REVIEW

Lupine, a source of new as well as hidden food allergens

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The present review summarizes current knowledge about lupine allergy, potential sensitization routes, cross-reactions between lupine and other legumes, and the respective IgE-binding proteins. Since the 1990s, lupine flour is used as a substitute for or additive to other flours, mostly wheat flour, in several countries of the EU. In 1994, the first case of an immediate-type allergy after ingestion of lupine flour-containing pasta was reported. Since then, the number of published incidents following ingestion or inhalation of lupine flour is rising. So far, the *Lupinus angustifolius* β-conglutin has been designated as the allergen Lup an 1 by the International Union of Immunological Societies Allergen Nomenclature Subcommittee. Initially, publications focussed on the fact that peanut-allergic patients were at risk to develop anaphylaxis to lupine due to cross-reactivity between peanut and lupine. At present, however, the ratio between cases of pre-existing legume allergy (mostly peanut allergy) to *de novo* sensitization to lupine seed is nearly 1:1. Although in December 2006, lupine and products thereof were included in the EU foodstuff allergen list according to the Commission Directive 2006/142/EC amending Annex IIIA of Directive 2000/13/EC in order to prevent severe reactions caused by ''hidden food allergens'', the majority of patients and medical personnel are still not aware of raw lupine seed as potentially dangerous food allergen.

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1 Introduction

The genus *Lupinus* belongs to the *Papillionaceae* subfamily in the *Leguminosae* family, which amongst others also contains peanut and soybean (Fig. 1) [1]. There are more than 450 *Lupinus* species that have been cultivated for over 4000 years. Mainly, four of them are still of agricultural significance: *Lupinus albus* in the Mediterranean area and Africa, *Lupinus angustifolius* (Lup an) in Australia, *Lupinus luteus* in Central Europe, these species belonging to the type of subgenus *L. albus*, and *Lupinus mutabilis* in South America. Their 1.5 m long roots are colonized with *Rhizobium* bacteria, thereby enriching the soil with nitrogen, which makes lupine a valuable fertilization plant [2].

Depending on the species, lupine may contain alkaloids, which taste very bitter and are mostly toxic. The respective disease, which is known in agriculture as *lupinism*, is characterized by symptoms such as restlessness, spasms, shortness of breath, somnolence, and eventually death due to respiratory arrest after ingestion of alkaloid-containing lupine plants [3].

However, in 1929, v. Sengbusch had screened approximately 1.5 million lupine plants and found three yellow and two blue lupines containing only 0.05% alkaloids. By selective breeding, he developed the so-called ''sweet lupines'' [4].

Sweet lupines are mainly harvested in Australia or France. Australia grows nearly 75% of the total world lupine crop and, consequently, is the world's main producer of this

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Abbreviations: Ara h, *Arachis hypogaea*; **Bet v,** *Betula verrucosa*; **DBPCFC,** double-blind placebo-controlled food challenge; **EC,** European Commission; **ED,** eliciting dose; **Gly m,** *Glycine maxima*; **IUIS,** International Union of Immunological Societies; **Lup an,** *Lupinus angustifolius*; **PR,** pathogenesis related; **SPT,** skin prick test

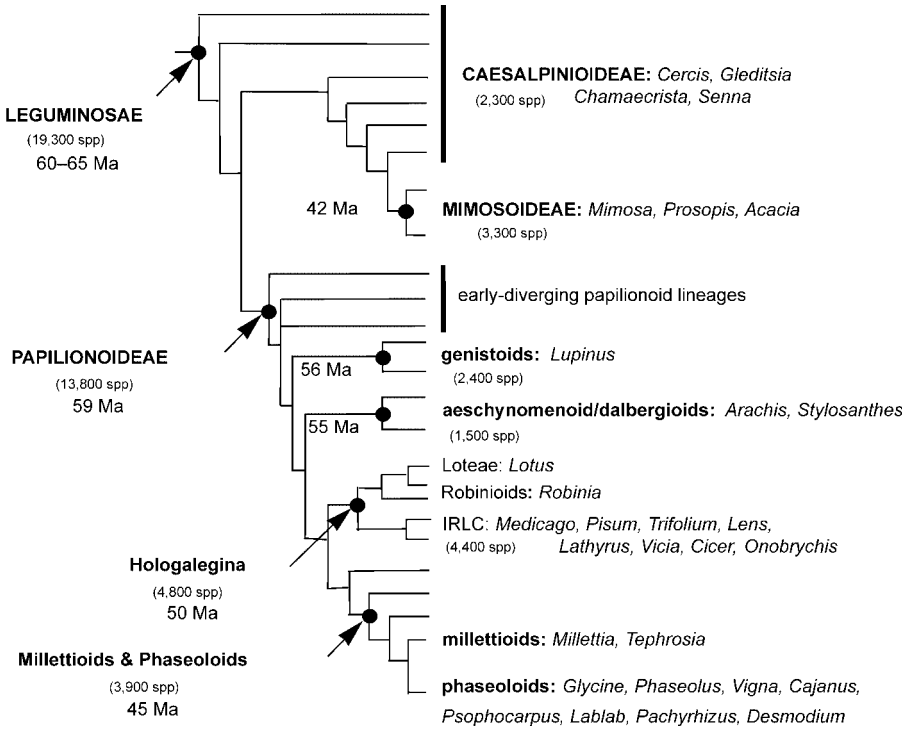

Figure 1. Simplified taxonomic tree of legume family, kindly provided by Paul Gepts *et al.* from their publication in Plant Physiology 2005 [1]. The three subfamilies (*Caesalpinioideae, Mimosoideae,* and *Papilionoideae*) and major subclades identified by molecular phylogenetic studies are shown in boldface. Their positions are indicated by black circles (estimated number of taxa from Lewis *et al.*, 2005 [98], and ages (in millions of years) from Lavin *et al.*, 2005 [99].) IRLC: inverted-repeat loss clade; Ma: million years ago.

particular legume [5]. The species *L. albus*, *L. luteus*, and Lup an, which belong to the ''sweet lupines'', are used as food.

2 Lupine as food

Lupine seed has traditionally been consumed in the Mediterranean area for ages. Flour of raw lupine seed is increasingly used as food ingredient because of its nutritional value. The major storage proteins of lupine are globulins (87%), which consist of two major protein types, β-conglutin (vicilin-like protein, accounting for 43.4%), α-conglutin (legumin-like protein, 33%) and two minor components, δ-conglutin (12.5%) and γ-conglutin (6%) [6]. Lupine is increasingly used as a protein source in European countries where lupine is used as replacement for potentially genetically modified soy products [5, 7]. An additional significance of lupine as food ingredient results from the wish of a subgroup of consumers to substitute animal proteins, *i.e.* milk and egg white, by plant proteins.

Ingestion of lupine-containing foods has been associated with the prevention of obesity, diabetes, and eventually cardiovascular disease [8]. Recently, hypocholesterolaemic properties have been demonstrated for lupine globulin proteins, which may decrease the risk of cardiovascular disease [9, 10]. The *in vitro* interaction between γ-conglutin and insulin and its effect after oral administration of γ-conglutin on the blood glucose levels of rats that were subjected to a significant glucose uptake was investigated by Magni *et al.* (2004) [11]. They showed a significant decrease of glycaemia similar to that induced by the anti-diabetes drug metformin.

Lupine products are considered valuable as additive to the human diet also because of their high protein and low oil content (Table 1) [12–14]. The composition of fatty acids is comparable to that of soy oil. The dietary value of lupine proteins is higher than that of beans or peas, which is mainly due to high concentrations of the essential amino acids lysine, leucine, and threonine, which are higher only in soy beans. Lupine does not contain lactose. It also does not contain gluten. Therefore, lupine is recommended for patients with wheat protein allergy and coeliac disease [12, 15].

However, lupine is not only appreciated for its nutritional value but also for its functional properties in meat products, bakery, and confectionary [16–18]. High water binding capacity and outstanding emulsifying capacities are additional relevant advantages that make lupine flour a valuable ingredient.

In 1996, lupine was officially introduced as a food ingredient in the UK, in 1997 in France, and in 2001 in Australia (www.efsa.europa.eu).

Wheat flour may contain up to 10 or even 15% of lupine flour. Its ground seeds are mixed with cereal flours and used to make pizza, pasta, cookies, and even milk substitutes.

Due to the fact that lupine species are not genetically modified, lupine flour is increasingly used as substitute for soy flour [7].

Table 1. Dietary values of lupine-, wheat- and rye flour

	Lupine flour	Wheat flour		Rye flour	
		Type 405	Type 1700	Type 815	Type 1370
Energy kJ/100 g	981	1409	1304	1355	1327
Proteins %	36–48	10.6	12.1	6.9	8.9
Digestible carbohydrates %	5	70.9	60.9	71.0	66.7
Roughage %	15–18	4.0	11.7	6.5	9.0
Fat %	4–7	1.0	2.1	1.0	1.4

[12] modified [13, 14].

3 Food allergy to lupine

Hypersensitivity to food consists of IgE-mediated and non-IgE-mediated syndromes, amongst which IgE-mediated reactions account for the majority of food-induced immune reactions [19].

Depending on the route of sensitization, food allergy can be divided into two categories, class I and class II food allergy. Class I food allergy occurs after sensitization *via* the gastrointestinal tract. Typical class I food allergens are, therefore, peanut, wheat, fish, cow's milk, and soybean. Class II food allergy is the result of a secondary sensitization to cross-reactive allergens in foods as a consequence of the initial immunoreactivity to homologous pollen- and other non-pollen-related allergens [20, 21], like, for example, birch-pollen related fruit allergy and the latex-fruit syndrome.

3.1 Lupine species as natural source of allergens

Lupine seeds are frequently eaten in Mediterranean countries (traditionally as ''lupini'', ''altramuces'', ''tremoços'') and have been consumed since ancient times. In case no ''sweet lupines'' are used, lupine seeds have to be boiled and an alkaloid extraction in hypertonic solution (salt and water) is needed before they can be safely ingested.

Since the early 1990s of the last century lupine is increasingly consumed as ingredient in several processed foods throughout Europe, USA, and Australia. The first published case report was a 5-year-old child with pre-existing peanut allergy who developed generalized urticaria with angioedema after the ingestion of lupine flour-containing pasta [22]. Since then, the number of reports on allergic reactions to lupine in the form of flour, seed, or dust, some of them severe, are rising in number. The symptoms mainly occur within a few minutes after ingestion. During the following years allergic reactions also after respiratory exposure mostly under occupational conditions with subsequent rhinoconjunctivitis and bronchial asthma have been described [5, 23–25]. A thorough search of the scientific literature in Pub Med (http://www.ncbi.nlm.nih.gov/sites/entrez?db = pubmed) was performed. Additionally, every case report available including abstracts from national and European conferences as far as possible revealed at least 151 cases of lupine allergy worldwide (Table 2). The broad range of symptoms consists of asthma/shortness of breath ($n = 38$), oral allergy syndrome ($n = 30$), generalized urticaria ($n = 24$), allergic rhinitis ($n = 24$), angioedema ($n = 23$), gastro-intestinal pain ($n = 14$), laryngeal oedema ($n = 10$), nausea ($n = 9$), conjunctivitis ($n = 8$), anaphylactic shock ($n = 8$), contact urticaria ($n = 1$), flush ($n = 4$), pruritus (generalized and palmpo-plantar pruritus) ($n = 4$).

Table 2. Lupine flour-allergic patients as reported in the literature

Country	Children	Adults	Documented pre-existing peanut allergy
France[a)] [24, 26–31]	15	5	13
Spain [25, 32–40]	1	11	1
Portugal[b)] [41]	0	2	0
Italy [15, 23, 42, 43]	11	1	5
The Netherlands [7, 44]	0	15	11
Switzerland [45, 46]	1	2	1
Germany [47–51]	2	6	2
Norway[c)] [52–54]	7	1	6
Finland [55]	1	5	1
Denmark [56]	3	0	3
USA [22]	1	0	1
Australia [5, 57]	0	10	0
Great Britain [58–60]	3	1	4

a) Additional 21 cases from France documented in a publication about results of the Reseau allergovigilance plus 14 cases from a study [27], which have not been included in the table due to the lack of demographic data [61].
b) Additional five cases documented in Portugal on lupine allergy without peanut allergy. However, no demographic data was provided [63].
c) Additional seven cases documented in the Register of Severe Allergic Reactions to Food, which have not been included in the table due to the lack of demographic data [62].

The number of cases and the severity of symptoms indicate the importance of this new up-coming allergen.

3.2 Lupine allergy and cross-reactivity with other legumes

Detection of allergen-specific IgE antibodies merely identifies sensitization to a particular allergen and does not allow

a decisive differentiation between clinically relevant IgE reactivity (*i.e.*, IgE antibody–allergen complex capable to cross-link FcεRI receptors and subsequent effector cell response leading to allergic symptoms) and IgE reactivity not accompanied by clinical symptoms (*i.e.* reactivity without an effector cell response) [64, 65]. IgE-positivity to several legumes basically indicates an immunological cross-reactivity *via* linear epitopes (sequence homology) or similar conformational epitopes without allowing estimation or prognosis as to the clinical significance.

The following legumes may cross-react with lupine as published by several groups [7, 22, 26, 27, 32, 44, 58, 63, 66–68]: peanut, soybean, lentils, beans, chickpeas, and peas. As far as peanut is concerned there are cross-reactivities between peanut and other legumes, *i.e.* soybean, pea, bean, and lentils in nearly 5% of the peanut-allergic patients [67]. According to Moneret-Vautrin *et al.* (1999) the risk of peanut-allergic patients to react to lupine with allergic symptoms is 28% [26]. An investigation from Great Britain with peanut allergic children and adolescents revealed a sensitization rate to lupine of 34% and an estimated prevalence of lupine allergy of 8% [58]. A recently published prospective prevalence study on lupine sensitization in 5366 patients revealed a frequency of cross-reactivity with lupine of 17.1% for patients with peanut allergy. Primary lupine sensitization was found in 3.7% of 1422 patients with current atopic disease and 1.8% of 226 patients with latent atopy [68]. Other investigations revealed cross-reactivity of up to 65% (15/23) with lupine in peanut-allergic or -sensitized patients as demonstrated *via* double-blind placebo-controlled food challenge (DBPCFC) or labial challenge [27]. No details were provided on the provocation test protocol. The dominant symptoms induced by the challenge with lupine flour were asthma followed by urticaria, rhinitis, and abdominal discomfort. The cumulative doses provoking a reaction were not significantly different for peanut and lupine (<1 g) [27]. Mazeyrat *et al.* (2004) consecutively investigated 515 children who admitted under the suspicion of food allergy. Of them 5% (26 of 515) were sensitized to lupine. 31% (8 of 26) of the children with lupine sensitization were allergic to other legumes (peas, lentils, beans). Peanut allergen-specific IgE-antibodies were found in significantly higher concentrations in children with peanut plus lupine sensitization compared with children who were exclusively sensitized to peanut. The results demonstrated that a history of reactions against legumes like peas, lentils, or beans is an important risk factor for the sensitization against lupine [66]. The authors hypothesized that the high concentrations of peanut allergen-specific IgE-antibodies in the sera of peanut-allergic children, who were additionally sensitized to lupine, could point to lupine sensitization as risk factor for the persistence of peanut allergy.

In a French investigation, 5/8 patients with a peanut allergy reacted positive to lupine flour in a DBPCFC, 2/8 in a labial challenge [26].

Reis *et al.* had investigated 1160 patients who had consulted an allergologist for lupine-specific IgE-antibodies and detected a sensitization rate of 4.1% (2007). In 75% a co-sensitization between lupine and other legumes, in 82.1% a co-sensitization between lupine and pollen could be detected. Five/39 lupine-sensitized patients (12.8%), who were questioned in detail, had a lupine allergy [63]. A Norwegian study had investigated the clinically relevant and non-relevant sensitization to lupine in children [52]. A total of 15 of 35 (43%) had a positive skin prick test (SPT) and 17 (49%) allergen-specific IgE-antibodies against lupine. A total of ten lupine-SPT-positive children were included in an open oral challenge test with lupine flour-containing pancakes, 1/10 reacted with urticaria, angioedema, and cough [52].

In their first study on this subject, published in 2007, Peeters *et al.* investigated six patients, three of them being allergic to lupine and peanut, the other three being allergic to lupine but tolerant to peanuts. All six patients were included in a DBPCFC with lupine flour and were positive [44].

In a second study on 39 unselected peanut-sensitized patients, Peeters *et al.* (2008) showed that 82% of the study population was sensitized to lupine, 55% to pea, and 87% to soy. Clinically relevant sensitization to lupine, pea, or soy occurred in 35, 29, and 33%, respectively, of the study population. None of the patients had been aware of the use of lupine in food [7].

Most studies on lupine allergy vary with regard to design, population, geographic origin, and endpoints. In addition, only few have used DBPCFC. Depending on the selection of the study population the percentage of clinically relevant and non-relevant lupine sensitization differs. However, although cross-reactivity between lupine and soybean or lentils, beans, and peas only rarely seems to be of relevance, the cross-reactivity between lupine and peanut more often is of clinical significance.

3.3 Current knowledge on single lupine allergens

Most food allergens of plant origin belong to a small number of protein families [20]: the cupin superfamily, which contains 11S and 7S seed storage proteins, the prolamine superfamily – non-specific lipid transfer proteins, pathogenesis-related (PR) proteins, which contain 2S albumins and hydrolase inhibitors. The PR proteins, amongst them the *Betula verrucosa* (Bet v) 1-related proteins belonging to the PR-10-family, are pollen-associated allergens as is the pan-allergen profilin. There are few related families of structural, metabolic, or defence proteins.

Since raw lupine in the form of seed, flour, or dust is known to induce not only allergic symptoms of different grades of severity but different disease entities (Baker's occupational asthma as well as food allergy), detailed knowledge on its single allergens and their clinical relevance is of immense importance. This concept is supported by

evidence obtained from other food allergies. The identification of proteins highly similar to the major birch pollen allergen Bet v 1 in pollen allergy-related food allergens like apple, hazelnut, cherry, carrot, celery, and soybean provides a molecule-based explanation for cross-reactivity [69]. A single allergen of soybean, the Bet v 1-homologous protein *Glycine maxima* (Gly m) 4, is an example for inhalant exposure to a pollen allergen (Bet v 1 from birch), which is able to induce severe allergic symptoms to food [70, 71]. Moreover, Gly m 4 closely resembles yellow lupine proteins [72].

The major allergens of the *Lupinus* species are storage proteins, the conglutins. The two main fractions are α-conglutin and β-conglutin. The α-conglutins consist of proteins with sedimentation coefficients of about 11S–12S. Therefore, they are also called 11S or legumin-like globulins. They consist of hexamers of two disulphide-linked heterogenous subunits indicated as acidic subunits (54–47 kDa) and basic subunits of 20 kDa, respectively [6]. The β-conglutins have sedimentation coefficients of 7S and are also referred to a 7S or vicilin-like globulins. They have a trimeric structure consisting of several polypeptides of 20–80 kDa without disulphide bridges.

The *L. angustifolius* allergen β-conglutin has been designated Lup an 1 by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee on the 29th of January 2008 [73].

Lupine species also contain γ-conglutins and δ-conglutins [74]. γ-Conglutin is a basic tetrameric 7S protein consisting of two polypeptides linked by disulphide bridges (17 and 30 kDa polypeptides). δ-Conglutin is a 2S monomeric minor protein consisting of two polypeptide chains (9 and 4 kDa) linked by disulphide bridges [6].

Some proteins belonging to the above-mentioned protein families have already been described as allergens in peanut and soybean, a fact that prompted investigations on potentially similar allergens in lupine species and cross-reactivity between lupine and other legumes.

Magni *et al.* [75] found a 20 kDa-protein that was IgE-reactive with sera from five lupine allergic patients in soybean, peanut, lupine, and pea extracts using defatted flours in one- and two-dimensional electrophoresis. Under reducing conditions these bands had a molecular weight of 17 and 30 kDa and were detectable in both, soybean and lupine extracts. They were identified as the lupine and soybean γ-conglutin and the 11S globulin basic subunits of soybean, peanut, lupine, pea, respectively. Cross-reactivity of anti-peanut-IgE with lupine γ-conglutin was described by members of the same group [76].

Poltronieri *et al.* described a polypeptide similar to γ-conglutin as one of the dominant allergens in almond seeds [77].

In soybean seed extracts, two similar polypeptides corresponding to the γ-conglutin homologous protein named Bg7S, also significantly reacted with IgE-antibodies from lupine-allergic patients [75].

The γ-conglutin polypeptides were also found to cross-react with *Arachis hypogaea* (Ara h) 3-specific IgG, Ara h 3 being a member of the 11S globulin family [76]. This finding speaks in favour of γ-conglutin being a major allergenic protein. In contrast, Holden *et al.* (2008) found γ-conglutin to be only poorly recognized by their population of Norwegian lupine-allergic patients with coexisting peanut allergy [53].

The basic subunits of the 11S lupine globulins may be allergenic: A sequence similarity as well as the respective cross-reactivity has been described between the 11S basic subunits of lupine, peanut, soybean, and pea globulins.

A high sequence identity and structural homology have been observed between the Bet v 1-homologous peanut allergen Ara h 8 and lupine allergens belonging to the PR-10 family [78]. A major peanut allergen, Ara h 1, shows high sequence identity with the precursor of the lupine β-conglutin [32, 78], whereas Ara h 2 revealed high sequence identity with the lupine δ-conglutin (Table 3) [27, 75, 79]. Among the cross-reactive proteins that have already been described for lupine and peanut, there is one with

Table 3. Comparison of IgE-binding lupine proteins with known cross-reacting peanut allergens

Potential lupine allergens (*Lupinus angustifolius)* Lup an	(kDa)	Peanut allergens (*Arachis hypogaea)* Ara h	(kDa)	Food allergens (protein families)
precursor of the lupine β-conglutin **Lup an 1**	20–80	**Ara h 1**	64	Vicilin-like storage protein
δ-Conglutin	9 4	**Ara h 2**	17 19	Conglutin-like protein
Unreduced γ-conglutin or α-conglutin	43	**Ara h 3/4**	61	Glycinin (Legumin)
Protein PR-10 of *Lupinus albus*	16.9[a)]	**Ara h 8**	17	PR-10 Bet v 1-family

In bold: IUIS documentation of the respective allergens [www.allergen.org]. To other identified peanut allergens such as Ara h 5, 9, 10, and 11 no homologous lupine proteins or allergens have been described so far.

a) The calculated molecular weight for the protein as deduced from the cDNA of the Bet v 1-homologue of lupine (accession no. FG091992).

a molecular weight of 43 kDa, which, according to Magni *et al.*, could be identical with the unreduced *L. albus* γ-conglutin [75], whereas the results of Holden *et al.* suggest it to correspond with α-conglutin (Table 3) [53].

Magni *et al.* (2005) observed that γ-conglutin could be responsible for the cross-reactivity with peanut allergen-specific IgE-antibodies [75], a result similar to that obtained by Moneret-Vautrin *et al.* (1999) [26]. These authors investigated allergen-specific IgE-antibodies of the lupine-allergic child reported by Novembre *et al.* (1999) [23] and other lupine allergic patients showing complete inhibition of IgE-binding to a 43 kDa lupine protein, with peanut extract [26]. Interestingly, the γ-conglutin polypeptides of lupine bound to IgG-antibodies that were specific for the basic subunit of Ara h 3, a known peanut allergen of the 11S globulin family, although no sequence similarity between both protein types could be demonstrated.

Magni *et al.* (2005) described a lupine 2S albumin of 14 kDa, a δ-conglutin that was not recognized by sera of five lupine-allergic patients whereas it is a strong allergen in other seed species [75].

Ara h 2, for example, a 2S protein of 17.5 kDa, is the most immunodominant peanut allergen causing over 85% allergic reactions [80]. It is not clear at present whether the lupine 2S protein is not allergenic as hypothesized by Magni *et al.*, or whether it is simply not reactive with sera of a certain subpopulation of lupine allergic individuals [75]. The results are controversial with regard to the observations as to whether the basic or the acidic subunits of 11S globulins were the relevant allergens for those allergic individuals who have been investigated in Europe: Whereas Magni *et al.* [75] according to their results favoured the basic subunits to be of relevance, the study of Holden and her group indicates the acidic subunits to be responsible for the IgE-binding [53]. This group showed by using immunoblot and ELISA that the majority of six lupine- as well as peanut-allergic patients had IgE-reactivity to more than one lupine conglutin. For each individual patient, IgE-binding to α-, β-, γ-, and δ-conglutins was specific and unique, so that lupine seems to express several important allergenic proteins with α-conglutin being a strong allergen [53]. Inhibition ELISA experiments supported observations of others on cross-reactivity to peanut and almond as well.

In a subsequent investigation, the Norwegian authors performed additional inhibition experiments, showing cross-reactivity between lupine β- and δ-conglutins and the peanut proteins Ara h 1 and Ara h 2, respectively. Lupine α-conglutin cross-reacted with Ara h 2 and γ-conglutin with Ara h 3. Lupine α-, β-, and δ-conglutin showed higher allergenicity than the basic γ-conglutin, which supports the results of Moneret 1999, where a 43 kDa band (probably α-conglutin) and a 13 kDa band (probably δ-conglutin) showed the highest IgE-binding capacity. However, β-conglutin (38 and 65 kDa) also seems to have allergenic potential [81].

The role of cross-reactive carbohydrate determinants for the development of lupine allergy has so far not been investigated in detail.

There is a large body of evidence that cross-reactivity between lupine, peanut, and soybean are mediated by similar proteins (Table 3). Although this may be suggestive for a similar clinical significance of the respective allergens in lupine, sensitization to a certain legume does not necessarily lead to the sensitization or an allergy to another legume. Peanut allergy seems to coexist with lupine allergy more often than is the case for lupine and soy allergy. These phenomena warrant further scientific investigations on the identification and characterization of lupine allergens, on the sensitization routes (see Section 3.6) and to clarify where and why cross-reactivity between lupine and other legumes become clinically relevant.

3.4 Food processing and its effect on lupine protein allergenicity

Somus de Castro Pinto *et al.* (2009) demonstrated the decrease of the antigenic activity of the globulins of lupine and other legumes like chickpea and lentil due to enzymatic hydrolysis with pepsin and trypsin as currently used in food processing industry [82] as did van Boxtel *et al.* for legumin allergens from peanuts and soybeans (Ara h 3 and Gly m 6, respectively) [83].

Thermal processing may influence plant protein allergenicity to a different extent, either increasing or decreasing IgE immunoreactivity, which has already been shown for legumes other than lupine, *e.g.* peanut, where thermal processing (roasting) enhanced the allergenic potency [84].

Lupine allergens are relatively stable with regard to heat treatment. Alvarez-Alvarez *et al.* investigated the allergenic characteristics of lupine seeds after boiling (up to 60 min), autoclaving (121°C at a pressure of 1.18 atmosphere up to 20 min and 138°C, 2.56 atmosphere up to 30 min) as well as microwave heating for 30 min and extrusion cooking. Neither microwave treatment nor cooking reduced IgE-binding capacity as confirmed *via* SDS-PAGE as well as immunoblotting. Only autoclaving at 138°C for at least 20 min (*i.e.* a treatment not used in customary food processing) significantly reduced IgE-binding capacity of a raw lupine seed extract [85]. Whereas in this study lupine seeds had been investigated, a Norwegian group applied heat treatment to lupine-containing foods and showed a subsequent decrease of IgE-binding to the respective lupine proteins, suggesting a matrix effect to be the cause for the discrepancy of results between these two different experimental designs [53].

Lupine cotyledons were treated with instantaneous controlled pressure drop at several pressure and time conditions (3, 4.5 and 6 bar for 1, 2, and 3 min). Subsequently, significant modifications of protein patterns were seen in SDS-PAGE analysis. Decreases in IgE-binding to lupine proteins as demonstrated by immunoblot were associated with the increases in steam pressure and time treatment suggesting a reduction in lupine allergenicity [86].

Rojas-Hijazo *et al.* (2006) used a different approach and investigated several manufactured foods by using the serum of a monosensitized lupine-allergic patient as a tracer to detect non-specified lupine proteins. Thereby lupine allergens with a molecular weight between 48 and 14 kDa were identified, out of which the 14 kDa-IgE-binding protein proved to be highly resistant to heat treatment [33].

Whereas the study of Alvarez-Alvarez was performed under artificial conditions that do not mirror realistic food processing procedures, the observations of Holden *et al.* as well as Rojas *et al.* may be relevant for lupine allergic patients.

3.5 Is lupine a novel and a hidden allergen?

Hidden allergens may be defined as:

(i) Allergen deliberately added to food but unlabelled in ingredient list and, therefore, not recognizable for the consumer
(ii) Allergen presence in food because of unintentional cross contact during food manufacturing itself.

A novel food allergen is characterized as binding specific IgE from patients who are allergic to the particular source material that has been introduced to human diet for the first time, *e.g.* kiwi at the beginning of the 1980s. A new allergen also can be an IgE-binding molecule that has never been described and identified before (for example by antisera, monoclonal antibodies, inhibition assays with the homologous protein, mass spectrometry) in a particular species as allergen source, for example, tropomyosins from previously not studied crustacean species.

Lupine and products thereof were included in the EU foodstuff allergen list, which according to the European Commission Directive 2006/142/EC (EC, European Commission) amending Annex IIIA of Directive 2000/13/EC must under all circumstances appear on the labelling of foodstuff in order to prevent severe reactions caused by ''hidden food allergens'' [87]. This has improved the situation of patients who may be at risk of developing a lupine allergy. However, those patients who are potential lupine-allergic individuals are still at risk of developing symptoms due to unintended contamination of food with lupine protein. According to a publication of the Norwegian National Reporting System and Register of Severe Allergic Reactions to Food [62, 88] a total of seven reports on cases with reactions most likely to lupine occurred in Norway. Some of the reported cases were caused by intended use of lupine flour in bakery products, other cases by inadvertent contamination by lupine, which had originally been used as ingredient for other products in the same bakery.

Furthermore, several food-allergic patients do not know that they are at risk of developing allergic symptoms after ingestion of lupine because they are unaware of the cross-reactivity between lupine and other legumes due to a pre-existing and well-known allergy to, for example, peanut. Therefore, the consumer would not actively avoid lupine consumption, even if he realized lupine to be an ingredient according to the declaration.

Surveillance systems depend on sensitive and specific detection methods that can accurately detect lupine traces in foods in order to evaluate the compliance with labelling regulations and allergen control strategies for maximal protection of consumers.

Lupine allergy has to be made known to a broad range of health care professionals, not only physicians but also dieticians in order to better advise potentially affected patients.

Lupine is a novel allergen for populations not accustomed to it as traditional ingredient in their diet. Moreover, lupine seems to be a food allergen of considerable significance. Even though lupine is recognized as important allergen and must be declared on the ingredient list it may still be hidden from the consumer because of unintentional cross-contact.

With regard to the fact that legume-allergic patients are mostly not aware of the risk of exposure to lupine, the term ''hidden'' gains on complexity.

3.6 Potential routes of sensitization for a lupine allergy

In some patients lupine allergy has developed *de novo* [15, 23, 34, 44, 45, 47, 57]. There are as many cases of pre-existing peanut allergy who suffered from lupine allergy due to cross-reactivity between lupine and peanut [22, 26, 54, 59].

According to the literature there are several sensitization routes possible.

There is good evidence for respiratory sensitization leading to inhalant as well as food allergy. There are also examples for a primary lupine sensitization *via* ingestion [15, 23, 34, 44, 45, 47, 57].

3.6.1 Sensitization *via* inhalation

Novembre *et al.* described a 3-year-old child with lupine allergy who had developed symptoms after the child had played with a lemon tree manured with lupine dust. The symptoms were asthma, rhinitis, conjunctivitis, cough, dyspnoea, and cyanosis [23]. Allergy diagnostic tests including an inhalation challenge test with lupine dust was positive.

Crespo had investigated seven patients who had an occupational exposure to lupine seed flour *via* inhalation [25]. Three out of seven showed symptoms during their work (conjunctivitis, rhinoconjunctivitis, and asthma). They revealed positive SPT results for lupine flour. Only those

two patients who had a rhinitis and conjunctivitis or asthma were positive in the inhalation challenge as well as in the oral food challenge and had allergen-specific IgE-antibodies against lupine. Both additionally revealed cross-reactivity of different degrees to other legumes. The inhalation of food allergens may induce respiratory symptoms particularly during occupational exposure. All three patients described by Crespo *et al.* (2001) had no history of food allergies but due to the inhalant sensitization to lupine seed flour had developed symptoms after ingestion of lupine flour-containing products. In particular, there was no pre-existing food allergy to peanut or other legumes [25].

Parisot *et al.* (2001) described a 30-year-old environmental technician, who had developed rhinitis, conjunctivitis, and angioedema occurring repeatedly after handling lupine flour for SPT [24]. She was non-atopic and tolerated peanut ingestion without symptoms. A prick-to-prick test with flour of raw lupine seed as well as lupine-specific IgE-detection was positive. This is also a case of occupationally induced inhalant allergy to lupine.

An Australian investigation on 54 workers in a food processing company revealed seven symptomatic out of 11 lupine-sensitized patients (65%) who had developed an occupational lupine allergy with rhinitis and asthma *via* inhalation [5]. A high sensitization rate detected by SPT was reported, mostly correlating with symptoms. The clinical significance of cross-reactivity between legumes in SPT, however, remained unclear.

3.6.2 Inhalant sensitization *via* inhalation of pollen?

Evidence for sensitization by pollen inhalation is particularly poor. Many patients who have lupine allergy also have pollen allergy, which may basically be due to the fact that pollen allergy is so common. However, as the legume soybean contains a clinically relevant allergen that is homologous to Bet v 1, the authors have screened the publications on lupine allergy for the according confirmation. Homologous proteins are known in lupine. In total, there are 21 cases with pollen allergy mostly due to grass and birch pollen in patients with lupine allergy. However, it has to be stated that not all published cases have undergone a complete allergy diagnostic procedure and are sometimes not well documented [24, 26, 28, 47].

In their publication in 1999, Moneret-Vautrin *et al.* considered a primary sensitization to lupine pollen possible and hypothesized that this phenomenon could evolve toward sensitization to lupine seed and flour and even to cross-reactivity to peanut [26]. There is, however, not enough evidence for an inhalant allergy induced by sensitization to lupine pollen from the literature and own observations. However, it is very likely that sensitization to cross-reactive components in pollen is able to cause food allergy to lupine.

3.6.3 Gastro-intestinal sensitization *via* cross-reactivity to other legumes?

At least 23 peanut allergic patients have been described in the literature, who accidentally reacted with allergic symptoms after the ingestion of lupine-containing food. Four studies confirmed this phenomenon *via* DBPCFC in patients with a clinically relevant peanut allergy [7, 26, 44, 58]. Reports in the literature suggest that primary sensitization to peanut *via* the gastro-intestinal tract may result in a cross-reactivity to lupine, leading to secondary sensitization and food allergy to lupine in peanut allergic subjects. However, the existing evidence for this phenomenon is still not comprehensive.

3.6.4 *De novo* sensitization?

A 26-year-old woman with Mediterranean family background had often ingested lupine as snack food (*lupini*). Once she had developed urticaria, angioedema, and shortness of breath and was admitted to the hospital for emergency treatment. She had lupine allergen-specific IgE-antibodies and was positive in SPT with a saline extract of lupine bran. She had no allergies to inhalant allergens and no food allergy [57].

A 23-year-old female patient with celiac disease had developed generalized urticaria as well as angioedema, vomiting, profound hypotension, and loss of consciousness 1 h after ingestion of gluten-free pasta with tomato sauce. There was no history of allergic asthma, allergic rhinitis, or food allergy. Whereas IgE detection and SPT to milk, egg, tomato, fish, fruit, and vegetables were negative, allergen-specific IgE-detection was positive to peanut (2.1 IU/mL; IU, international unit) and lupine (> 100 IU/mL). SPT also was positive for fresh lupine as well as for peanut. The ingestion of peanut (> 50 g), however, was well tolerated. On the basis of these data the authors draw the conclusion of a primary sensitization to lupine [15], which seemed to be in contrast to the often published assertion that most cases of lupine allergy occur in peanut-allergic individuals. This phenomenon has been investigated in animals. In 2005, Lifrani *et al.* demonstrated in a mouse model that although antibody cross-reactivity can be induced between peanut and lupine by intraperitoneal and oral sensitization with peanut, not necessarily cross-allergenicity occurs [89]. More recently, Vinje *et al.* (2009) established a mouse model of lupine allergy. There, C3H/HeJ mice received sensitization and challenge by oral administration with lupine using cholera toxin as adjuvant. The mice showed a shift towards a T-helper cell type 2 response, confirmed by a high level of lupine-specific IgE antibodies in serum and predominant interleukin-4 production by splenocytes. In titration experiments (0.1–10.0 mg lupine protein) the optimal challenge dose to induce anaphylactic reactions was 5.7 mg [90]. Although immunological processes cannot directly be

transferred from mouse to man this model is interesting and may probably be used for pre-clinical evaluation of novel approaches to immunotherapy of lupine allergy.

Several studies in which additional immunoblot investigations were performed revealed IgE-reactivity to the following lupine proteins in mono-sensitized patients: 13; 14; 17; 21; 24; 29; 34; 38; 50; 35–55; 59; 60; 65; 66; 71 kDa in contrast to sera of peanut allergic patients: 14–21; 43; 66 kDa. Since the methods have not been exactly identical, these results have to be interpreted with caution, but it seems that the allergen profiles of lupine and peanut are not identical. The identity of the allergens is not yet known [22, 24, 26, 34, 44, 47].

3.6.5 Transcutaneous sensitization?

With regard to the respective observations in peanut allergy, there is also a possibility of the transcutaneous route of sensitization, although there is no evidence from the literature at present.

3.6.6 Intrauterine sensitization?

Frequent consumption of peanuts during pregnancy has been reported to be associated with an increased risk of peanut sensitization in young children [91]. This has not been observed so far for lupine sensitization.

The sensitization routes for lupine are still not entirely understood. The synopsis of all cases published so far reveals a nearly similar number of *de novo* sensitizations to lupine when compared with those individuals who clinically reacted allergic to lupine due to an underlying peanut allergy. This contradicts the often published statement that a lupine allergy is mostly resulting from a pre-existing peanut allergy.

However, peanut allergic patients are highly at risk of reacting to lupine-containing food with severe allergic symptoms. Therefore, those patients will definitely avoid peanut but not necessarily lupine when they are oblivious to cross-reactivity between peanut and lupine.

3.7 Allergy diagnostic procedures in cases of suspected lupine allergy

Lupine allergy belongs to the potentially life-threatening food allergies. Up to the 27th of September 2009 only one lupine allergen has been identified and admitted to the IUIS Allergen Nomenclature Subcommittee.

The sensitization routes as well as the significance of the cross-reactivity between lupine and other highly allergenic legumes are still not entirely elucidated. Investigations on threshold level(s) at which allergic symptoms develop are sparse but of great importance for the patients as well as the medical personnel diagnosing and treating allergies.

In order to obtain clinically relevant knowledge on lupine allergy it is essential to detect clinical symptoms to this emerging cause of food allergies:

A lupine allergy should be suspected if patients develop allergic symptoms after ingestion:

(i) of lupine-containing products
(ii) of food, which could probably contain lupine (Table 4)
(iii) of food allergens not immediately obvious as being causative
(iv) of flours in general
(v) of other legumes, particularly peanuts and soybean
(vi) or if they suffer from celiac disease with additional symptoms of food allergy [15]
(vii) or from idiopathic anaphylaxis (in the case of recent food ingestion)

In the case of the ingestion of new or hidden allergens the (false) diagnosis of idiopathic anaphylaxis may be made. Therefore, cases with this diagnosis should also be re-evaluated. As far as new allergens are concerned, in general no standardized and commercially available prick test solutions exist, meaning that allergologists depend on testing raw materials (prick-to-prick tests).

However, lupine as well as lupine pollens are available for allergen-specific IgE-antibody detection *via* a fluorescent enzyme immunoassay ImmunoCAP (Phadia, Uppsala, Sweden). To the best of our knowledge there is an SPT solution available in France. Diagnostic allergy testing for the confirmation of a lupine seed-sensitization or manifest food allergy for lupine should, therefore, consist of the detection of lupine seed-specific IgE-antibodies and an SPT (prick-to-prick test, respectively) with raw lupine flour or

Table 4. Lupine flour-containing foods (exemplified)

Regular bakery goods
Pasta
Pizza
Gingerbread
Rolls (*i.e.* ''hot-dog'' rolls)
Waffles
Gluten-free bakery products and foods for patients suffering from coeliac disease
Foods for patients allergic to milk proteins
Food products, mainly for vegetarians: *e.g.*
Cream cheese
Tofu
Sausages
Liquid flavour / spices
Veal or pork cutlet
Jam
Noodles
Coffee substitute
Ketchup
Traditional consumption of lupine
Lupini (cooked or dried lupine seeds served as snack food), ''altramuces'', ''tremoços'')

ground raw lupine seed or a commercially available lupine seed prick test solution. In case of confirmation, the diagnostic procedures should be extended to other legumes, particularly peanut and soybean. In order to prove or to exclude the clinical relevance of a lupine seed sensitization, challenge tests should be performed. DBPCFC is strongly advised because depending on the respective challenge protocol this procedure additionally allows the determination of threshold levels.

3.8 Lupine allergy and threshold levels

Available data on threshold levels vary considerably in quality, with relatively few studies providing the best quality individual data, using the low-dose DBPCFC. Amongst those, the most reliable data on threshold levels result from investigations in peanut-allergic patients [92].

All published data on this subject with regard to lupine are inconsistent, and mostly data on the differentiation between subjective and objective symptoms are lacking. According to an investigation of Moneret-Vautrin *et al.* (1999) the lowest (cumulative) eliciting dose (ED) was 265 mg of lupine flour inducing asthma and abdominal pain in peanut-allergic patients [26]. A study from The Netherlands has performed DBPCFC and detected subjective symptoms with concentrations of 1 mg or less up to 3 mg and objective symptoms at concentrations between 300 and 1000 mg [44]. The objective symptoms varied between a decrease of the forced expiratory volume (FEV 1) of about 50%, hoarseness, and rhino-conjunctivitis. The amount of 965 mg lupine flour, which in oral challenge test induced allergic symptoms, may easily be contained in 100 g bread and, therefore, is indeed relevant for lupine-allergic patients [26]. When compared with the threshold levels for other legumes as allergen sources, the data are the following: Ballmer-Weber *et al.* (2007) found the lowest observed adverse effect level for subjective symptoms with soy flour to be 10 mg with a corresponding protein content of 5.3 mg [93] and the lowest observed adverse effect level for objective symptoms with soy flour to be 454 mg (soy protein = 240.6 mg). Hourihane *et al.* detected subjective symptoms with 0.1 mg of peanut protein (0.2 mg of peanut) and objective symptoms with 2 mg peanut protein (corresponding with 4 mg of peanut) and more [94]. In a recent study from The Netherlands, Peeters *et al.* (2009) detected the lowest ED for lupine, inducing mild subjective symptoms, as being 0.5 mg, and the no observed adverse effect level as being 0.1 mg. The ED for lupine of 0.5 mg is low, and is only fivefold higher than for peanut. No predictive factors for lupine allergy were found [7].

Lupine is a new allergen with increasing significance and an ED, which is only fivefold higher than for peanut. Although the EC Directive has been amended and lupine has to be labelled there still may be a lot of undetected lupine allergy cases, which is due to the lack of awareness. In order to assess the risk and protect consumers, scientific studies, education, the quality of allergy diagnostic tests as well as the knowledge of threshold levels have to be improved to provide concise data on the basis of which law regulators and food industry can adapt their procedures of risk management.

4 Determination of *Lupinus* species in food

The content of lupine proteins in processed foods can be investigated by public food control laboratories. The first methods for the detection of lupine proteins by ELISA are based upon the serum of one lupine-allergic patient [22]. Since then sandwich-ELISA for the detection and quantification of lupine proteins in processed food have been developed, which are based upon polyclonal rabbit sera. This technique has a detection limit of 1 μg/g, is able to detect lupine protein in different food matrices [95], and is commercially available (Diagnostic Innovations, St. Asaph Business Park, UK; http://www.hallmarkav.com/page8.html). Demmel *et al.* developed a hybridization probe-based real-time-PCR for the detection of lupine DNA (*Lupinus* species) in foods. The method reliably detected 0.01 pg of lupine DNA, whereas 0.001 pg did not give any positive signals. The limit of detection of the method was reported as 0.1 mg/kg. The assay was shown to be suitable for the analysis of frequently encountered food matrices and was sensitive enough to successfully detect the presence or absence of lupine DNA [96]. As the threshold concentration responsible for the development of lupine-allergy is not known as yet, it is not certain whether the sensitivity of the above-mentioned detection methods is sufficient or not. A recently published study from Kaw *et al.* (2008) describes the development of a sandwich ELISA for the detection of lupine residues in foods with a limit of quantification of 1 ppm based upon rabbit antisera to *L. albus* as the capture reagent and sheep anti-serum as the detector reagent [97]. Minor cross-reactivity was detected with soybean and black bean. The authors claim this test to be an effective analytical tool to detect and quantify lupine residues in processed food. They, however, admit that further validation studies and investigations with additional model foods apart from beef frankfurter and apple cinnamon muffin have to be performed to finally consolidate the assay [97].

5 Concluding remarks

Lupine allergy belongs to the potentially life-threatening food allergies. Since the early 1990s of the last century lupine is increasingly consumed as ingredient in several processed foods. At least 151 cases of lupine allergy have been described worldwide, comprising a broad range of symptoms occurring after ingestion. However, all published

data on lupine threshold levels are inconsistent, and in particular data on the differentiation between subjective and objective symptoms are lacking. Furthermore, allergic reactions due to respiratory exposure mostly under occupational conditions with subsequent rhino-conjunctivitis and bronchial asthma have also been described, indicating that lupine may induce different entities of allergic diseases.

Lupine allergens are relatively stable with regard to thermal processing. According to the literature several sensitization routes are possible. Cross-reactions have been observed between lupine and other legumes like peanut, soybean, lentils, beans, chickpeas, and peas. Whereas cross-reactivity between lupine and soybean or lentils, beans and peas only rarely seems to be of relevance, the cross-reactivity between lupine and peanut more often is of clinical significance.

The major allergens of the *Lupinus* species are storage proteins, the conglutins. The *L. angustifolius* allergen β-conglutin has been designated Lup an 1 by the IUIS Allergen Nomenclature Subcommittee.

Since December 2006 in the EU the presence of lupine products in foods has to be labelled on ingredient lists as food that in sensitive persons may induce reactions.

This has improved the situation of patients who may be at risk of developing a lupine allergy. Sandwich-ELISA for the detection and quantification of lupine proteins in processed food have been developed, which are based upon polyclonal rabbit sera. This technique has a detection limit of 1 μg/g and is able to detect lupine protein in different food matrices.

However, those patients who are potential lupine-allergic individuals are still at risk of developing symptoms due to unintended contamination (cross-contact) of food with lupine protein. It is highly probable that lupine allergy will develop an increasing clinical significance. Therefore, it is strongly recommended to perform a differentiated diagnostic allergy procedure and investigations in order to obtain standardized allergens and test procedures leading to the correct diagnosis. Inclusion of lupine in allergen analysis as part of risk management plans of the food industry may contribute to reduce the risk for food allergic consumers.

This work is dedicated to Dr. Wolf-Meinhard Becker, Research Centre Borstel, Borstel, Germany, for his 65th birthday. Material (Fig. 1) is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

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