, peanut and almond used in this study, SDS-PAGE was performed and the proteins were stained with Coomassie blue (fig. 1). In the lupin flour and processed lupin protein extracts, components with molecular weights from 4 to 80 kDa were present. A decrease in

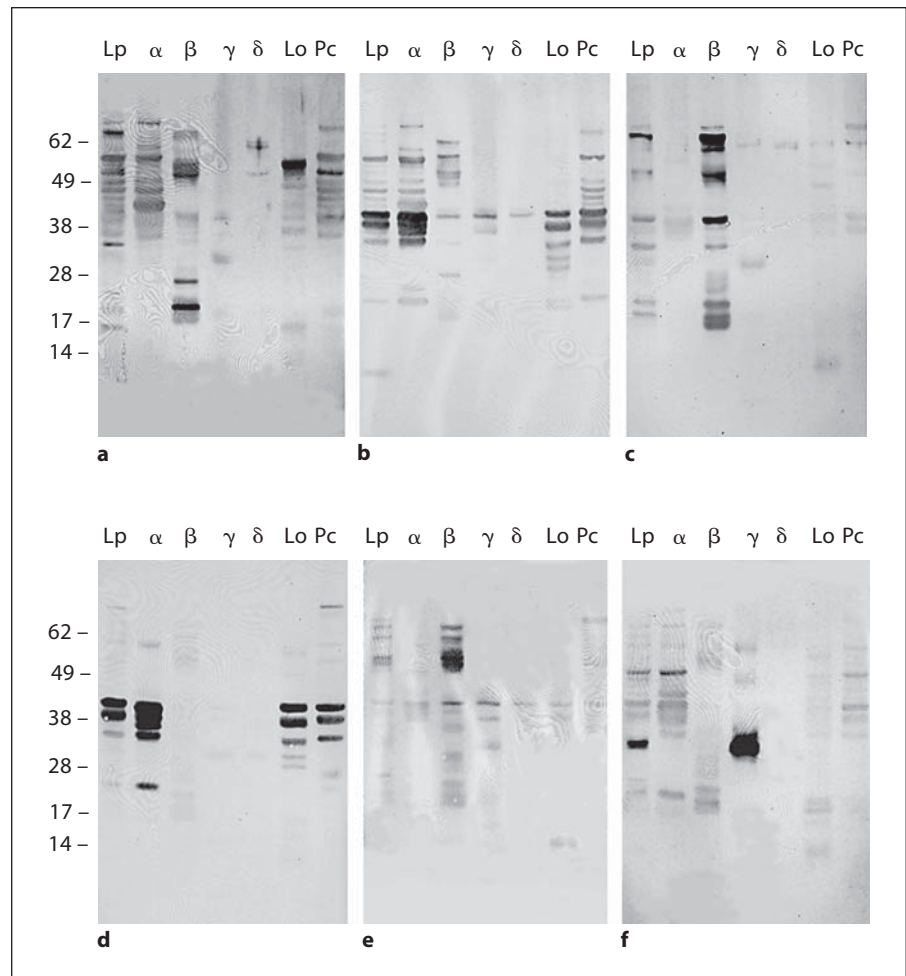


**Fig. 2.** Immunoblot analysis for IgE binding to lupin allergens, using serum from lupin-allergic patients P1–P6 (**a–f**, respectively; see table 1). Protein sizes (in kDa) are indicated on the left side of the blots. Lp = Lupin flour (Lupipan);  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  =  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -conglutinin; Lo = highly processed lupin proteins (Lopino); Pc = moderately processed lupin proteins (pancake).

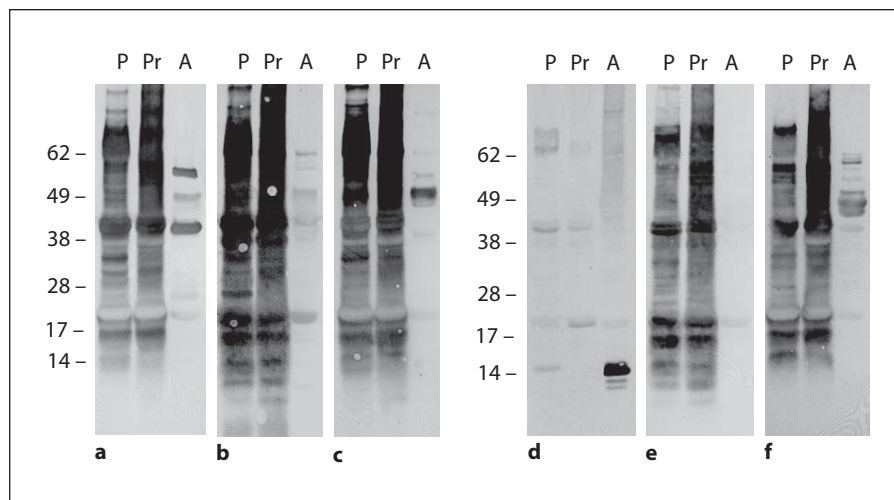
intensities of protein bands above 49 kDa and between 16 and 38 kDa were seen in the processed lupin extracts compared to the untreated lupin flour extract. In the raw and processed peanut extracts, proteins with molecular weights between 4 and 98 kDa were observed. The number and intensity of protein bands >49 kDa and between 28 and 34 kDa were less in the roasted peanut extract compared to the raw extract. However, a protein band at 36 kDa was more intense in the roasted than in the raw peanut extract, in addition to a protein band of approximately 98 kDa that appeared in the roasted peanut extract.

#### *Identification of IgE-Binding Proteins with Immunoblotting*

In order to investigate IgE binding to lupin allergens, immunoblotting with lupin flour, conglutins and processed lupin proteins was performed with sera from 6 lu-

pin-allergic patients (table 1, fig. 2). Several IgE-binding protein bands were detected in the range from 10 to 80 kDa. Although the IgE-binding patterns differed somewhat between the patients, two bands of approximately 40 and 43 kDa were recognized in lupin flour by 5 patients (P1, P2, and P4–P6). The specificity towards the different conglutins varied among the individuals. P4 recognized solely  $\alpha$ -conglutinin, P3 had high IgE binding to  $\beta$ -conglutinin, and low IgE binding to  $\gamma$ -conglutinin, while P1, P2, P5 and P6 recognized  $\alpha$ -conglutinin as well as  $\beta$ - and  $\gamma$ -conglutinins. Of these, P1 had similar IgE binding to  $\alpha$ - and  $\beta$ -conglutinins, P2 had highest IgE binding to  $\alpha$ -conglutinin and P5 had highest IgE binding to  $\beta$ -conglutinin. P6 demonstrated a remarkably high IgE binding towards  $\gamma$ -conglutinin, compared to the others. Two common proteins of approximately 40 and 43 kDa were recognized in  $\alpha$ -conglutinin by P1, P2, P4 and P6. No IgE binding towards  $\delta$ -conglutinin was observed in the immunoblot analyses.

**Fig. 3.** Immunoblot analysis for IgE binding to peanut and almond allergens, using serum from lupin-allergic patients P1–P6 (a–f, respectively; see table 1). Protein sizes (in kDa) are indicated on the left side of the blots. P = Native peanut; Pr = roasted peanut; A = almond.



The effects of processing on the allergenicity of lupin proteins were investigated by comparing IgE-binding to extracts from lupin flour (unprocessed), a lupin flour-containing pancake (moderately processed) and Lopino (highly processed). The pancake was included as it was used in the clinical food provocation tests of the patients. Only P4 had similar IgE-binding patterns to native and processed lupin, whereas the remaining patients had different binding patterns to proteins in these extracts. Generally, fewer and less intense IgE-binding bands were seen in the processed extracts, especially the highly processed Lopino extract.

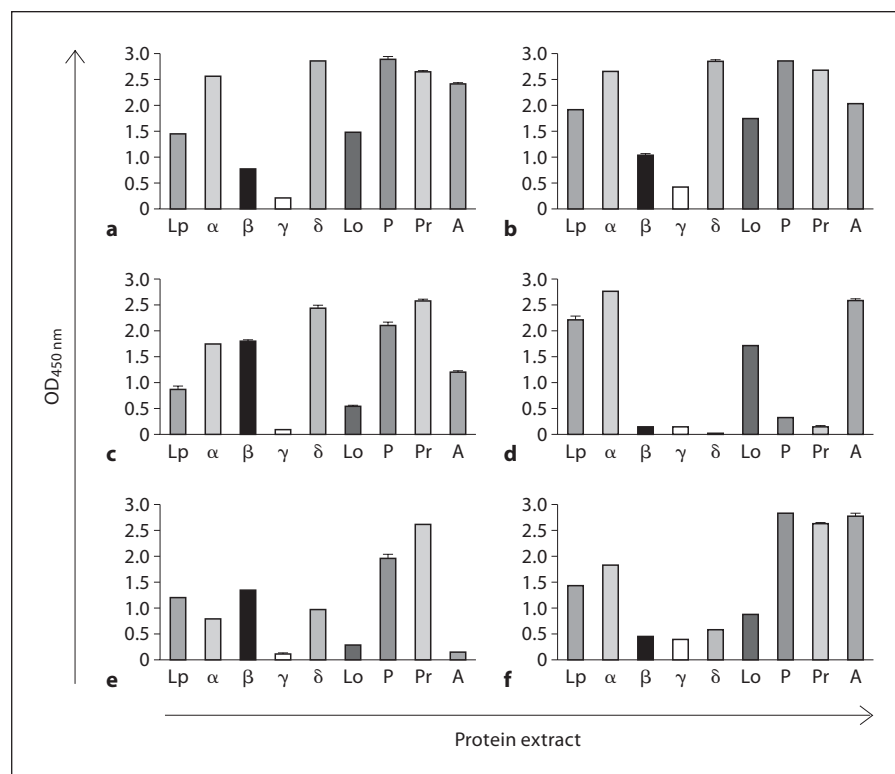
Immunoblots of raw and roasted peanuts were performed. Sera from 5 of the 6 patients (P1, P2, P3, P5 and P6) exhibited numerous IgE-binding protein bands (fig. 3). The few observable differences seen in the IgE profiles between raw and roasted peanut extracts were similar to those revealed in the pattern of the Coomassie-stained proteins. In addition, IgE cross-reactivity to almond was investigated and 5 of the lupin-allergic patients had serum IgE that recognized almond proteins (P1–P4 and P6). The IgE-binding patterns and the intensity of detected bands in almond were distinctive for each patient. Notably, serum IgE from P4 bound to only one almond protein, while the remaining sera bound to several proteins. None of the patients recognized the control protein  $\beta$ -lactoglobulin (data not shown). Furthermore, the negative control sera did not show any IgE binding to the protein extracts used in this study (data not shown).

#### *Indirect ELISA for Serum IgE Responses to Lupin, Peanut and Almond*

Indirect ELISA was performed to confirm the IgE reactivity and to screen the patient sera against protein extracts for further use in inhibition ELISA (fig. 4). The IgE-binding reactivity towards the individual congenitins varied among the 6 lupin-allergic patients, as previously observed with immunoblotting. P4 showed high IgE binding exclusively to  $\alpha$ -conglutin, while the other patients demonstrated reactivity to more than one conglutin. P1 and P2 had higher IgE binding to  $\alpha$ - and  $\delta$ -conglutin than to  $\beta$ -conglutin, and weak binding to  $\gamma$ -conglutin. P3 and P5 had slightly higher IgE binding to  $\beta$ -conglutin than to  $\alpha$ - and  $\delta$ -conglutin, while P6 had higher IgE binding to  $\alpha$ -conglutin than for  $\beta$ -,  $\gamma$ - and  $\delta$ -conglutin. Overall, the indirect ELISA results confirmed the immunoblot analyses, although the IgE binding to individual congenitins differed somewhat between the two analyses. However, reactivity towards  $\delta$ -conglutin was only demonstrated by indirect ELISA. Furthermore, the strong IgE binding of P6 towards  $\gamma$ -conglutin seen in the immunoblot analysis was not observed in the indirect ELISA analysis.

All patients had similar or slightly higher IgE binding to lupin flour than for processed lupin proteins in the indirect ELISA. In addition, high IgE binding to raw and roasted peanut were demonstrated in 5 patients (P1–P3, P5 and P6). P1, P2 and P6 had similar IgE binding to raw and roasted peanuts, while P3 and P5 had slightly higher IgE binding to roasted peanuts. Furthermore, 5 of the 6 patients (P1–P4 and P6) demonstrated IgE cross-reactivity to almond in the indirect ELISA, thus confirming the

**Fig. 4.** Indirect ELISA of specific IgE reactivity to lupin, peanut and almond, using serum from lupin-allergic patients P1–P6 (a–f, respectively; see table 1). Lp = Lupin flour (Lupipan);  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  =  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -conglutin; Lo = highly processed lupin proteins (Lopino); P = native peanut; Pr = roasted peanut; A = almond. The mean values for triplicates are shown and the standard error of the mean is indicated by error bars.



immunoblot analyses. None of the sera reacted towards the control protein human serum albumin. No binding to any protein extract used in this study was seen in sera from atopic and non-atopic control individuals (data not shown).

#### Serum IgE Inhibition ELISA

For further investigation of the presence of cross-reactive allergens in the protein extracts, inhibition ELISA was performed (fig. 5), in which the capacity of lupin flour, lupin conglutins, native peanut and almond extracts to inhibit IgE binding to immobilized lupin flour extract was examined. Patient sera demonstrating an OD reading  $\leq 0.2$  (450 nm) towards a protein extract in the indirect ELISA were considered negative to this protein and these extracts were therefore not studied in the inhibition assay. Dose-dependent inhibition by lupin and/or peanut extracts (positive controls) was seen in all patient sera, while the negative control extract ( $\beta$ -lactoglobulin) did not inhibit IgE binding to lupin flour extract (data not shown). The efficiency of inhibition by the individual conglutins varied between patients. Strikingly,  $\alpha$ -conglutin strongly inhibited IgE binding to lupin flour in all patient sera, while  $\beta$ -conglutin was a strong inhibitor in

2 of the 6 patient sera (P3 and P5). In 2 patient sera (P1 and P6),  $\gamma$ -conglutin was the second most potent inhibitor, although higher concentrations than for  $\alpha$ -conglutin were needed to give similar inhibitions. Interestingly, almond inhibited IgE binding to lupin flour in all patient sera analyzed.

The variations in the degree of inhibition observed with the protein extracts were further demonstrated by determining the  $IC_{50}$  of IgE binding to lupin flour extract (table 2). Compared to peanut and almond, lupin had the lowest  $IC_{50}$  (i.e. the strongest inhibitory effect) in 3 of the 6 patients (P3, P5 and P6). Peanut had the lowest  $IC_{50}$  in 2 patients (P1 and P2) and, interestingly, almond was the most potent inhibitor for 1 patient (P4). These low  $IC_{50}$

**Fig. 5.** ELISA for inhibition of IgE binding to lupin flour by various protein extracts. Potential inhibitors include lupin flour (Lp),  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -conglutin ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , respectively), peanut (P) and almond (A). Inhibition of IgE binding was assessed using serum from lupin-allergic patients P1–P6 (a–f, respectively; see table 1). The mean values for triplicates are shown and the standard error of the mean is indicated by error bars. ND = Not determined.

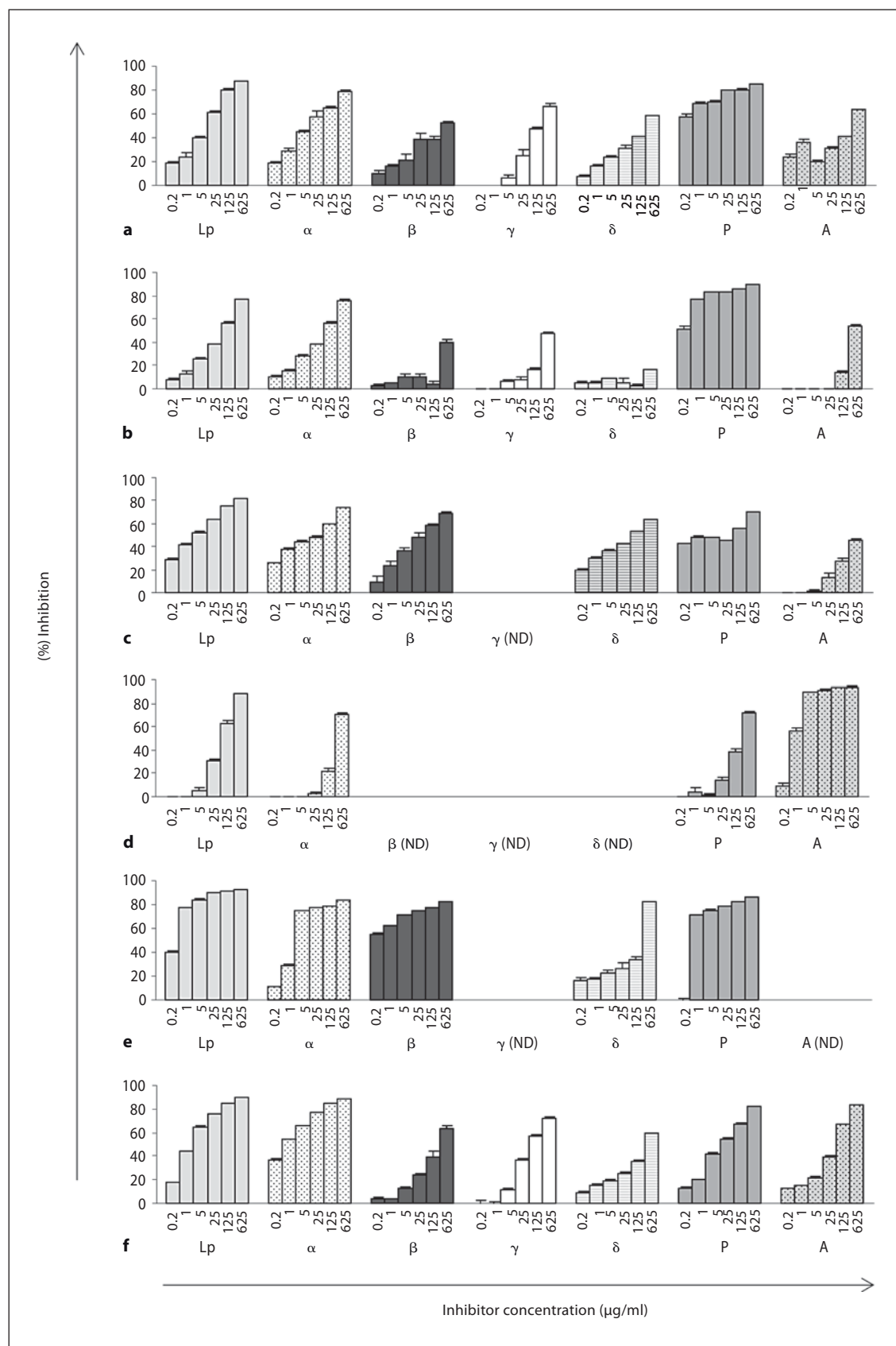


**Table 2.** Inhibitor concentration ( $\mu\text{g/ml}$ ) required for 50% inhibition of IgE binding to lupin flour

| Patient, No. | Lupin | $\alpha$ -Conglutin | $\beta$ -Conglutin | $\gamma$ -Conglutin | $\delta$ -Conglutin | Peanut            | Almond            |
|--------------|-------|---------------------|--------------------|---------------------|---------------------|-------------------|-------------------|
| 1            | 10    | 12                  | 527                | 166                 | 351                 | <0.2 <sup>a</sup> | 201               |
| 2            | 58    | 59                  | >625 <sup>b</sup>  | >625 <sup>b</sup>   | >625 <sup>b</sup>   | 0.2               | 528               |
| 3            | 3.7   | 33                  | 38                 | ND                  | 64                  | 50                | >625 <sup>b</sup> |
| 4            | 67    | 318                 | ND                 | ND                  | ND                  | 204               | 0.9               |
| 5            | 0.3   | 2.1                 | <0.2 <sup>a</sup>  | ND                  | 215                 | 0.6               | ND                |
| 6            | 1.7   | 0.8                 | 234                | 76                  | 335                 | 17                | 45                |

ND = Not determined.

<sup>a</sup> Inhibition was >50% at minimum inhibitor concentration of 0.2  $\mu\text{g/ml}$ .

<sup>b</sup> Inhibition was <50% at maximum inhibitor concentration of 625  $\mu\text{g/ml}$ .

values of lupin flour, peanut and almond ranged from <0.2 to 3.7  $\mu\text{g/ml}$ . Among the conglutins,  $\alpha$ -conglutin was the most potent inhibitor in 5 patients (P1–P4 and P6), while for the remaining patient (P5),  $\beta$ -conglutin was the strongest inhibitor. Although a 50% inhibition of IgE binding to lupin flour was also obtained with  $\gamma$ - and/or  $\delta$ -conglutins in some of the patients, these concentrations were generally higher than for lupin flour and/or peanut.

## Discussion

IgE-mediated allergic reactions against lupin have been documented, but few immunological studies have addressed specific IgE binding towards separated lupin conglutins. In the present study, 6 lupin-allergic patients, who reacted in SPTs and oral food challenge tests, were studied in detail by immunoblotting, indirect ELISA and inhibition ELISA. The majority of patients demonstrated IgE reactivity to more than one lupin conglutin. The IgE-binding profiles towards  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -conglutins were specific for each individual patient, indicating the presence of several important allergenic proteins in lupin, rather than one major allergen. Although IgE reactivity to all four conglutins was demonstrated, the reactivities towards  $\alpha$ -conglutin, and to a lesser extent  $\beta$ -conglutin, were the most prominent. Interestingly, only 1 patient was monospecific, i.e. had IgE binding solely to  $\alpha$ -conglutin.

The immunoblotting experiments revealed a large variation in the protein subunits recognized by human IgE. However, in total lupin flour extract and  $\alpha$ -conglutin, two proteins of approximately 40 and 43 kDa were detected by IgE from 5 of the 6 lupin-allergic patients. This indicates the presence of potential major allergens in  $\alpha$ -

conglutin, which is in agreement with previous reports suggesting a 43-kDa protein as a dominant allergenic protein in lupin [8, 9]. Poltroni et al. [20] suggested that this 43-kDa IgE-binding protein coincides with lupin  $\gamma$ -conglutin. The results from the present study suggest however that this protein rather corresponds with  $\alpha$ -conglutin.  $\alpha$ -Conglutins are 11S globulins, which consist of higher-molecular-weight acidic subunits and lower-molecular-weight basic subunits [13]. A predominant IgE binding towards the subunits of higher molecular weights was seen in the present study, indicating IgE binding towards the acidic subunits. This is in contrast to a previous study by Magni et al. [15], who reported a main IgE reactivity directed towards the basic subunits. Nonetheless, in general our IgE-binding studies strongly suggest the presence of two lupin  $\alpha$ -conglutin allergens that might be classified as 11S globulins. In addition to the strong IgE binding towards  $\alpha$ -conglutin, IgE binding to  $\beta$ -conglutin was observed with 5 of the 6 sera. To our knowledge, this finding has not previously been reported for human sera, although similar results were reported in a recently described mouse model for lupin [14].  $\gamma$ -Conglutin, a minor conglutin in lupin, was poorly recognized by the patients' IgE in the current study, as only 1 of the sera had high IgE binding to  $\gamma$ -conglutin. This finding is in contrast to Magni et al. [15], who described strong IgE binding to  $\gamma$ -conglutin with sera from 5 lupin-sensitized patients. The indirect ELISA confirmed the results from the immunoblotting with respect to  $\alpha$ -,  $\beta$ - and  $\gamma$ -conglutins, although the affinities towards the individual conglutins varied between the two analyses. In particular, the high IgE binding to  $\gamma$ -conglutin seen in 1 patient serum with immunoblotting was not seen in the indirect ELISA, indicating the presence of linear epitopes in  $\gamma$ -conglutin, which were exposed during protein denaturation.

The 2S albumin,  $\delta$ -conglutin, which is claimed to be a potent allergen in other seeds, did not react with any of the patients' sera in the immunoblot analyses, which is in accordance with other studies with fractionated lupin proteins [14, 15]. Unlike the results from the immunoblotting,  $\delta$ -conglutin was recognised by 5 of the 6 sera in the ELISA. In the lupin mouse model [14], similar IgE binding to  $\delta$ -conglutin was demonstrated by ELISA, but not by immunoblot. This difference could be explained by the denaturing conditions in SDS-based electrophoresis, which may destroy IgE-binding epitopes in  $\delta$ -conglutin. This suggests that  $\delta$ -conglutin may have a less stable structure than  $\alpha$ -,  $\beta$ - and  $\gamma$ -conglutin or that conformational epitopes might be involved.

In the present study, and, to our knowledge, for the first time specific IgE inhibition ELISA has been performed with different lupin conglutins. Differences in the degree of inhibition among the conglutins as shown by  $IC_{50}$  were found.  $\alpha$ -Conglutin, and to a lesser extent  $\beta$ -conglutin, inhibited IgE binding to lupin flour more potently than  $\gamma$ - and  $\delta$ -conglutins. This confirms the strong allergenicity of both  $\alpha$ -conglutin and  $\beta$ -conglutin and suggests a lower allergenicity of  $\gamma$ - and  $\delta$ -conglutins.

One of the clinical characteristics of lupin allergy, which has become apparent in the literature, is the induction of allergic reactions in peanut-allergic individuals by ingestion of lupin [2–4, 6, 9]. All patients in this study had peanut allergy in addition to lupin allergy. Inhibition assays demonstrated IgE cross-reactivity between lupin and peanut, which might suggest the presence of closely related allergens in these foods, with similarities in their IgE-binding epitopes. Alignment studies have recently described a structural homology between lupin  $\alpha$ -conglutin and peanut Ara h 3, and between lupin  $\beta$ -conglutin and peanut Ara h 1 [21, 22]. These proteins might thus be responsible for some of the reported cross-reactivities with lupin in peanut-allergic individuals. To confirm this, IgE-binding studies with purified peanut allergens (Ara h 1 and Ara h 3) can be performed with sera from the lupin-allergic patients. However, the possible existence of other lupin allergens that cross-react with peanut allergens, such as the peanut allergens Ara h 8 and the lupin pathogenesis-related protein PR-10 protein [21], cannot be excluded.

The effect of processing on the allergenicity of lupin proteins was also addressed in the present study. The majority of sera demonstrated less IgE binding to heat-treated lupin, indicating that the IgE-binding epitopes are, at least to some degree, heat labile or that the protein solubility is altered by heat treatment. This finding is in con-

trast to a previous study, which described that heat treatment of lupin seeds resulted in minimal changes in IgE recognition, except when prolonged autoclaving was applied [23]. The use of treated seeds, in contrast to the lupin-containing foods used in the current study, may explain the differences in results, because the presence of a matrix might affect the processing and thereby the allergenicity of lupin proteins [14]. In addition, in the present study IgE binding to processed lupin proteins differed among the lupin-allergic patients, implying that specific lupin allergens are differently affected by heat treatment.

In a previous study, a monoclonal antibody produced against lupin unexpectedly cross-reacted with almond [18], which indicated recognition of a well-conserved epitope or structure between lupin and almond. This prompted us to investigate IgE binding to almond in the sera from the 6 lupin-allergic patients. Interestingly, IgE binding to almond was seen in 5 of the 6 patients. ELISA inhibition experiments showed that almond inhibited IgE binding to lupin flour in all these individuals, especially P4, suggesting the presence of cross-reactive allergens in lupin and almond. Phylogenetically, lupin and almond are only distantly related. Lupin belongs to the legume family (Fabaceae), while almond belongs to the rose family (Rosaceae). These plants are, however, members of the same plant class (Magnoliopsida) and may therefore contain homologous proteins belonging to related protein families that are involved in allergic cross-reactivity (i.e. pan-allergens). Based on immunoblotting and the strong inhibition of IgE binding to lupin by almond in the inhibition ELISA with serum from P4, a possible cross-reactivity could be expected between lupin  $\alpha$ -conglutin and an almond protein approximately 14 kDa in size, for example 2S albumin or the profilin Pru du 4 [20, 24]. However, neither of these two almond proteins has, to our knowledge, been described to have homology to lupin  $\alpha$ -conglutin. Another protein in almond that possibly could cross-react with lupin  $\alpha$ -conglutin is amandin (prunin) [25]. These proteins are both 11S globulins and contain conserved  $\beta$ -barrel domains [13] that might allow IgE binding. Overall, our results may suggest a possible clinical cross-reactivity between lupin and almond, but this needs to be investigated further by oral provocation tests in almond- and lupin-allergic patients.

In summary, IgE-binding studies using serum from confirmed lupin-allergic patients show that  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -conglutins are candidate allergens in lupin and indicate the presence of cross-reactive allergens in lupin, peanut and almond. Although differences in the patients' IgE

binding in the lupin conglutins were seen, a prominent reactivity to  $\alpha$ -conglutins and to a lesser extent  $\beta$ -conglutins was observed throughout the study. However, IgE binding and serological reactivity of the conglutins are not proof of clinical allergy. Evaluation of clinical reactivity of these proteins should be confirmed by open or double-blind food challenges with purified allergens.

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