

Monoclonal Antibodies against the Candidate Lupin Allergens α -Conglutin and β -Conglutin

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Key Words

Conglutins • Enzyme-linked immunosorbent assay •
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Abstract

Background: The ingestion of dietary products containing sweet lupin (such as *Lupinus albus* or *Lupinus angustifolius*) has been reported to cause IgE-mediated allergic reactions. Recent studies have indicated lupin globulins as important IgE binding proteins. The aim of the present study was to generate and characterize monoclonal antibodies (mAbs) against lupin seed proteins. **Methods:** Mice were immunized with a protein isolate from *L. albus* and mAbs were obtained by hybridoma techniques. Albumins and globulins were extracted, and the globulin fraction was separated further into conglutins by anion exchange chromatography. Specificities, binding patterns and applications of the mAbs were investigated by immunochemical methods. **Results:** Five mAbs were produced: Lu11 (an IgG2b antibody), Lu8, Lu18, Lu34 and Lu35 (all IgM antibodies). The mAbs reacted strongly with protein isolates from both *L. albus* and *L. angustifolius*. All mAbs are directed towards the lupin globulin fraction; Lu11 and Lu18 recognize α -conglutin, while Lu8, Lu34 and

Lu35 recognize β -conglutin. In addition, Lu11 inhibited the binding of IgE from patients with positive skin prick tests to lupin proteins in a competitive ELISA by approximately 30%. Furthermore, preliminary results show that Lu11 can be used to develop a sensitive method for the detection of α -conglutin in foods. **Conclusions:** Lupin globulins are immunogenic and α -conglutin is a potential allergen. This is the first study describing mAbs against the candidate lupin allergens, emphasizing the importance of additional studies on conglutins in lupin allergy.

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Introduction

Sweet lupins, such as the white lupin (*Lupinus albus*) and the blue lupin (*Lupinus angustifolius*), are widely cultivated legumes. The use of lupins in food products has increased notably in many European countries during the past decade. Like peanuts, soy, peas, beans and lentils, lupins belong to the Leguminosae plant family. Because of their high protein content and uncomplicated cultivation, lupin seeds provide a suitable substitute for the use of proteins from other sources such as soybeans, and offer a way to circumvent the use of genetically modified food products [1]. Lupin flour is used in bread, pastry, biscuits,

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pasta, sauces and dietary products sold as milk and soy substitutes [2, 3]. The seeds are consumed as a snack food termed lupini (i.e. boiled and dried seeds). With the growing use of lupin proteins in dietary products, an increase in allergic reactions against lupins mediated by immunoglobulin (Ig) E has been reported. The allergic responses range from urticaria and angioedema [4], contact urticaria [5], oral allergy syndrome [6] and rhinoconjunctivitis [7, 8] to anaphylaxis [9, 10]. Lupin allergy apparently arises either by primary sensitization [7, 11] or by clinical cross-reactivity in persons who are allergic to peanut [12].

Seed storage proteins have been characterized as major allergens in several legume seeds and tree nuts [13–17]. In individuals with lupin allergy, globulins have been suggested to be important allergens [8, 18]. As in other legumes, globulins, and to a lesser extent albumins, represent the majority of storage proteins found in lupin seeds [19]. Lupin globulins consist of 2 major globulins named α -conglutin (11S and 'legumin-like') and β -conglutin (7S and 'vicilin-like'), and an additional globulin of minor quantity called γ -conglutin [20, 21]. Both α - and β -conglutin, as well as γ -conglutin, have been suggested as candidate allergens in lupins [8, 22, 23]. Mature 11S and 7S globulins are hexamers and trimers, respectively. However, the subunit structure of these conglutins appears to be complex and differences in the subunits in different *Lupinus* cultivars and species have been described [21, 24]. In *L. angustifolius*, α -conglutin was found to consist of 4 subunits which are non-covalently linked. The subunits range in size from 55 to 80 kDa and contain a disulphide-bound moiety of 20 kDa. Upon reduction, the 20-kDa moiety detaches, and the 4 larger subunits are split into polypeptides of 36–63 kDa. β -Conglutin contains 4 major non-covalently-linked subunits, as well as various minor components, which range in molecular weight between 20 and 60 kDa. The subunit proteins are not disulphide linked, which explains why β -conglutin patterns are identical before and after reduction. γ -Conglutin contains monomers of approximately 40 kDa, which, upon reduction, are split into 2 polypeptides of 30 and 17 kDa [21, 24, 25].

Until now, limited information has been available on the antibody-binding characteristics and allergenicity of the different lupin seed storage proteins. In this respect, monoclonal antibodies (mAbs) are useful tools to study antibody-antigen interactions. Therefore, in the present study mAbs to lupin proteins were generated, and their specificities towards different lupin seed fractions as well as their possible applications are described.

Materials and Methods

Materials

Processed proteins from *L. albus* seeds (Lopino®: Lupina, Visbek, Germany) were purchased from a local store in Germany. This product consists of mainly heat-treated proteins, i.e. seeds were soaked, blended and the filtrate was boiled and pressed into a tofu-like product. Lupin flour, milled from *L. angustifolius* seeds, was kindly provided by Soja Austria (Lupipan®: Vienna, Austria). Dry, mature *L. angustifolius* seeds were kindly provided by Frank & Miedendorp Food Products (Twello, The Netherlands). Hazelnut (*Corylus avellana*), white bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), peanut (*Arachis hypogaea*), common pea (*Pisum sativum*), soy (*Glycine max*) and lentil (*Lens culinaris*) were all obtained from local stores in Norway.

Protein Extractions

Protein extracts from processed lupin proteins, used for immunizations, boosters, enzyme-linked immunosorbent assays (ELISAs) and immunoblots were prepared as described before [26]. In brief, processed lupin proteins were resuspended by vortexing, extracted overnight at 45°C in 100 mM Tris-glycine/1 mM dithioerythritol (Sigma-Aldrich, St. Louis, Mo., USA) and centrifuged. Proteins in the supernatant were precipitated using 65% ammonium sulphate, centrifuged and resuspended in 50 mM Tris/1 mM dithioerythritol, pH 8.0. Finally, the extract was dialysed against water and concentrated by freeze drying. The protein extract was stored at –20°C.

Protein extracts from lupin flour, hazelnut, white bean, chickpea, peanut, common pea, soy and lentil were prepared as described previously [27]. In brief, samples were extracted with 0.1 M Tris/0.5 M glycine (pH 8.7, 1:5 w/v) overnight at 45°C, and centrifuged at 39,200 g for 25 min at 4°C. Fat and larger particles from the matrix were removed by filtration through glass wool. The protein extracts were stored at –20°C or 4°C.

Protein Measurements

Protein concentrations were measured according to the Lowry dye-binding assay (DC Protein Assay: Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (BSA; Sigma-Aldrich) as a standard [28].

Preparation of mAbs

Three 6-week-old female BALB/c mice were kept in a minimal disease unit. The mice were injected intraperitoneally with 100 µg of the processed lupin protein extract in the presence of aluminium sulphate (Pierce Biotechnology, Rockford, Ill., USA) in a total volume of 100 µl. Two weeks after immunization, the mice received an intraperitoneal booster injection of 50 µg of the processed lupin protein extract dissolved in phosphate-buffered saline (PBS). On the 11th day after the booster, the mice were bled by cardiac puncture under anaesthesia (1:1 hypnorm:dormicum mixture subcutaneously administered) and sacrificed by cervical dislocation. Spleens were removed under sterile conditions and splenocytes were obtained using a 0.7-µm nylon cell strainer (BD Biosciences, Erembodegem-Aalst, Belgium). Spleen cells were fused with mouse plasmacytoma cells [29] (OURI; available at our laboratory) using polyethylene glycol 1500 (Roche, Penzberg, Germany) according to the literature [30], and cultured in RPMI 1640 containing 10% fetal bovine serum and 100 U/ml penicil-

lin/streptomycin (all obtained from Life Technologies, Paisley, UK) supplemented with hypoxanthine-aminopterin-thymidine (Sigma-Aldrich). After 7 days, the medium was replaced by a culture medium containing hypoxanthine-thymidine (Sigma-Aldrich). Ten days after the fusion, cell culture supernatants were screened for anti-lupin-specific antibodies by indirect ELISA, as described below. Clones were obtained by limited dilutions.

The animal study was approved by the Committee for Experiments on Animals, Rikshospitalet University Hospital, Oslo, Norway.

Antibody Isotyping and Purification of mAbs

The isotype of the mAbs was determined by indirect ELISA as described below, using anti-mouse IgG or IgM alkaline phosphatase (AP) conjugates (Sigma-Aldrich) and dinitrophenol phosphate substrate (Sigma-Aldrich), or biotinylated anti-mouse IgG1, IgG2a and IgG2b antibodies (BD Biosciences Pharmingen, San Diego, Calif., USA), extravidin AP (Sigma-Aldrich) and dinitrophenol phosphate substrate (Sigma-Aldrich).

For the purification of mAbs, clone supernatants were centrifuged at 1,500 g for 30 min and filtered through a 0.45- μ m filter (Sarstedt, Numbrecht, Germany). The purification of murine IgG mAbs was performed using a 2-ml protein A column (PROSEP®-A, Bioprocessing Ltd., Durham, UK) according to the manufacturer's recommendations.

Production of Polyclonal Anti-Lupin Antibodies

A polyclonal antiserum was raised against the processed lupin protein extract in a rabbit as previously described [26]. Antibody purification was performed as previously described [27] and the antibodies were stored at -80°C until use.

Isolation of Lupin Flour Globulins, Albumins and Prolamins

The extraction of total albumins and globulins was performed according to a modified protocol of two published procedures [21, 31]. In brief, lupin flour was defatted with n-hexane (35 ml/g of dry weight) under vigorous vortexing for 10 min at room temperature (RT), followed by centrifugation at 6,000 g for 10 min at RT. The supernatant was removed and the defatted lupin flour was dried in air. The albumin fraction of the proteins was extracted with water (adjusted to pH 8.0) containing 10 mM CaCl₂ and 10 mM MgCl₂ (34 ml/g) under vigorous vortexing for 10 min at RT. The suspension was centrifuged at 30,000 g for 15 min at 4°C. The supernatant, which contained the albumins, was collected, dialyzed against PBS (pH 7.6) and stored at 4°C until use. In order to minimize the residual amounts of albumin present in the pellet, a second albumin extraction was performed, and the supernatant was discharged. The globulin fraction was extracted from the pellet with 100 mM Tris-HCl (pH 7.5) containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 34 ml/g), by vigorous vortexing for 10 min at RT. The globulin-containing solution was centrifuged at 30,000 g for 15 min at 4°C and a portion of the supernatant was dialyzed against PBS (pH 7.6) and stored at 4°C until use. For the extraction of the prolamins, 75% ethanol was added to the pellet and the solution was vortexed vigorously for 10 min at RT. The solution was centrifuged at 30,000 g for 10 min at 4°C and the supernatant was stored at 4°C until use.

Separation of Conglutins

A portion of the globulin fraction was desalted by dialysis with 50 mM Tris-HCl (pH 7.5). The α -, β - and γ -conglutins were purified by anion exchange chromatography on a Resource Q column (Amersham Biosciences AB, Uppsala, Sweden) as described by Melo et al. [21] using fast protein liquid chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden) with automated fraction collection. The column was equilibrated with 50 mM Tris-HCl (pH 7.5), loaded with approximately 5 mg of the globulin fraction and eluted with a continuous, linear gradient of NaCl (0–1 M). The mobile phase flow was 1.5 ml/min and the detection wavelength was 280 nm. γ -Conglutin did not bind to the anion exchanger at 0 M NaCl. β -Conglutin eluted from the Resource Q column with approximately 0.3 M NaCl. Finally, α -conglutin eluted from the Resource Q column with 0.40–0.45 M NaCl. Fractions were collected every minute and stored at 4°C until use.

γ -Conglutin Isolation

The release of γ -conglutin was induced by the heat treatment of mature *L. angustifolius* seeds as described in the literature [32]. In brief, dry seeds were soaked in deionized H₂O (1:10 w/v) and heated for 4 h at 60°C under gentle shaking. The supernatant was collected, dried by rotary evaporation, and the proteins were redissolved in 50 mM Tris-HCl (pH 7.5). The solution was stored at 4°C until use.

Protein Electrophoresis and Immunoblotting

The NuPAGE gel system (Invitrogen, Carlsbad, Calif., USA) was used for the electrophoretic separation of protein extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in accordance with the manufacturer's instructions. The protein sample concentrations were applied as mentioned in the respective figure legends. Separation was performed under reducing conditions for 40 min at 200 V in 2-(N-morpholino) ethane sulfonic acid (MES) SDS running buffer, using 4–12% Bis-Tris gels and Mark12 unstained or SeeBluePlus2 prestained reference standards. Samples were prepared with lithium dodecyl sulfate sample buffer and dithiothreitol reducing agent (all from Invitrogen). The gels were either stained with SimplyBlue SafeStain (Invitrogen) or the proteins electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad) for 60 min at 30 V with transfer buffer using a XCell II blot module (Invitrogen). For the immunostaining of the Western blots, Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.6) was used as washing buffer and TBST containing 1% BSA was used as blocking and assay buffer. After blocking for 30–60 min, the blot was incubated overnight at 4°C with the crude clone supernatants or the purified mAb, diluted in assay buffer as indicated in the respective figure legends. The blot was washed (3 $\times$ 15 min) and incubated for 1 h with horseradish peroxidase (HRP) conjugated anti-mouse Ig (Dako Cytomation, Glostrup, Denmark). After washing (3 $\times$ 10 min), the membrane was developed with 3,3',5,5'-tetramethylbenzidine substrate solution (Zymed, San Francisco, Calif., USA) until bands of sufficient intensity appeared (2–15 min). All washing and incubation steps were performed at RT with gentle shaking, if not otherwise stated.

Patients

Human sera used in this study were obtained from children with known or suspected food allergy (n = 5, median age = 8 years,

range 2.5–17) referred to the Voksentoppen Center for Asthma and Allergy at the National Hospital. All the children demonstrated positive skin prick tests (median wheal = 5 mm, range 4.5–11) and specific serum IgE (median = 6.2 kU/l, range 2.0–20.2) to lupin flour.

ELISA Procedures

For this study, sera from individuals that responded positively in a skin prick test to lupin flour were used. Flat bottom, high-binding polystyrene 96-well microplates (Corning Inc., Corning, N.Y., USA) were coated with 100 µl of 0.05 M carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) containing 2 µg/ml of the processed lupin protein extract (indirect, competitive and inhibition ELISAs) or purified polyclonal anti-lupin antibody (sandwich ELISA) for 90 min at 37°C. All washing steps were carried out with PBS containing 0.005–0.05% Tween 20 (PBST; pH 7.6). The plates were blocked with PBS containing 0.1% Tween 20 or 1% BSA for 30–60 min at RT or 37°C.

Indirect ELISA. For hybridoma screening, the supernatants were diluted 1:1 in PBST. For other studies, crude clone supernatants, purified mAbs or mouse sera were diluted in PBST as indicated in the respective figure legends and incubated for 1 h at RT under gentle shaking. An anti-mouse Ig AP conjugate (Sigma-Aldrich) and a dinitrophenol phosphate substrate (Sigma-Aldrich) were used to detect bound antibodies. Optical densities (ODs) were read at 405 nm using a reference filter at 630 nm.

IgE Inhibition ELISA. The mAb Lu11 (5–0.16 µg/ml) and the respective patient serum (n = 5, diluted 1:25) were added (50 µl of each/well) and incubated simultaneously overnight at 4°C. For the detection of bound IgE, the wells were first incubated with rabbit anti-human IgE (Dako Cytomation), followed by incubation with goat anti-rabbit IgG HRP conjugate (Zymed). Both incubations were performed for 1 h at 37°C. The substrate for HRP was K-Blue 3,3',5,5'-tetramethylbenzidine (Neogen, Lexington, Ky., USA), the reaction was stopped with 2 M H₂SO₄ and OD was read at 450 nm.

Competitive ELISA. The mAb Lu11 (0.1 µg/ml) and relevant protein extracts (1,000–0.5 µg/ml) were added (50 µl of each/well) and incubated simultaneously for 2 h at RT. The final steps were performed as described for the indirect ELISA.

Sandwich ELISA. Protein extracts (1,000–0.49 ng/ml) were added and incubated for 1 h at RT under gentle shaking. For the detection of bound lupin proteins, the wells were first incubated with mAb Lu11 (1 µg/ml), followed by incubation with HRP-conjugated anti-mouse Ig (Dako Cytomation). Both incubations were performed for 1 h at RT under gentle shaking. The final steps were performed as described for the IgE inhibition ELISA.

Results

Binding Characteristics of the mAbs

Five mAbs were obtained and designated Lu8, Lu11, Lu18, Lu34 and Lu35. Antibody isotyping demonstrated that Lu11 belonged to the IgG2b subclass, while the remaining mAbs all belonged to the IgM class. In all experiments, Lu11 was used as a purified antibody, whereas the IgM mAbs were tested as crude clone supernatants.

The reactivity of the mAbs towards processed lupin protein (*L. albus*) and lupin flour (*L. angustifolius*) extracts and the control protein ovalbumin was studied by titrations in the indirect ELISA format (fig. 1). Among the mAbs, some differences were observed in their reactivity towards processed lupin proteins and lupin flour. Lu11 and Lu18 showed a higher affinity for processed lupin proteins than for lupin flour. Lu8 and Lu35 had equal affinities for both protein extracts, however, Lu8 expressed some unspecific binding towards ovalbumin. Lu34 showed a high affinity for processed lupin proteins, but had a low response to lupin flour.

The binding patterns of the mAbs towards processed lupin proteins were investigated by immunoblotting (fig. 2). All mAbs reacted against several proteins. Lu11 and Lu18 had similar binding patterns and recognized a number of proteins with molecular sizes between 32 and 49 kDa. Lu8 mainly showed reactivity against 2 proteins, with molecular sizes of approximately 20 and 40 kDa. Lu34 bound to a number of proteins ranging in sizes from approximately 20 to 49 kDa. Lu35 recognized several proteins between 20 and 43 kDa.

Specificities towards Globulins

In order to characterize the proteins that are recognized by the mAbs, lupin flour was fractionated into albumins, globulins and prolamins (fig. 3a). The amount of prolamins obtained was below the detection limit in the Lowry assay and they were not visible on the gel. All the mAbs showed a strong affinity for the lupin flour globulin fraction in an indirect ELISA (data not shown) as well as by immunoblotting (fig. 3b). Additionally, Lu8 and Lu35 recognized the albumin fraction to a minor degree. However, the number and intensity of the bands were lower than those observed for the globulin fraction. Attempts were made to fractionate the processed lupin proteins, but the results were unsatisfactory and not usable for further studies.

Specificities against Conglutins

In order to characterize the specificity of the mAbs towards the different conglutins, the globulin fraction of lupin flour was separated into the individual conglutins by anion exchange chromatography (fig. 4a). The chromatogram showed that the majority of proteins were β- and α-conglutins, whereas the amount of γ-conglutinin was small. Therefore, dry mature *L. angustifolius* seeds were heated in deionized H₂O to induce active secretion of γ-conglutinin. This resulted in the isolation of significant amounts of γ-conglutinin (fig. 4b). Immunoblotting

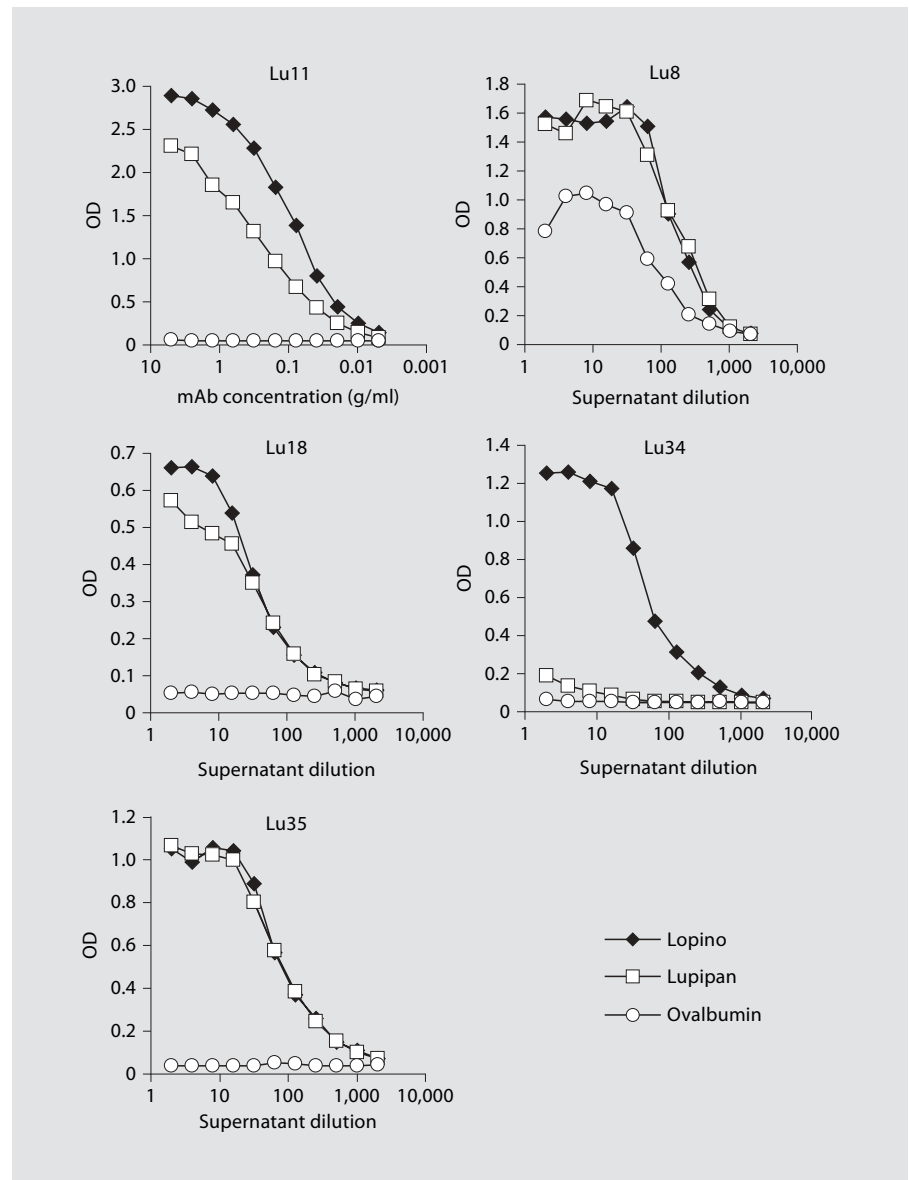


Fig. 1. Titrations of mAbs (Lu8, Lu11, Lu18, Lu34 and Lu35) against processed proteins from *L. albus* (Lopino), lupin flour from *L. angustifolius* (Lupipan) and control protein (ovalbumin) in indirect ELISA.

with the conglutins showed that Lu11 and Lu18 recognized α -conglutin, while Lu8 recognized β -conglutin (fig. 5). Lu34 and Lu35 recognized α -conglutin as well as β -conglutin, although both demonstrated a higher affinity for β -conglutin. In addition, none of the antibodies reacted towards γ -conglutin, even when larger amounts of the protein isolate were applied onto the gels prior to immunoblotting (fig. 5).

IgE Inhibition

In order to investigate whether Lu11 competes with human IgE for binding to lupin proteins, a competitive

ELISA was performed, using sera from 5 individual patients with skin prick tests positive to lupin proteins. Lu11 was shown to inhibit the binding of human IgE to processed lupin proteins by an average of 30% (fig. 6). Maximum inhibitions for individual sera ranged from 12 to 65%. Although all patients demonstrated IgE binding to processed lupin proteins, lupin flour as well as α - and β -conglutins in indirect ELISA and immunoblot, Lu11 showed no inhibition of the binding of human IgE to either lupin flour nor α -conglutin (data not shown).

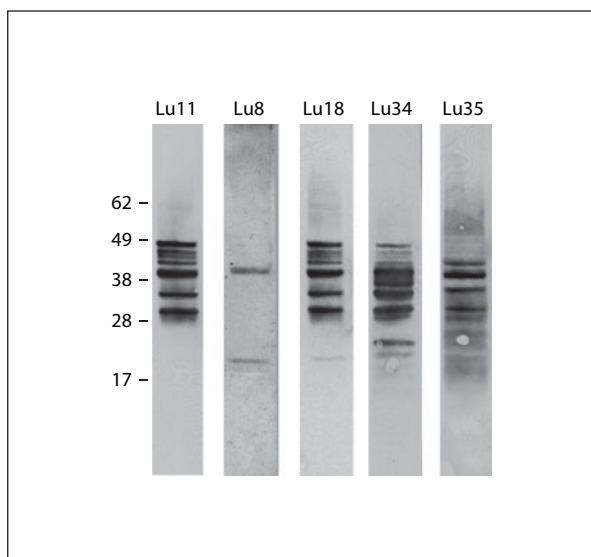


Fig. 2. Reactivities of mAbs against processed lupin protein extract from *L. albus* (Lopino) by immunoblotting. Protein sizes (in kDa) are indicated on the left side of the blots. The following mAb concentration, clone supernatant dilutions and lupin protein concentrations were applied: Lu11, 0.05 µg/ml mAb, 1 µg lupin protein; Lu8, 1:10 diluted, 5 µg lupin protein; Lu18, 1:100 diluted, 1 µg lupin protein; Lu34, 1:5 diluted, 5 µg lupin protein; Lu35, 1:500 diluted, 5 µg lupin protein.

Application of Lu11

It was examined whether Lu11 can be used for the development of a detection method for lupin α-conglutinin. First, a competitive ELISA was performed using several relevant and control protein extracts (hazelnut, bean, chickpea, peanut, pea, soy and lentil). No cross-reactivity to proteins other than the extracts containing lupin globulins was observed (fig. 7a). Furthermore, Lu11 was tested in a sandwich ELISA format using a purified anti-lupin antibody from a rabbit for capture and Lu11 as a detection antibody. Preliminary results indicate that that this combination of antibodies results in a highly specific ELISA for the detection of lupin proteins (fig. 7b).

Discussion

Allergic symptoms against lupin proteins have not been described until recently [4–10], and so far only a few studies have addressed the antibody binding towards fractionated lupin seed proteins [8, 22]. mAbs are valuable tools in the investigation of antigen-antibody interactions and have shown to be useful for diagnostic and therapeutic purposes. Furthermore, mAb-based meth-

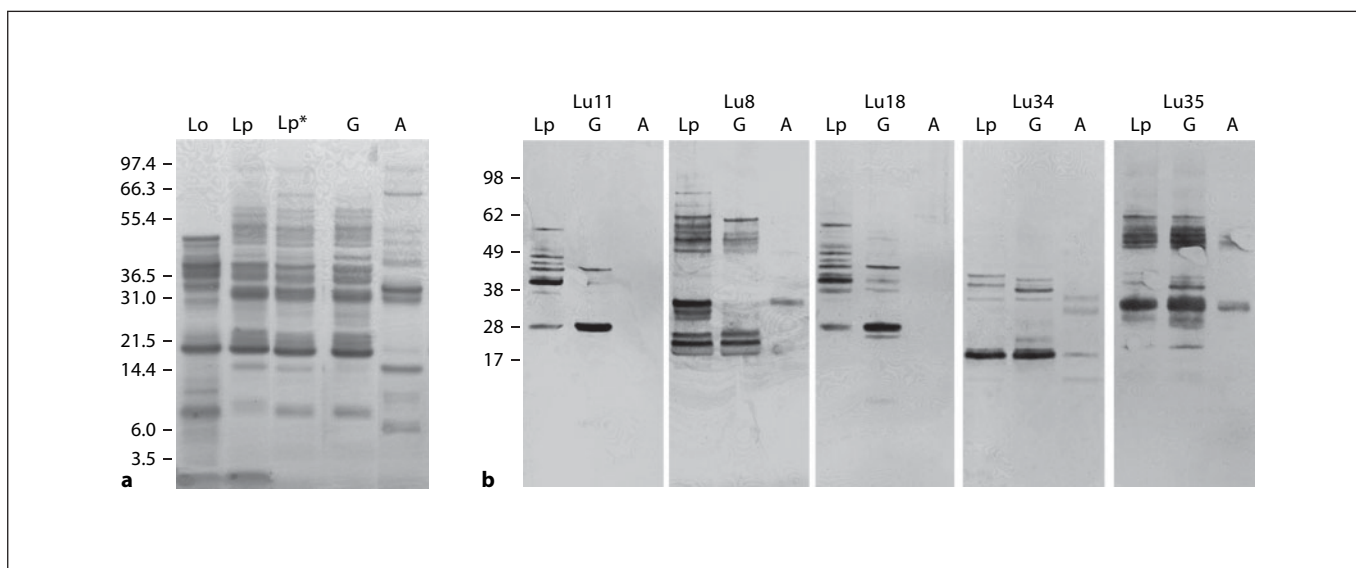


Fig. 3. SDS-PAGE of processed lupin proteins (Lopino), lupin flour (Lupipan) and the lupin flour fractions (a), and immunoblots of mAbs against the lupin flour fractions (b). In the SDS-PAGE, 10 µg of each fraction was applied. The following mAb concentration, clone supernatant dilutions and lupin protein concentrations were applied in the immunoblots: Lu11, 0.05 µg/ml mAb, 1 µg protein; Lu8, 1:10 diluted, 5 µg protein; Lu18, 1:50 di-

luted, 1 µg protein; Lu34, 1:10 diluted, 5 µg protein; Lu35, 1:1,000 diluted, 1 µg protein. Protein sizes (in kDa) are indicated on the left side of the gel and blots. Lo = Lopino extract; Lp = Lupipan Tris-glycine buffer extract; Lp* = defatted untreated Lupipan; G = total Lupipan globulin fraction; A = total Lupipan albumin fraction.

ods are also applied to allergen quantification. In the present study, and to our knowledge for the first time, mAbs against lupin seed proteins were generated and characterized. The 5 mAbs obtained reacted against the predominant type of storage proteins in lupin, the globulins. Two of the antibodies recognized α -conglutin, the remaining 3 recognized β -conglutin.

The mAbs were generated in mice by immunization with the same processed lupin protein preparation from *L. albus* seeds that we had previously used to produce polyclonal antibodies in rabbits [26]. The choice of processed lupin protein over native lupin flour as the immunogen was made with respect to the mAbs' intended application as food allergen identification tools. The characterization of the mAbs demonstrates that they have a strong affinity to both processed and native lupin proteins from *L. albus* and *L. angustifolius*, respectively, and that they are able to detect lupin in foods. Our strategy is supported by recently published data, demonstrating that conventional food processing techniques (such as microwave heating and boiling) only result in minor changes in the lupin proteins' composition and allergenicity [33]. Their immunochemical binding properties were only decreased after prolonged autoclaving.

Of the 5 mAbs generated, 1 belonged to the IgG class of antibodies and 4 belonged to the IgM class. mAbs of the IgM class are generally considered as less favourable for usage in immunological studies due to the large pentameric structure. However, IgM mAbs have been applied successfully in several methods. The majority of mAbs used for human blood grouping in clinical practice, for example, are of the IgM isotype, and these methods clearly require and deliver an extremely high degree of specificity. Moreover, a two-site IgM-based sandwich ELISA was recently described for the detection of the recombinant *Alt a1* [34]. A careful evaluation of the specificity/cross-reactivity of any mAb is clearly important, and that is what we have aimed to do in the present work. In addition, the fact that a majority of the mAbs generated in this study were IgM is not explained by a weak in vivo IgG response to lupins, since high IgG antibody titres were measured in the sera of the mice (data not shown). We neither detected lupin-like constituents in the legume-rich diet of the mice (measured by a quantitative lupin ELISA [27]), which otherwise might have caused an oral tolerance to the immunogen.

All mAbs mainly reacted against the globulin fraction of lupin flour, which confirms the observation made for other legumes that globulins have considerable immunogenic potency and are important food allergens [13–16,

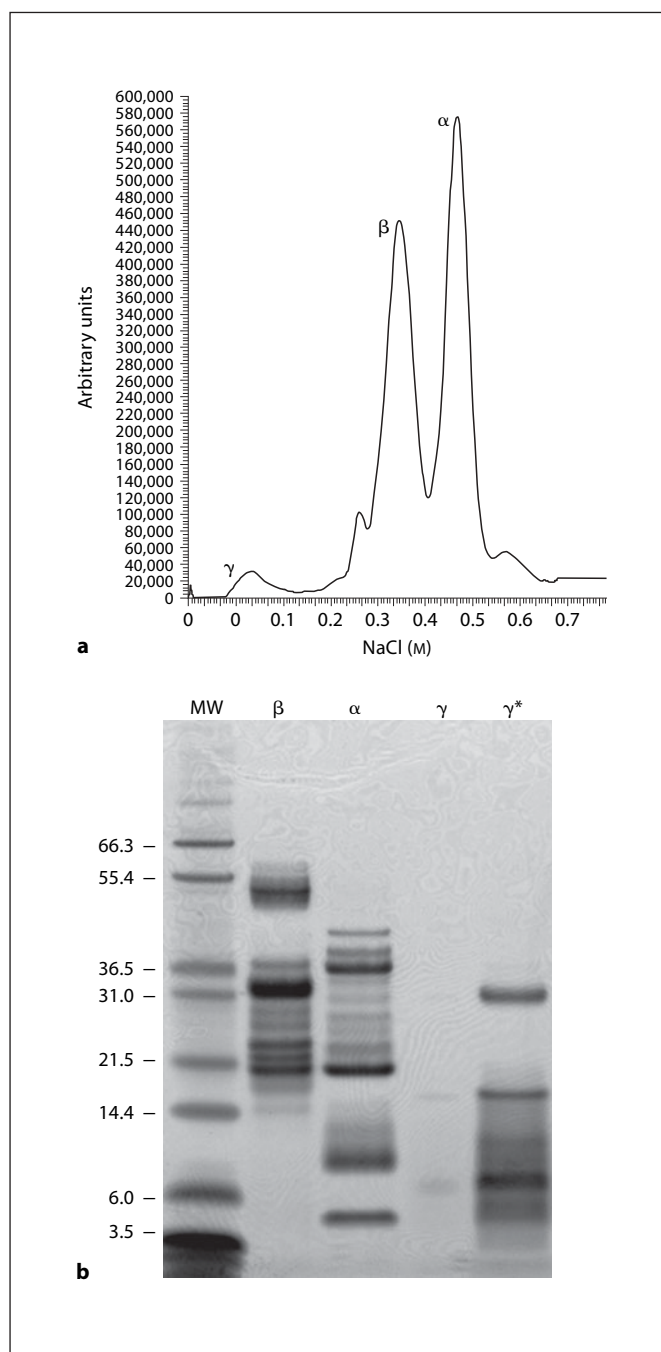


Fig. 4. Isolation of α -, β - and γ -conglutins. Chromatogram (a) and SDS-PAGE (b) of lupin flour globulins separated into conglutins. Protein sizes (in kDa) are indicated on the left side of the gel. MW = Molecular weight marker; β = β -conglutin fraction (10 μ g); α = α -conglutin fraction (10 μ g); γ = γ -conglutin fraction obtained by heat treatment of *L. angustifolius* seeds (10 μ g); γ^* = higher concentration of γ -conglutin fraction (45 μ g).

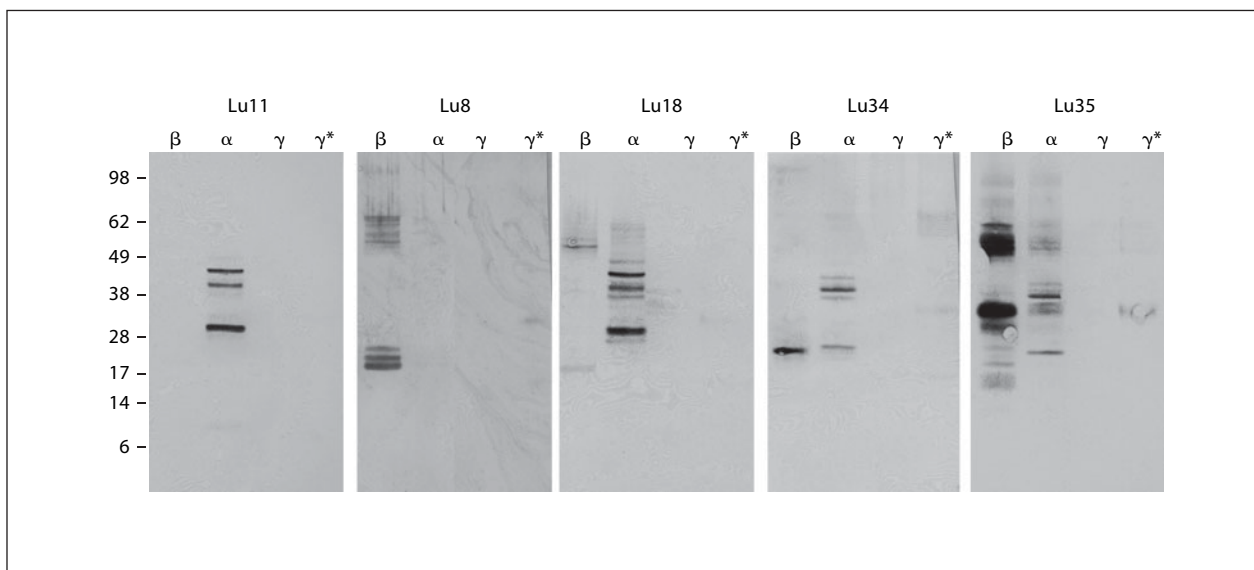


Fig. 5. Specificity of mAbs against the β -, α - or γ -conglutins by immunoblotting. One microgram of β -, α - or γ -conglutins was applied, in addition to 5 μ g of γ -conglutins (γ^*). Protein sizes (in kDa) are indicated on the left side of the blots. The following mAb concentration and clone supernatant dilutions were applied in the immunoblots: Lu11, 0.05 μ g/ml mAb; Lu8, 1:10 diluted; Lu18, 1:100 diluted; Lu34, 1:10 diluted; Lu35, 1:1,000 diluted.

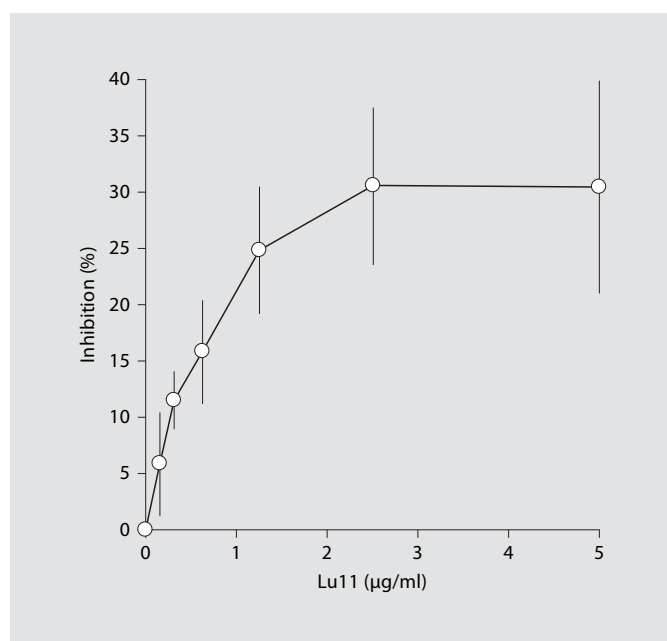


Fig. 6. Inhibition of IgE binding to processed lupin proteins by mAb Lu11. The results are shown in a representative curve (from 3 individual experiments). Bars indicate the mean values $\pm$ SEM for sera ($n = 5$, 1:25 diluted) from patients with positive skin prick tests to lupin proteins.

35]. Additionally, 2 of the mAbs also showed a weak binding to the albumin fraction. This may be due to the recognition of possible residues of globulins in the albumin fraction or a certain degree of unspecific binding of these IgM mAbs.

The globulin fraction of lupin seeds primarily consists of α - and β -conglutins, and to a lesser extent of γ -conglutins [20]. In the present study, the α - and β -conglutins were successfully separated from the total globulin fraction by anion exchange chromatography. Since the amount of γ -conglutins obtained by this procedure was small, a heat treatment of the lupin seeds was performed [32], resulting in sufficient amounts of the protein for subsequent studies. Furthermore, the isolated globulin fractions corresponded in their molecular size to those reported by others [24, 31, 32, 36]. Using these fractions, the affinity profiles of the mAbs were investigated. Lu11 and Lu18 recognized α -conglutins in a similar manner, which might indicate that these antibodies recognize a similar peptide and/or epitope. The other 3 mAbs preferentially recognized β -conglutins, all in distinct fashions, indicating binding to 3 different proteins. The fact that these mAbs showed some additional binding to α -conglutins might indicate an affinity to structurally related proteins that are present in both α - and β -conglutins [24]. Moreover, it ap-

peared that all mAbs recognized a number of polypeptides in conglutins. This could be the result of binding to similar epitopes present on distinct polypeptides, or the presence of several protein isoforms.

All mAbs except Lu34 had high reactivities towards proteins from both *L. albus* and *L. angustifolius* in indirect ELISA, which is probably a consequence of conserved globulin peptide sequences within the lupin species [37]. The somewhat weaker affinity of Lu34 for *L. angustifolius* than for *L. albus* might be explained by a reduced expression of the target protein in *L. angustifolius* compared to *L. albus*, a difference in epitope structures or a higher affinity to processed lupin proteins.

The anti-lupin mAb Lu11 was shown to inhibit human IgE binding to processed lupin proteins by approximately 30%, which indicates that these antibodies recognize corresponding or overlapping epitopes on α -conglutin. Epitope characterization using Lu11 might thus identify an allergenic domain present in α -conglutin. Surprisingly, Lu11 did not inhibit human IgE binding to unprocessed lupin flour. This indicates that these antibodies recognize an epitope, or part of an epitope, in processed lupin proteins that is not (entirely) exposed in native lupin flour. The processing of lupin proteins might result in protein agglutination, causing a change in epitope structure or availability. Agglutination may also explain why our attempts to fractionate processed lupin proteins were not successful. However, the discrepancy in inhibition rates might also be due to the fact that the 2 lupin products were derived from different lupin species. The fractionation of native *L. albus* proteins might therefore provide more information about the cause of the discrepancy.

Previous studies on the specificities of IgE from patients with lupin allergy have identified various IgE-reacting polypeptides in lupin seeds, which were all identified as globulins [22]. Our observation that all patients' sera demonstrated IgE binding to α -conglutin supports the previous proposition of α -conglutin as an allergen in lupin [22]. However, Parisot et al. [8] described a dominant allergen with a size of approximately 43 kDa, which corresponds to the non-reduced form of γ -conglutin, while others suggested that an important part of the IgE is directed against α -conglutin as well as γ -conglutin [22]. Apparently, there seems to be large variation in the binding patterns of IgE to lupin proteins between individuals [8, 33], which indicates that there might be several important allergenic proteins remaining to be characterized.

Currently, Lu11 is being used in our laboratory in the development of a specific, sensitive and quantitative ELISA method for the determination of α -conglutin in food prod-

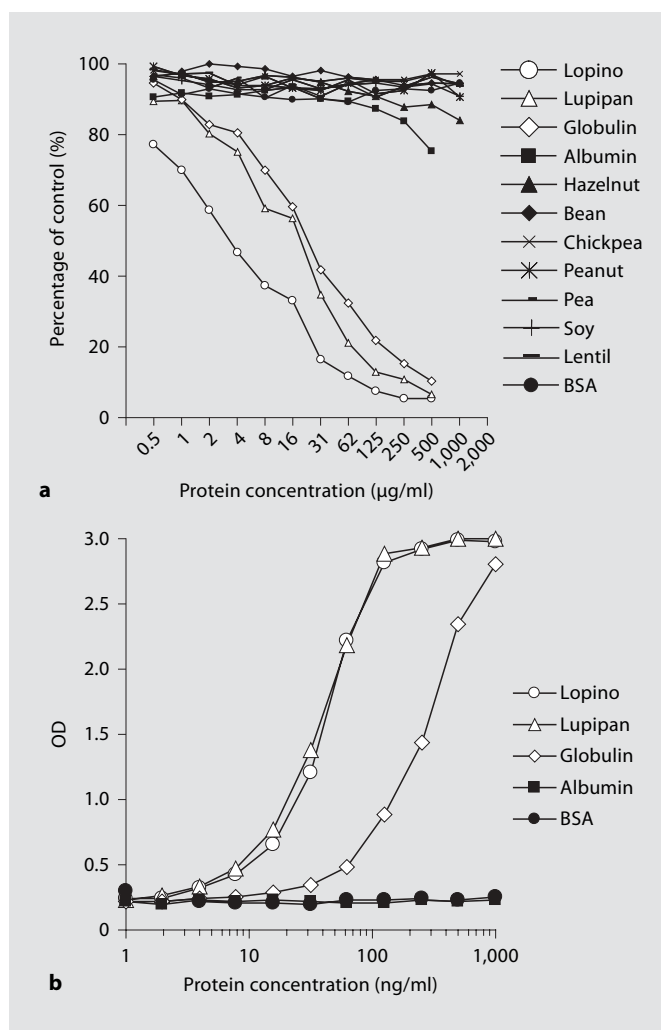


Fig. 7. Competitive ELISA with mAb Lu11 against potential cross-reactive protein extracts (a) and sandwich ELISA for detection of lupin globulins using polyclonal anti-lupin antibody and mAb Lu11 (b).

ucts, as briefly described in this study. As part of that work, the features of the anti-lupin polyclonal antibody [27] and mAb will be compared. Such an assay is a valuable tool to specifically detect lupin residues in foods, and contributes to increased food safety for lupin-allergic patients.

In conclusion, in the current study 5 mAbs against *Lupinus* spp. with specificities towards α - and β -conglutins are presented, which can be used for further investigations of the allergenicity and detection of lupin proteins. Moreover, the results of the present study emphasize the importance of additional studies on conglutins as candidate allergens in lupin allergy.

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