CHARACTERISATION OF ALLERGENIC PROTEINS IN LUPIN SEEDS AND THE RELATIONSHIP BETWEEN PEANUT AND LUPIN ALLERGENS

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ABSTRACT

Lupin seeds are high in protein and dietary fibre but low in fat and starch and a number of health benefits have been associated with their consumption. As a result of these positive characteristics lupin seed components are increasingly being used as foods and food ingredients. However, allergic reactions to lupin have been reported on ingestion or inhalation of lupin seed proteins. In this study we have characterised the proteins from *Lupinus angustifolius* (narrow-leafed lupin) seeds that are allergens and also analysed genes encoding major seed storage proteins.

To identify the allergenic proteins, *L. angustifolius* seed proteins were separated by two dimensional (2D) gel electrophoresis. Western blots of these gels were screened with serum from individuals allergic to lupin and the proteins that bound IgE (allergens) were identified by mass spectrometry. IgE reactive spots were positively identified (had two or more peptide matches to the same protein) and 32 corresponded to conglutin β, one of the major seed storage proteins.

In the initial part of the study, sera from individuals allergic to lupin but not peanut was used to characterise allergenic proteins. There is evidence that some individuals who are allergic to peanuts are more likely to be allergic to lupin and it is possible that these people react to different allergens. We are now investigating which lupin proteins are allergens for these people who react to lupin and peanut and conducting challenge tests to determine the identity of the cross-reactive allergens.

KEYWORDS

Lupinus angustifolius, lupin, allergen, conglutin β, conglutin α, peanut, cross-reactivity

INTRODUCTION

Lupin seeds are high in protein and dietary fibre but low in fat and starch and a number of health benefits have been associated with their consumption (Magni *et al*. 2004; Sitori *et al*. 2004; Hall *et al*. 2005). As a result of these positive characteristics lupin seed components are increasingly being used as foods and food ingredients. However, allergic reactions to lupin have been reported on ingestion or inhalation of lupin seed proteins (e.g. Hefle *et al*. 1994; Smith *et al*. 2004). In is not clear what the prevalence of allergy to lupin is in the general population but it appears to be low (Lindvik *et al*. 2007; Shaw *et al*. 2008). However, it has been variously estimated that 30% (Moneret-Vautrin *et al*. 1999) or 4% (Shaw *et al*. 2008) of peanut-allergic individuals react to lupin.

In this study we have characterised the proteins from Lupinus angustifolius (narrow leafed lupin) seeds that bind IgE and investigated whether lupin allergens cross-react with those from peanut. We have used oral food challenges to investigate the prevalence of lupin allergy in people with allergy to peanut. We have also analysed the genes encoding the major seed storage proteins.

METHODS

PATIENT SERA

Serum was collected from volunteers with either a clinical reaction to lupin or a clinical reaction to peanut and a positive skin test for lupin. All volunteers had a positive skin test (> 3 x 3 mm wheal) with lupin extract (*L. angustifolius* and/or *L. albus*) or showed a high level of lupin specific IgE in their sera. Peanut allergic individuals underwent a lupin food challenge to determine if they reacted clinically to lupin. The studies included in this paper were approved by the Human Ethics Committees at University of Western Australia, University of Sydney, Royal Prince Alfred Hospital or Princess Margaret Hospitals, and all participants (or their parents) gave informed consent.

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SDS-PAGE AND 2D-GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) or 2D-gel electrophoresis and either stained with Coomassie Brilliant Blue G250 or transferred to nitrocellulose. Western blots were probed with IgE from serum of allergic individuals to identify potentially allergenic proteins. Detailed methods for isolation of lupin proteins, electrophoresis and immunoblotting are as described in Goggin *et al*. (2008).

INHIBITION ASSAYS

To identify proteins in lupin that share cross-reactive epitopes with peanut assays in which peanut proteins were used to inhibit binding of IgE to lupin proteins were done. Serum was diluted 1/10 in TTBS (50 mM Tris, 150 mM NaCl, pH 8, 0.1% Tween 20) containing 0.2% BSA and pre-incubated with 100 µg of either peanut seed total protein, total lupin flour protein (positive control) or β-lactoglobulin (negative control) overnight to block IgE epitopes that recognise proteins in these extracts. The serum was then used to probe a Western Blot of total lupin flour proteins. IgE binding was detected by incubation with a mouse monoclonal anti-human IgE antibody conjugated to alkaline phosphatase [Sigma, Sydney, Australia] diluted to 1/15000. Binding of the secondary antibody was detected using the Immun-Star Chemiluminescent Protein Detection System (BioRad, Hercules, California) on a G-Box HR gel documentation and analysis system (Syngene).

MASS SPECTROMETRIC ANALYSIS OF PROTEIN SPOTS

The immunoblots of 2D-gels screened with IgE from lupin allergic individuals were compared with the Coomassie stained gels and protein spots that showed IgE-binding were excised for mass spectrometric analysis. Detailed methods are as described in Goggin *et al*. (2008).

CONSTRUCTION OF LUPIN SEED cDNA EXPRESSION LIBRARY

A SMART cDNA library construction kit (Clontech) was used to make the lupin seed library using the long-distance PCR method for cDNA synthesis, starting with 2 µg total RNA. The cDNA was ligated into pDNR-LIB (Clontech). Random clones were selected from the pDNR-LIB library for sequencing. Clones were sequenced by Macrogen (South Korea).

RESULTS AND DISCUSSION

Sequencing of expressed sequence tags from a lupin seed cDNA Library

The sequence of approximately 100 randomly chosen clones from a *L. angustifolius* seed cDNA library was determined and used in a blastx search of the NCBI NR database to identify any clones with similarity to seed storage proteins. Clones encoding conglutin α, β, δ and γ were identified. Sequences of two full-length conglutin β clones were submitted to Genbank (EF455725, EU352876). EF455725 contained a full open reading frame but the protein it encodes is truncated at the C-terminus compared to other conglutin β (or vicilin-like proteins) in the database.

CHARACTERISATION OF REACTIVITY OF VOLUNTEERS

One group of volunteers that were recruited for this project reacted primarily to lupin (termed lupin-allergic). None reacted clinically to peanut although three had a positive skin test. The second group included people who were allergic to peanut and had a positive skin test to lupin (sensitised to lupin). This group were offered an oral food challenge to determine whether they reacted to lupin. Forty-one of the 134 (30.6%) individuals who took part had a clinical reaction to lupin (termed peanut/lupin allergic). Their symptoms included urticaria, gastrointestinal symptoms and anaphylaxis. To our knowledge, this study is the largest to investigate the prevalence of lupin allergy in peanut allergic individuals. We estimate that 19 to 25% of peanut allergic individuals are sensitised to lupin meaning that 5.8 to 7.5% of peanut allergic individuals react to lupin. Other smaller scale studies have made similar estimates and our results are more in line with those of Shaw *et al*. (2008; 9 individuals tested) who estimated 4% of peanut allergic individuals reacted to lupin than with Moneret-Vautrin (1999; 8 individuals tested) whose estimate was 30%.

IDENTIFICATION OF ALLERGENIC PROTEINS FOR LUPIN-ALLERGIC AND LUPIN/PEANUT ALLERGIC INDIVIDUALS

We investigated whether the difference in reactivity of the two groups of volunteers to lupin and peanut was a result of each group reacting to different lupin allergens. Serum (containing IgE) from lupin-allergic and peanut/lupin-allergic individuals was used to screen Western blots of lupin flour proteins separated by SDS-PAGE. The IgE from the serum of the two groups recognised different proteins in the lupin seed extract (Fig. 1). IgE from lupin-allergic individuals bound strongly to seed proteins of 49–90 kDa (Fig. 1a). IgE from peanut/lupin-allergic individuals showed much weaker reactivity to lupin seed proteins (although most had strong reactivity to peanut seed proteins) and in consequence blots were 'exposed' for longer to obtain an image. Where IgE binding could be detected it was predominantly to 25 and 34 kDa proteins but for some individuals there was binding to proteins of approximately 50 kDa (Fig. 1b).

Serum from lupin-allergic individuals was used to probe a Western blot of a 2D-gel of lupin seed proteins. Spots that bound IgE (results not shown, see Goggin *et al*. 2008) were analysed by mass spectrometry and matches to known sequences were

made using MS/MS ion searches (Matrix Science) of the MSDB database and an in-house library (lupin database) of all lupin EST sequences (including those above). The lupin database included many *L. angustifolius* sequences and was relied upon for most of the matches. Of the 48 *L. angustifolius* IgE binding spots analysed for which data was obtained, 32 were identified as conglutin β. The ability of conglutin β to bind IgE was confirmed when sera from lupin-allergic individuals was used to screen purified protein (results not shown, see Goggin *et al*. 2008). This suggests that for lupin-allergic individuals this is the major allergen with eight of the twelve volunteers reacting to this protein.

Two spots on the 2D-gel probed with lupin-allergic patient sera were identified as conglutin γ. However, as the secondary antibody we used bound to conglutin γ (when used without sera) the identity of conglutin γ as an allergen in *L. angustifolius* cannot be confirmed. For 12 of the spots there was no matching protein in the databases searched so it is possible that there are allergens we did not identify because the sequences were not in our database.

Serum from a peanut/lupin-allergic individual was used to screen a 2D-gel of lupin proteins. One of the IgE binding spots analysed corresponded to conglutin α and another to conglutin γ (Fig. 2). We are continuing our characterisation of the IgE-binding proteins.

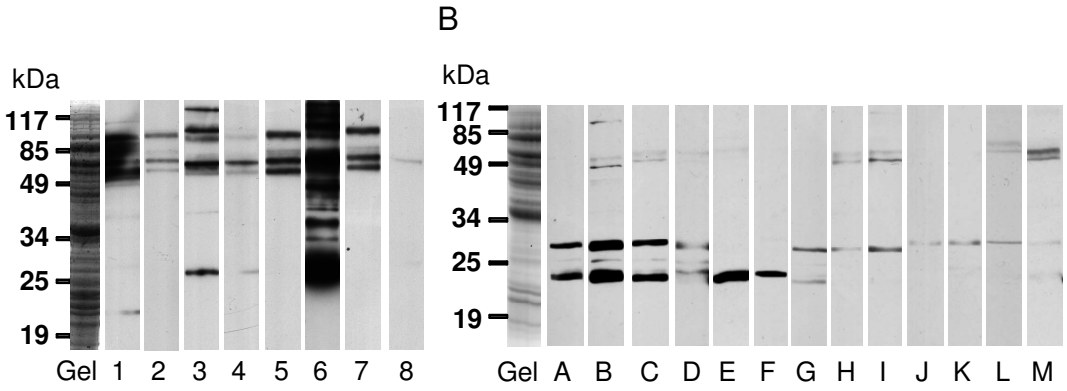

Fig. 1. Immunoblot of *L. angustifolius* flour probed with sera from a. lupin-allergic individauls (lanes 1–7), serum from an individual who was not allergic to lupin (lane 8) and b. peanut/lupin allergic individuals (lanes A-M). The total protein pattern of lupin flour is shown to the left (gel).

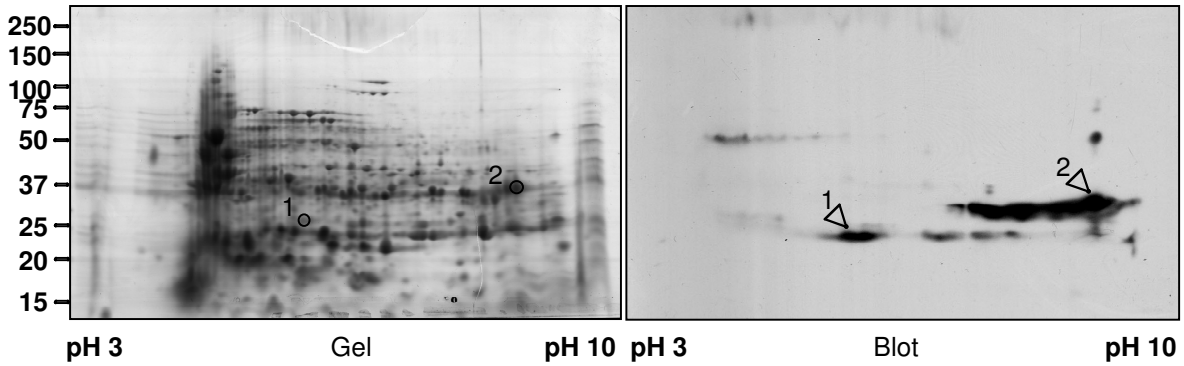

Fig. 2. 2D-electrophoretic analysis of *L. angustifolius* seed protein, showing total protein and immunoblot probed with sera from a peanut/lupin-allergic individual. Protein spots for which identifications were made are enclosed by circles. Spot identified as: 1. conglutin α. 2. conglutin γ.

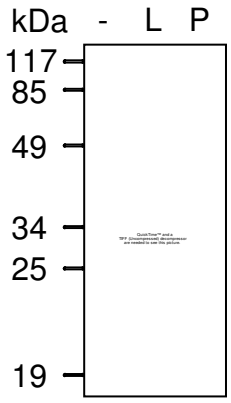

Fig. 3. Inhibition assay to identify proteins in lupin that cross-react with peanut proteins. Serum from a peanut/lupin-allergic individual was incubated with either β-lactoglobulin (-), lupin seed flour extract (L) or peanut seed extract (P) and then used to probe an immunoblot of *L. angustifolius* flour.

INHIBITION ASSAYS TO IDENTIFY PROTEINS IN LUPIN THAT SHARE EPITOPES WITH PEANUT

Assays where peanut proteins were used to inhibit binding of IgE from peanut/lupin-allergic individuals to lupin seed protein were performed to try to identify whether epitopes are shared by lupin and peanut allergens. An example of an inhibition assay is shown in Fig. 3. When serum was pre-incubated with an unrelated protein, beta-lactoglobulin, IgE bound strongly to 25 and 34 kDa proteins in the lupin flour extract separated by SDS-PAGE. These bands correspond in size to those for which spots were sequenced above. When pre-incubated with lupin seed proteins, IgE binding was almost completely inhibited, although some IgE binding to the 25 kDa protein could still be detected. A similar result was observed when the serum was incubated with peanut seed proteins suggesting that the lupin and peanut proteins share common IgE epitopes (Fig. 3). Given that a 25 kDa IgE-binding protein was identified as conglutin α and a 34 kDa protein as conglutin γ it is possible that one or both of these proteins share a cross-reactive epitope with a peanut allergen. Other recent studies have suggested Conglutin α cross-reacts with peanut allergens (Peters *et al*. 2008; Holden *et al*. 2008). This cross-reactivity may explain why the peanut allergic individuals in this study react to lupin.

Our results suggest that individuals that react clinically to lupin but not peanut react predominantly to conglutin β. Those that react to peanut and lupin may react to conglutin α and this protein may share cross-reactive epitopes with Ara h3/4 from peanut. More research is required to determine whether this cross-reactivity is clinically significant.

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