



Molecular characterisation of Lac s 1, the major allergen from lettuce (*Lactuca sativa*)

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Abstract

Background: IgE sensitisation to non-specific lipid transfer proteins (nsLTP), e.g., Pru p 3 the major allergen from peach and most important allergenic LTP, is strongly associated with severe symptoms in food allergic patients. Lac s 1, a member of the nsLTP protein family, was recently identified as major allergen in lettuce (*Lactuca sativa*), but has not yet been investigated on the molecular basis.

Objective: Molecular characterisation and immunological comparison of Lac s 1 to peach allergen Pru p 3.

Methods: Lac s 1 cDNA was cloned by RT-PCR and natural (n) Lac s 1 was purified by a two-step chromatography. Protein structure was verified by N-terminal sequencing, mass spectrometry, and circular dichroism spectroscopy. Immunoblotting, ImmunoCAPTM, and competitive IgE binding experiments were performed to study the IgE sensitisation pattern and cross-reactivity with Pru p 3. Allergenic potency was analysed by histamine release assay.

Results: Twenty-nine lettuce allergic patients, with or without concomitant peach allergy, and 19 peach allergic patients without lettuce allergy were included in this study. IgE reactivity to lettuce was due to mono-sensitisation to Lac s 1 or cross-reactive glycan structures. Two Lac s 1 isoforms were identified which showed amino acid identity (aa-id) of 62% to each other, up to 66% to Pru p 3, and 72% to the N-terminal peptide of plane pollen LTP Pla a 3. The prevalence of IgE binding to nLac s 1 was 90% using lettuce extract in immunoblotting experiments. Enhanced sensitivity was observed in ImmunoCAPTM using purified nLac s 1 in comparison to extracts (93% versus 76%). Although IgE sensitisation to Lac s 1 and Pru p 3 was strongly associated, the two LTPs showed different IgE binding properties. Sensitisation to LTPs does not necessarily reflect the clinical disease, but Lac s 1 was capable of triggering histamine release as shown by positive skin test results in Lac s 1 mono-sensitised patients and by *in vitro* mediator release assays.

Conclusion: Purified nLac s 1 will enhance the sensitivity in component resolved diagnosis of lettuce allergy. Similar to other cross-reactive food allergies, exclusive testing of IgE reactivities to LTP cannot be used as biomarker for clinical relevance. Our data provide indirect evidence that Pru p 3 might act as the primary sensitising agent in patients allergic to both lettuce and peach.

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1. Introduction

Like many other common vegetables (e.g., endives, chicory, and artichokes) or sources of weed pollen allergens such as mugwort and ragweed, lettuce (*Lactuca sativa*) belongs to the

Compositae (Asteraceae) family. Lettuce allergy is not frequently reported in the literature, but exists in clinical practice, predominantly in the Southern part of Europe. Several case reports have described lettuce as a cause of occupational contact dermatitis combining delayed and immediate type reactions (Krook, 1977; Paulsen et al., 1998), lip and facial swelling after ingestion, and aggravation of pre-existing dermatitis (Oliwiecki et al., 1991). Recent studies reported systemic reactions including anaphylactic reactions after ingestion of lettuce

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(Miguel-Moncin et al., 2003; Olive-Perez and Pineda, 2003). Enrique et al. reported that approximately 22% of the Spanish patients with plane (*Platanus*) pollinosis and food allergy present allergy to vegetables including lettuce (Enrique et al., 2002).

Initial case reports have shown that multiple proteins of lettuce exhibit IgE binding properties (Helbling et al., 1994; Vila et al., 1998), whereas others reported a predominant reactivity with a protein of 42–48 kDa (Franck et al., 2000; Escudero et al., 1999; Cadot et al., 1996), which has not been identified so far. Very recently, Miguel-Moncin et al. (2003) described a 9 kDa protein as major allergen in 10 out of 14 lettuce allergic patients, which was identified by N-terminal sequencing as non-specific lipid transfer protein (nsLTP) and was denominated as Lac s 1. All lettuce allergic patients reported concomitant allergy to *Platanus* pollen and most of them to several other foods including peach. Interestingly, 50% of the subjects experienced anaphylactic reactions to lettuce. In addition, the authors showed a partial cross-reactivity of IgE directed to Lac s 1 with *Platanus* pollen, but a more pronounced cross-reactivity with the lipid transfer proteins from the Rosaceae family, e.g., Pru av 3 from cherry (Scheurer et al., 2001) and peach. Pru p 3, the major allergen from peach (Lleonart et al., 1992; Pastorello et al., 1999), showed broad IgE cross-reactive properties with walnut and peanut extract (Asero et al., 2002), LTPs from mugwort and chestnut (Pastorello et al., 2002a; Díaz-Perales et al., 2000), Vit v 1 from grape (Pastorello et al., 2003), Zea m 14 from maize (Pastorello et al., 2000), Cor a 8 from hazelnut (Pastorello et al., 2002b), and Mal d 3 from apple (Sanchez-Monge et al., 1999). Although the highest degree of sequence identity of the N-terminus of Lac s 1 was found with Mal d 3, only one subject of the lettuce allergic patient group reported apple allergy (Miguel-Moncin et al., 2003). Therefore, the authors postulated a lack of correlation between sequence identity and clinical cross-reactivity.

Non-specific LTPs constitute a class of conserved proteins belonging to the PR-14 group of pathogenesis related proteins (van Loon and van Strien, 1999). In numerous studies, they have been described as major allergens in foods and pollen (reviewed by Ballmer-Weber, 2002; Pastorello and Robino, 2004; Salcedo et al., 2004). They appear as pan-allergens and possess a high stability against thermal processing and gastrointestinal digestion. It is generally believed that these properties are responsible for the high rate of severe food allergies caused by LTPs. So far, LTPs have mainly been described as predominant allergens in the Mediterranean area. Few studies have reported sensitisation to this protein family in Central and Northern Europe (Schad et al., 2005; Reuter et al., 2006) and food allergies caused by LTPs have not been described in the USA. The reasons for the differences in geographical distribution of LTP sensitisation are still unknown. One hypothesis is a primary sensitisation to Pru p 3, caused by high consumption of peaches over a long period each year, starting very early in life in the Mediterranean area and a subsequent cross-reactivity with LTP proteins present in other foods. Moreover, some researchers have discussed the possibility that nsLTPs from certain pollens, which are less abundant in Central and Northern Europe, may act as primary sensitising

allergens (Breiteneder and Mills, 2005; Pastorello and Robino, 2004).

In our study, we identified the primary structures of Lac s 1 isoforms and analysed the allergenic potency of natural Lac s 1 purified from lettuce. Since an association between lettuce and peach allergy is known (Miguel-Moncin et al., 2003) and the peach LTP Pru p 3 is one of the most clinically relevant members of this protein family, we investigated the co-sensitisation, IgE cross-reactivity, and allergenic potency in comparison to nPru p 3 from peach.

2. Methods

2.1. Patients

Three study groups were recruited at the Allergy Department of the Institut Universitari Dexeus in Barcelona, Spain: lettuce allergic patients with (A) and without (B) peach allergy, and peach allergic patients without concomitant lettuce allergy (C). Inclusion criteria were a clear clinical history of type I allergic reactions after ingestion of lettuce and/or peach and specific IgE against the respective food as determined by *in vivo* and *in vitro* tests. A detailed clinical history of symptoms related to lettuce and peach ingestion was recorded for all patients. Skin prick tests (SPT) were performed using commercial extracts of *Romaine* lettuce (LETI Laboratories, S.L., Barcelona, Spain) and peach (ALK-Abello Laboratories, Madrid, Spain) (Bruijnzeel-Koomen et al., 1995). For prick-to-prick techniques, performed according to the standard method of Dreborg and Foucard (1983), a lancet was pricked into fresh lettuce leaves of *Romaine*, iceberg, mini-*Romaine* and butterhead lettuce varieties just before pricking the skin. Histamine dihydrochloride and phosphate-buffered saline (PBS) solution served as positive and negative controls, respectively. The skin tests were considered positive if the wheal diameter was 3 mm greater than the negative control, 15 min after testing. Specific IgE was quantified using ImmunoCAPTM (Phadia, Uppsala, Sweden), a fluorescence enzyme immunoassay, for lettuce (f215) and peach (f95). Oral challenge tests were not performed due to the risk of severe reaction in patients with sensitisation to nsLTPs. Written informed consent was obtained from all patients and the study was approved by the local ethical committee of the Institut Universitari Dexeus of Barcelona (Spain).

2.2. Preparation of protein extracts

L. sativa var. butterhead was obtained from the local market. Leaves were homogenised (Bio-Homogeniser M133/2280, ESGE, Switzerland) followed by the previously described low temperature extraction method (Vieths et al., 1992). Briefly, 10 g of the homogenised material was extracted for 4 h by 4 °C in approximately 10–15 ml phosphate buffered saline (PBS, 20 mM phosphate, 150 mM NaCl, pH 7.4) containing 2% suspended polyvinyl polypyrrolidone (PVPP), 2 mM ethylenediamine tetraacetic acid disodium salt (EDTA), and 10 mM sodium diethyl dithiocarbamate (DIECA) (Björkstén et al., 1980) and solid material was then removed by centrifugation

(12,000 × g, 4 °C for 30 min). For purification by FPLC, the supernatant was first dialysed against PBS for 12 h and subsequently for 48 h against 50 mM sodium acetate pH 5. The extract was filtrated through a 0.22 µm pore diameter membrane (Sartorius, Göttingen, Germany).

2.3. Amplification, cloning, and sequencing of *Lac s 1* cDNA

Total RNA was prepared from butterhead lettuce by the RNeasy Plant Kit (Qiagen, Hilden, Germany) and subjected to reverse transcription with oligo Not d(T) oligonucleotides. cDNA was amplified by PCR using oligo Not d(T) and a degenerated forward primer (5'-AAG TGY GGY GTY ARC ATC CC-3') derived from the conserved sequence of ragweed LTP Amb a 6 (Acc. No. U89793). For identification of the unknown 5'-cDNA end, a 5'RACE was performed using the Gene Racer Kit (Invitrogen, Carlsbad, CA, USA) and the degenerated antisense primers 5'-CAR TCR GTG SWR GGR CTG TC-3' and 5'-GTG SWG GRC TGAT CTT GTA AGG-3', respectively. To obtain cDNA containing for a complete *Lac s 1* open reading frame, a PCR was performed using 5'-GTA AAC TTG AAA AAT GGC AAG GAT GGC A-3' (sense) and 5'-TCA CTG CAC CTT GGA GCA ATC AGT G-3' (antisense) or 5'-GTG ACA TGT GGT CAG GTG GTG GGC GC-3' (sense) and 5'-TCA CTG GAT CCT ATT GCA GTC GGT G-3' (antisense) as oligonucleotide primers (stop codons are underlined). PCR products were cloned and analysed by sequencing (MWG, Munich, Germany).

2.4. Purification of natural *Lac s 1* from lettuce and *Pru p 3* from peach

Natural *Lac s 1* (n*Lac s 1*) was purified as described by Pastorello et al. (1999) with some modifications. Protein extract (850 ml) derived from 680 g of butterhead lettuces was loaded on a cation exchange Mono S HR 5/5 column (GE Healthcare Biosciences, Uppsala, Sweden) with a flow rate of 0.7 ml/min. Proteins were fractionated by using a gradient of ammonium bicarbonate (0–1 M) at a flow rate of 1 ml/min. *Lac s 1* containing fractions were identified by SDS-PAGE, Coomassie Brilliant Blue or silver staining, and immunoblotting with a cross-reactive rabbit antiserum raised against *Pru p 3*, pooled and lyophilised. After reconstitution in 1 ml H₂O, the partially purified protein was applied to Superdex 30 HR 16/60 (GE Healthcare Biosciences) equilibrated in 20 mM MOPS, 0.5 M NaCl, pH 7.4 and fractions containing pure n*Lac s 1* were pooled. Protein concentration was determined using the BCA assay (Pierce, Cheshire, UK) using BSA as standard.

Peels from approximately 1.5 kg of peaches were used for the purification of natural peach LTP n*Pru p 3*. The purification was performed identically to the method described for n*Lac s 1* except for some modifications. Approximately 600 ml protein extract was applied to a Mono S HR 5/5 column (GE Healthcare Biosciences) with a flow rate of 1 ml/min. For the subsequent step of size exclusion chromatography, a Superdex 75 HR 10/30 (GE Healthcare Biosciences) was used. Peach LTP contain-

ing fractions were identified with an antiserum raised against *Pru p 3*.

2.5. Structural analysis

For N-terminal sequencing aliquots of purified nsLTPs were subjected to SDS-PAGE and Coomassie Brilliant Blue staining. The protein bands were excised and analysed using a 492 Procise sequencer (Applied Biosystems, Foster City, USA). Electrospray ionisation mass spectrometry (ESI/MS) by nano LC-MSD XCT plus analyser (Agilent) was performed to determine the molecular mass of nsLTPs. Purified proteins were dialysed against 25 mM ammonium hydrogen carbonate buffer. The final protein concentration was 50 µg/ml. Circular dichroism (CD) spectrometry was performed using a Jasco J-710 spectropolarimeter (Jasco, Groß-Umstadt, Germany) with a step width of 0.2 nm and a bandwidth of 1 nm to verify the secondary structure of *Lac s 1* and *Pru p 3*. LTPs (250 µg each) were dialysed against 10 mM KH₂PO₄ and measured within a spectral range of 185–255 nm at 50 nm/min.

2.6. SDS-PAGE and determination of specific IgE

Protein extracts, protein fractions, or purified *Lac s 1* were applied to SDS-PAGE under non-reducing conditions (*T* = 15%) and transferred by semi-dry blotting (45 min, 150 V) onto a nitrocellulose (NC) membrane (0.2 µm, Schleicher und Schuell, Dassel, Germany). NC membranes were blocked with skim milk (5% in Tris buffered saline, 0.05% Tween 20). For detection of IgE binding to lettuce extract (60 µg/cm slot), patient sera were diluted 1:6.7. For analysis of IgE binding to purified n*Lac s 1* (1 µg/cm slot), patient sera were diluted 1:10. Detection was performed with a biotin-labelled anti-human antibody (1:750, KPL, Gaithersburg, Maryland, USA) and visualisation was performed with alkaline phosphatase-conjugated streptavidin (1:3000, Caltag Laboratories, Burlingame, CA, USA) and BCIP/NBT (Bio-Rad, Munich, Germany) as substrate. For immunoblot inhibition assays, sera were preincubated with lettuce extract (100 µg), skim milk (100 µg), MUXF (Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc)-, or MM (Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc)-glycopeptides (25 µg each).

Monitoring of nLTPs purification by chromatography was performed using SDS-PAGE, Coomassie Brilliant Blue and silver staining, and immunoblotting with a cross-reactive rabbit anti-*Pru p 3* antiserum (1:20,000, Dr. D. Barber, ALK-Abello, Madrid, Spain) and a biotin-labelled anti-rabbit IgG antibody (1:6000, Dako, Hamburg, Germany). Visualization was done as described above.

Quantification of specific IgE to n*Lac s 1* and n*Pru p 3* was performed by experimental ImmunoCAP™ testing. For this approach, approximately 5 µg of each purified allergen was coupled to the solid phase as described previously (Marknell et al., 2002). Specific IgE was quantified in kU/L and ranked in CAP classes: class 0: ≤0.35 kU_A/L; class 1: 0.35–0.7 kU_A/L; class 2: 0.7–3.5 kU_A/L; class 3: 3.5–17.5 kU_A/L; class 4: 17.5–50 kU_A/L; class 5: 50–100 kU_A/L; class 6: >100 kU_A/L.

For linear relationships of two parameters the Pearson's correlation coefficient (R) analysis was applied, two-tailed probabilities were calculated. p -Values <0.05 were regarded as significant.

2.7. IgE inhibition experiments

IgE cross-reactivity between LTPs was assessed by an ELISA inhibition assay. The assay was optimized by titration of antigens on the solid phase and serial dilution of patient sera. Inhibition of IgE binding to nLac s 1 was performed with three different patient sera: EDP 1:10 (0.125 μ g Lac s 1), MSCH 1:16 (0.25 μ g Lac s 1), and SGR 1:16 (0.25 μ g Lac s 1). Inhibition of IgE binding to Pru p 3 was determined by the same patient sera: EDP 1:10 (0.25 μ g Pru p 3), MSCH 1:20 (0.25 μ g Pru p 3), and SGR 1:20 (0.25 μ g Pru p 3). For the inhibition assay, 96 well microtiter plates were coated overnight at 4 °C with purified nLTPs ranging from 0.125 to 0.25 μ g/ml (indicated above). After washing with 0.05% Tween-20 in PBS, the coated wells were blocked for 30 min with PBS buffer including 1% bovine serum albumin (BSA) followed by a washing step and incubation of 50 μ l diluted serum (indicated above) mixed with 50 μ l specific inhibitor (overnight, room temperature). The inhibitor concentration ranged from 0.001 to 10 μ g/ml. Thereafter, the wells were washed and incubated with 100 μ l peroxidase-labelled anti human IgE antibody (No. 9160-05, SBA, Birmingham, AL, USA, 1:2000 dilution) for 2.5 h at room temperature. After washing, the colorimetric reaction was started by addition of 100 μ l peroxidase substrate buffer (210 mM citrate buffer, pH 3.95; 3 mM H₂O₂; 1 mM tetramethylbenzidine). This reaction was stopped by adding 50 μ l of 3 M H₂SO₄. The optical density was measured at 450 nm. Indicated values are mean values of a two independent determinations.

2.8. Histamine release assay

Basophils isolated from blood of a non-allergic donor were stripped of their unspecific IgE by lactic acid treatment as described previously (Foetisch et al., 2003). The basophils were then resensitised with IgE of patients' serum, followed by stimulation with lettuce extract (10–10,000 ng/ml), or purified allergens (in the range of 0.1–10,000 ng/ml), or with BSA (10 μ g/ml) as negative control. Antigen induced release of histamine was quantified by a commercial competitive enzyme immunoassay (Immunotech, Marseille, France). Histamine release was calculated as percent of the total amount of histamine determined after lysis of the basophils by two-fold freezing and thawing. After subtraction of the spontaneous release, the result was considered to be positive if a release $>10\%$ of total histamine was obtained.

3. Results

3.1. Patients

Forty-eight Spanish patients were included in the study: 29 lettuce allergic patients, of which 23 had a concomitant peach allergy (group A), and 6 did not react to peach (group B), and

19 peach allergic patients without lettuce allergy (group C). Anamnestic and serological data are given in Table 1.

Of the 29 patients in groups A and B, 21 (72%) reported systemic symptoms, including anaphylactic reactions ($n=15$), urticaria ($n=3$), angioedema ($n=3$), and gastrointestinal symptoms ($n=2$) after ingestion of lettuce. Isolated Oral Allergy Syndrome (OAS) was diagnosed in 8 patients. 3 out of 6 lettuce allergic patients without peach allergy (group B) reported lettuce induced anaphylactic reactions. Among the 42 patients in groups A and C, peach allergy was associated with systemic reactions in 32 (76%) patients, while 10 (24%) exclusively reported OAS after ingestion of peaches. In addition to peach allergy, immediate hypersensitivity reactions to hazelnut ($n=18$, 62%), peanut ($n=17$, 59%), and walnut ($n=15$, 52%) were the most frequently reported concomitant food allergies in the lettuce allergic patients (groups A and B). Among the same patients, 23 (72%) were allergic to plane pollen, whereas only 5 suffered from an associated mugwort pollen allergy.

As an inclusion criterion additional to clinical allergy, all patients displayed sensitisation to lettuce and/or peach as determined by *in vivo* and/or *in vitro* assays. All patients except one have been evaluated by skin prick test and showed positive results with the respective allergens. One lettuce allergic patient (YSF) was not subjected to *in vivo* testing because of severe atopic dermatitis. In comparison to skin prick tests performed with commercial lettuce extract solution, prick-to-prick testing with fresh lettuce revealed stronger reactivities in 7/17 lettuce allergic patients who were tested with both methods. In addition, 9 patients who were not reactive using commercial lettuce prick test solution were positive after prick-to-prick testing. A positive skin result with peach was obtained for one of five tested lettuce allergic patient without peach allergy (LMC). In addition, a positive skin result with lettuce was obtained in at least 6 out of 19 subjects who tolerated lettuce (group C). IgE sensitisation to lettuce and peach was confirmed by commercial ImmunoCAPTM with respective food extracts for 22/29 (76%) lettuce allergic patients and 39/42 (93%) peach allergic patients.

3.2. IgE sensitisation pattern to *L. sativa*

Analysis of IgE binding patterns of lettuce allergic patients by immunoblotting of butterhead lettuce showed a frequency of IgE binding to nLac s 1 of 90% (26/29) (data not shown). The profiles were characterized by either, a mono-sensitisation to nLac s 1, or co-sensitisation to high molecular weight (HMW) proteins with molecular masses above 50 kDa and a protein with an apparent molecular mass of 24 kDa in single cases. Two out of 3 patients without reactivity to Lac s 1 exclusively reacted with HMW proteins and were positive (ImmunoCAPTM class 2) to lettuce extract. IgE binding to HMW proteins and the 24 kDa protein was completely inhibited after preincubation of patient sera with MUXF-glycopeptide, whereas no competition of IgE binding was obtained using MM-glycopeptide and skim milk as inhibitors (data not shown). These results show that IgE reactivity to a 24 kDa protein and HMW proteins, which have not been identified so far, is determined by cross-reactive glycan structures.

Table 1
Clinical history and specific IgE determination (CAP class/kU/L) of lettuce and peach allergic patients

Patient	Age/sex	Lettuce				Peach				Other food allergies
		Symptoms	SPT/prick-prick [mm ²]	Extract CAP	nLac s 1 CAP	Symptoms	SPT	Extract CAP	nPru p 3 CAP	
(A) Lettuce and peach allergic patients										
MLR	33/F	A	25/150	1 (0.65)	4 (23.4)	A	+	3 (4.19)	3 (7.64)	Asparagus
MAE	34/F	A	20/35	2 (0.97)	3 (5.12)	A	+	3 (4.50)	3 (5.57)	Walnut peanut
LCGH	25/F	G	9/348	2 (2.72)	3 (16.1)	OAS	+	3 (16.4)	3 (11.2)	Apple, pear, peanut
STV	30/F	A	neg/35	0	3 (5.95)	U	+	2 (0.89)	2 (0.80)	Green beans
MNF	28/F	OAS	35/nd	2 (2.18)	3 (13.2)	OAS	+	4 (18.6)	3 (16.6)	Cherry, hazelnut, mugwort, walnut, peanut
AMA	27/F	A	20/nd	2 (1.30)	4 (20.4)	U	+	4 (19.0)	4 (27.2)	Hazelnut, peanut, sunflower seeds
SGR	33/F	A	neg/nd	2 (2.96)	3 (15.9)	U	+	3 (11.3)	3 (13.1)	Hazelnut, walnut, sunflower seeds, apple
SSG	28/F	A	30/60	2 (2.72)	3 (16.8)	OAS, U	+	4 (19.6)	3 (15.1)	Hazelnut, walnut, peanut, green bean
EDP	25/F	OAS	20/nd	2 (0.71)	3 (7.48)	AE	+	3 (5.00)	3 (6.68)	Cherry, peanut, melone
AFC	34/M	A	20/nd	2 (0.79)	3 (5.77)	OAS	+	2 (2.50)	2 (2.87)	Cherry, hazelnut, onion, peanut, tomato, walnut, apple
NSR	31/F	A	24/90	0	5 (61.9)	OAS		3 (13.4)	3 (15.0)	pomegranate, apple
EMB	35/F	A	nd/45	1 (0.52)	2 (2.73)	OAS	+	2 (2.32)	2 (1.63)	Walnut
MSCH	27/F	U	neg/30	2 (1.34)	3 (9.84)	U	+	3 (10.5)	3 (15.3)	Hazelnut
NMM	32/F	OAS	20/nd	1 (0.65)	3 (4.51)	U	+	2 (3.20)	2 (3.17)	Hazelnut
PAL	6/F	A, U	neg/120	1 (0.46)	3 (5.14)	AE	+	3 (16.0)	3 (10.8)	Apple, nuts
PGR	24/F	OAS	35/90	1 (0.36)	2 (1.38)	CU, Rh	+	2 (1.37)	2 (1.56)	Cherry, apple, pear, apricot, nectarine, kiwi, hazelnut, almond, walnut, celery, peanut
EMG	30/F	A	neg/126	2 (2.00)	3 (9.07)	U	+	3 (12.1)	3 (12.0)	Cherry, hazelnut, walnut, peanut, rice, maize
ABR	35/F	OAS	neg/30	2 (0.94)	3 (5.15)	U	+	3 (3.70)	3 (4.83)	Grapefruit, tomato, maize, walnut, sunflower seeds, green beans
RRM	38/M	AE, U	neg/54	2 (0.73)	3 (6.64)	U	+	3 (6.77)	3 (5.28)	Cherry, hazelnut, walnut, peanut, cereals, pistachios
DPM	26/F	AE	neg/110	1 (0.67)	2 (2.19)	AE	+	3 (5.82)	2 (3.19)	Cherry, hazelnut, apple, pear, almond, walnut, peanut, kiwi
MMM	15/F	OAS	30/nd	0	2 (3.22)	OAS	+	3 (4.00)	3 (6.02)	Hazelnut, peanut, walnut
AGG	7/F	AE	20/nd	0	2 (1.58)	A	+	3 (5.32)	3 (4.25)	Cherry, peanut
EBO	30/F	A	25/25	1 (0.42)	3 (3.78)	U	+	3 (4.50)	2 (3.44)	Hazelnut, tomato, peanut
(B) Lettuce allergic patients without peach allergy										
MMG	24/F	A	neg/28	0	3 (4.69)	NO	neg	nd	2 (1.80)	Hazelnut, peanut, maize, apple
GGG	37/F	OAS	20/nd	0	0	NO	neg	nd	3 (6.08)	Hazelnut, peanut, green bean, mugwort
NPT	28/F	OAS	neg/36	2 (0.75)	0	NO	neg	nd	1 (0.66)	Hazelnut, fish
GVC	23/F	A	25/nd	2 (2.10)	3 (11.4)	NO	neg	nd	3 (11.4)	Hazelnut, peanut, tomato, banana, walnut
YSF	36/F	G	nd*/nd*	2 (1.68)	2 (1.17)	NO	nd*	nd	2 (0.79)	Tomato, hazelnut, banana
LMC	38/F	A	9/348	0	5 (66.4)	NO	+	4 (20.2)	5 (60.4)	Walnut, apple
(C) Peach allergic patients without lettuce allergy										
OSN	22/F	NO	nd/18	nd	0	U	+	3 (13.5)	4 (21.5)	Cherry, hazelnut, chestnut
MLM	34/M	NO	neg	nd	3 (4.77)	U	+	2 (1.10)	3 (5.38)	Cherry, grape, soybean
FGT	26/M	NO	neg	nd	3 (14.1)	U	+	2 (1.95)	3 (8.05)	Hazelnut, peanut, walnut
RFJ	42/F	NO	nd/nd	nd	2 (2.56)	OAS	+	2 (1.24)	2 (0.73)	
EMM	43/F	NO	neg/35	nd	0	U	+	2 (0.79)	2 (0.71)	Cherry, hazelnut, apricot, walnut, peanut, bean
LME	31/F	NO	neg	nd	0	OAS	+	0	1 (0.47)	Cherry
MJBC	24/F	NO	neg	nd	2 (0.84)	AE	+	2 (1.12)	1 (0.64)	Cherry, peanut, walnut, melone
OPN	24/M	NO	neg	nd	0	U, AE	+	2 (1.39)	1 (0.61)	Cherry, apple, kiwi, plum, peanut
OMS	19/M	NO	35/35	nd	3 (4.60)	U, AE	+	3 (4.05)	3 (5.30)	Cherry, apple
MDC	17/F	NO	neg	nd	1 (0.53)	OAS, AE	+	2 (2.05)	2 (2.05)	Cherry, apple, melone
IGB	30/F	NO	neg/25	nd	0	U, AE	+	0	0	Cherry, walnut, peanut
SCP	26/F	NO	30/24	nd	4 (37.4)	AE	+	4 (37.5)	4 (44.8)	Walnut, peanut
MRO	38/F	NO	nd/nd	nd	3 (5.47)	OAS	+	1 (0.38)	0	Cherry, hazelnut, kiwi, apple, pear

Patient	Age/sex	Lettuce				Peach				Other food allergies
		Symptoms	SPT/prick–prick [mm ²]	Extract CAP	nLac s 1 CAP	Symptoms	SPT	Extract CAP	nPru p 3 CAP	
RGP	24/F	NO	neg	nd	3 (4.64)	U	+	3 (3.90)	3 (5.70)	Cherry, hazelnut, tomato, kiwi, sunflower seeds, rice, maize, pineapple
RMJ	10/F	NO	neg	nd	0	U	+	2 (1.28)	2 (1.05)	
CMC	34/M	NO	neg	nd	0	U	+	2 (0.79)	1 (0.37)	Hazelnut
LGB	14/F	NO	24/nd	nd	2 (1.63)	U, OAS	+	2 (1.00)	2 (1.43)	
MPC	26/F	NO	neg/nd	0	0	OAS	+	0	0	Peanut
DGP	7/F	NO	neg	nd	Empty	U	+	2 (1.63)	Empty	Peanut

SPT: commercial skin prick test using protein extracts from lettuce or peach. Prick-prick: prick-to-prick test with native material. A, anaphylaxis; G, gastrointestinal symptoms; OAS, Oral Allergy Syndrome; U, urticaria; AE, angioedema; CU, contact urticaria; Rh, rhinoconjunctivitis; nd*, not determined because of severe atopic dermatitis.

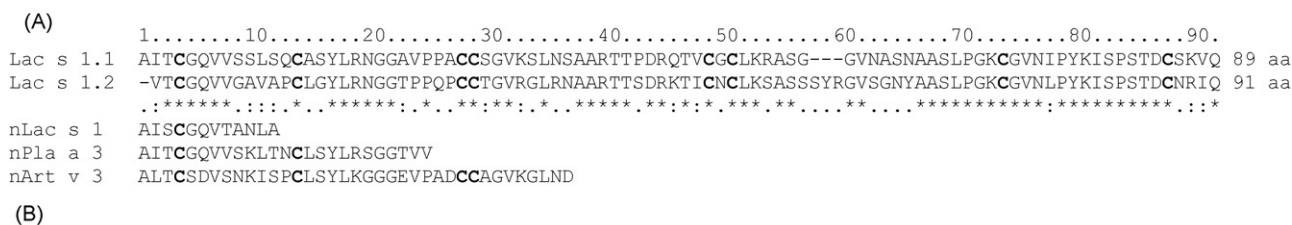
dicted molecular masses are 8891 Da for Lac s 1.1 and 9410 Da for Lac s 1.2.

Deduced amino acid sequences of two cloned cDNAs encoding distinct isoforms of Lac s 1 are shown in Fig. 1A. The open reading frames correspond to mature polypeptides of 89 amino acids for Lac s 1.1 and 91 amino acids for Lac s 1.2. Lac s 1.1 and Lac s 1.2 matched at 7/12 (58%) and 5/12 (42%) positions with the N-terminal sequence of purified nLac s 1, respectively. Both isoforms are characterised by 8 conserved cysteine residues and an amino acid identity of 62% between each other. In comparison with other food LTPs, both Lac s 1 isoforms showed the highest amino acid identity (aa-id) with Pru p 3 from peach (56% and 66%) (Fig. 1B). Aa-id up to 27% were observed with nsLTPs from pollen, Amb a 6 from ragweed, and Par j 1 and Par j 2 from pellitory. Notably, 72 and 56% aa-id was observed with a N-terminal peptide from plane pollen LTP, Pla a 3 (Lauer et al., 2007) for Lac s 1.1 and Lac s 1.2. Lac s 1 isoforms showed 46 and 54% aa-id with the known N-terminal amino acid sequence of Art v 3 from mugwort pollen (Fig. 1A). An isoelectric point of 9.22 was calculated for Lac s 1.1 and 9.5 for Lac s 1.2. The pre-

Approximately 2 mg nLac s 1 could be purified from 680 g butterhead lettuce, and approximately 10 mg nPru p 3 were obtained from 1.5 kg peaches. Obviously no process or product derived impurities were observed after purification of nLac s 1 and nPru p 3. Secondary structure analysis by CD-spectrometry of nLac s 1 and nPru p 3 yielded identical spectra showing α -helical proteins with typical minima at 208 and 222 nm, indicative of a tightly folded structure typical of members of this protein family (Fig. 2).

3.5. IgE binding to *Lac s 1* is strongly associated with sensitisation to *Pru p 3*

Three selected patient groups were compared by IgE binding to nLac s 1 and nPru p 3 using experimental ImmunoCAPs



	Lac s 1.1	Lac s 1.2	Pru p 3	Amba 6	Par j 1	Par j 2
Lac s 1.1	100	62	66	25	19	22
Lac s 1.2		100	56	27	19	20
Pru p 3			100	27	19	18
Amba 6				100	25	24
Par j 1					100	35
Par j 2						100

Fig. 1. (A) Amino acid sequences of Lac s 1.1 (Acc. No. EF101532), Lac s 1.2 (Acc. No. EF101531), and the N-terminal amino acid sequence of nLac s 1, Pla a 3 from plane (Lauer et al., 2007) and Art v 3 from mugwort pollen (Acc. No. P0C088). "Bold" cysteine residues, "*" identical, ":" similar, "." non-conserved residues. (B) Amino acid identities (%) between Pru p 3 (Acc. No. AJ277163), Amb a 6 (Acc. No. U89793), Par j 1 (Acc. No. P43217), Par j 2 (Acc. No. P55958) and both Lac s 1 isoforms.

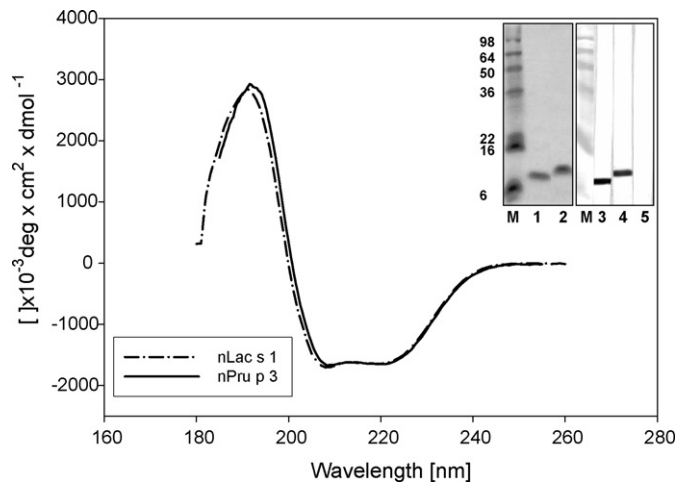


Fig. 2. CD-spectra of purified nLac s 1 and nPru p 3. Upper right: Coomassie Brilliant Blue staining of nLac s 1 (1) and nPru p 3 (2), and IgE binding of nLac s 1 (3) and nPru p 3 (4). (M) Molecular weight marker, (5) buffer as negative control.

(Table 1, Fig. 3). The prevalence of IgE sensitisation was 93% (27/29) for nLac s 1 in lettuce allergic patients and 90% (38/42) for nPru p 3 in peach allergic patients. The frequency of IgE binding was 100% for nLac s 1 and nPru p 3 in patients with lettuce and peach allergy (group A), 67% for nLac s 1 and 100% for nPru p 3 in lettuce allergic patients without peach allergy (group B), and 56% for nLac s 1 and 83% for nPru p 3 in peach allergic patients without lettuce allergy, respectively (group C). Sensitisation to nLac s 1 was associated with IgE binding to nPru p 3 in all cases except one (MRO). In contrast, 8 subjects sensitised to nPru p 3 showed no IgE binding to nLac s 1. All study groups showed a significant linear correlation of specific IgE titres for nLac s 1 and nPru p 3 (A: $p=0.007$, B and C: $p<0.0001$, Fig. 3).

3.6. Cross-reactivity between Lac s 1 and Pru p 3

Three sera from patients of group A were used to study cross-reactivity between Lac s 1 and Pru p 3 by reciprocal IgE inhibition experiments. In ELISA inhibition experiments using nLac s 1 on the solid phase, nPru p 3 and nLac s 1 (self-inhibition) showed identical inhibition curves for all sera (Fig. 4A). In contrast, preincubation of patient sera with nLac s 1 was found to result in incomplete inhibition of IgE binding to nPru p 3 on the solid phase (Fig. 4B). Maximal inhibition values of 52–61% were calculated for nLac s 1 as inhibitor, whereas 75–82% inhibition of IgE binding was obtained with the same concentration (10 $\mu\text{g/ml}$) of nPru p 3. Hence, the data indicate that nLac s 1 contains a reduced number of epitopes recognized by the IgE antibodies of the sera studied, as compared to nPru p 3 and/or that its IgE binding is of lower affinity than that of nPru p 3.

3.7. Allergenic potency

The histamine release assay was used to assess the biological activity of the allergens studied. Using sera of patients SGR and SSG, who were both allergic to lettuce and peach, nLac s 1 trig-

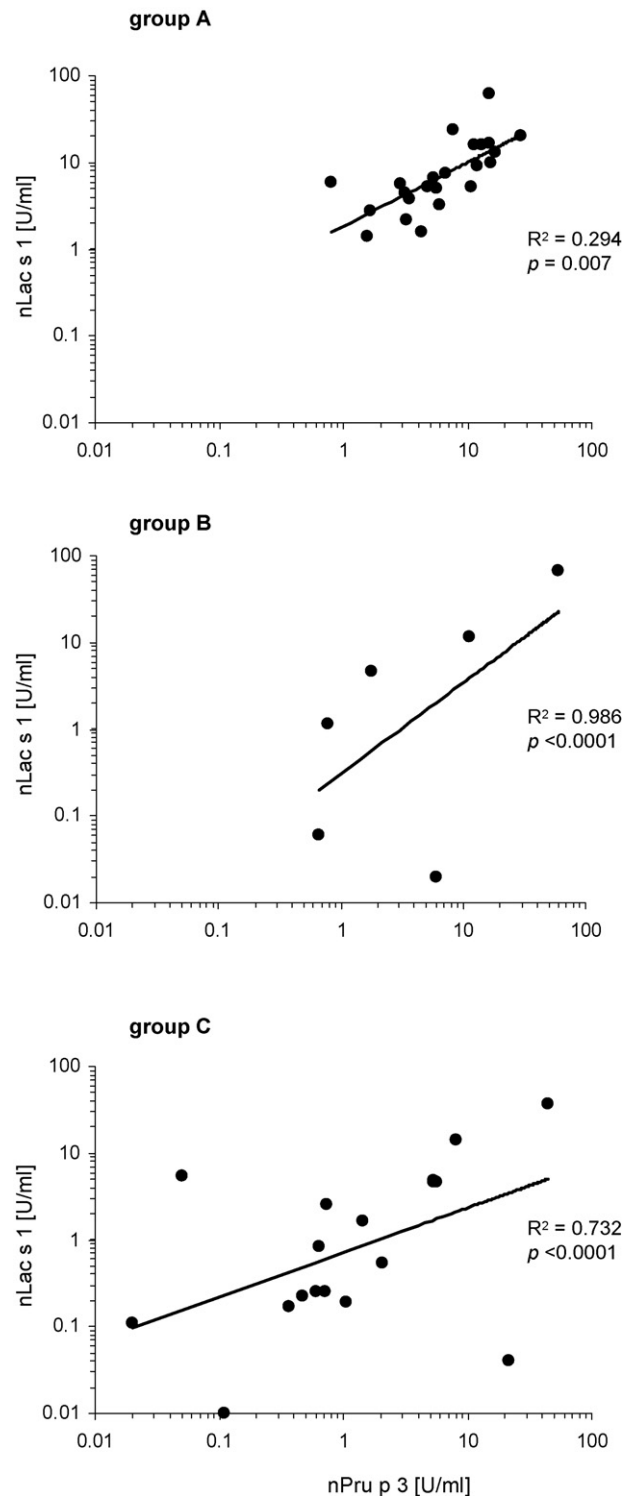


Fig. 3. Comparison of CAP values [U/ml] of nLac s 1 and nPru p 3 from lettuce allergic patients with peach allergy (A, $n=23$) and without peach allergy (B, $n=6$), and peach allergic patients without lettuce allergy (C, $n=18$). Trend lines, coefficients of determination (R^2), and two-tailed probabilities (p) are given.

gered histamine release up to approximately 20% while nPru p 3 triggered up to 50% (SSG) (Fig. 5). The high histamine release values obtained with nPru p 3 supported the strong allergenic potency and clinical relevance of the peach allergen. In case of the serum of patient LMC, who was allergic to lettuce but not

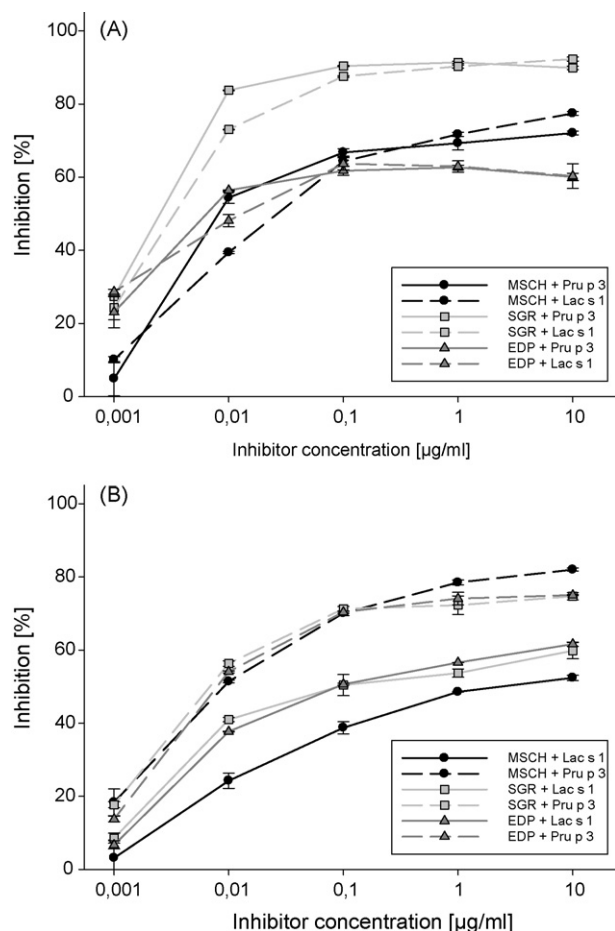


Fig. 4. Analysis of IgE cross-reactivity between nLac s 1 and nPru p 3 by competitive ELISA experiments. Dose-dependent inhibition of IgE binding to (A) Lac s 1 and (B) Pru p 3 after preincubation of individual patient sera (MSCH, SGR and EDP) with Lac s 1 and Pru p 3, respectively. The experiment was performed twice, error bars are depicted.

to peach, the CAP testing was negative with lettuce extract, but showed extremely high values for nPru p 3 (60.4 kU/L) and comparable values for nLac s 1 (66.4 kU/L). Instead, the histamine release induced by nPru p 3 was very weak (maximum values at approx. 15%), whereas nLac s 1 triggered a maximum histamine release of >50%. Moreover, maximum mediator release values (15–35%) were determined for lettuce extract only for the highest concentration used, which was in concordance with the analysis of low or negative specific IgE against lettuce extract (Table 1).

4. Discussion

Although the frequency of immediate hypersensitivity reaction to lettuce is considered to be low, we were able to recruit 29 Spanish patients with allergic symptoms after ingestion of lettuce, including 15 patients who had experienced anaphylactic reactions. Clinical history of lettuce allergy was supported by detectable sensitisation in all subjects. Prick-to-prick testing of lettuce was more sensitive than skin prick tests with commercial skin prick solutions, presumably due to reduced LTP content in the prick solution.

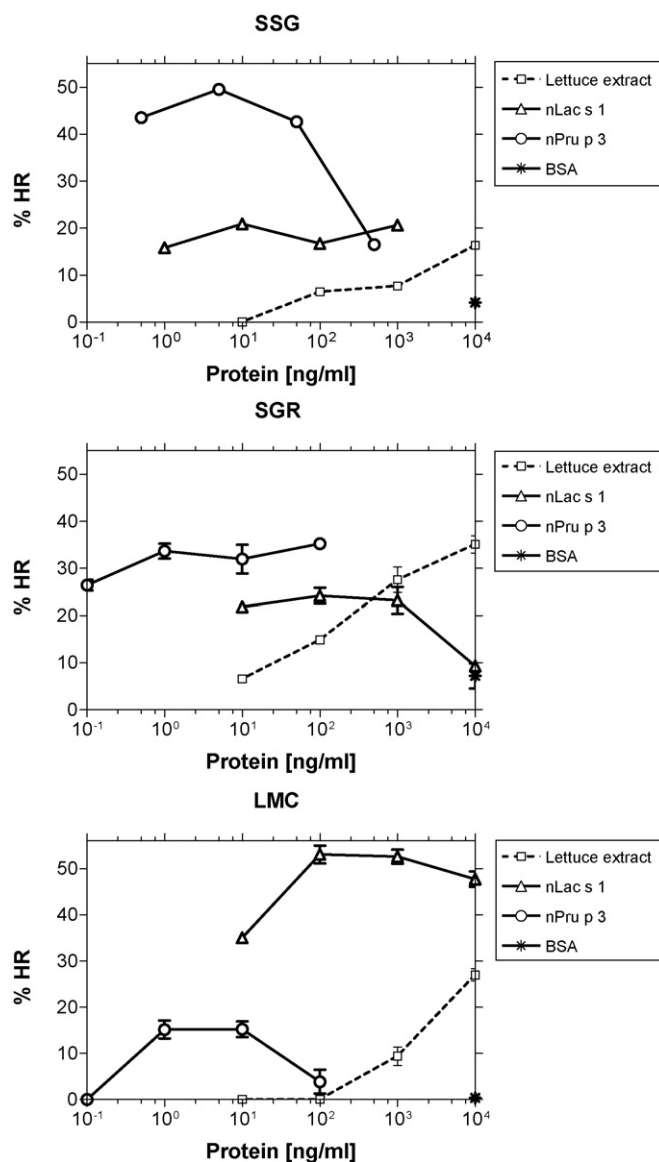


Fig. 5. *In vitro* histamine release values after stimulation of basophils passively sensitised with IgE from three lettuce allergic patients (SSG, SGR and LMC). Cells were stimulated dose-dependently with lettuce (butterhead) extract, nLac s 1, and nPru p 3. BSA (10 μg/ml) was used as negative control.

We did not find differences in the protein profile of several lettuce varieties (chicory, butterhead, iceberg, (mini)-*Romaine*, frisee) or between younger (inner) and older (outer) leaves, but we cannot exclude slight differences in the level of LTP accumulation in the leaves (data not shown). Lac s 1 was confirmed as the major allergen in *L. sativa* var. butterhead and mini-*Romaine* (Miguel-Moncin et al., 2003). IgE binding to a 24 kDa protein and HMW proteins (>50 kDa) (Miguel-Moncin et al., 2003; Escudero et al., 1999; Franck et al., 2000; Cadot et al., 1996), was due to cross-reactive carbohydrates, CCDs (data not shown).

The generation of oligonucleotides for the cloning strategy was based on the known cDNA of ragweed LTP Amb a 6, which belongs to the same botanical family (Asteraceae). We identified two Lac s 1 isoforms in butterhead lettuce by conventional

RT-PCR and subsequent 5'RACE. Aa-id up to 66% with Pru p 3 from peach and up to 72% with the N-terminal peptide of Pla a 3 from plane pollen explains IgE cross-reactive properties of homologous LTPs in lettuce allergic patients with concomitant peach and plane pollen allergy. Interestingly, the identified Lac s 1 isoforms showed relatively high aa-id with the N-terminal peptide of mugwort LTP Art v 3, also from Asteraceae, whereas the aa-id with Amb a 6 was lower. Moreover, the identified Lac s 1 sequences showed only a lower aa-id with the N-terminal sequence of nLac s 1 obtained from butterhead lettuce, indicating that both isoforms are members of a multigene family. The presence of both LTP-cDNAs in the preparation using material from one plant was confirmed by screening a cDNA library which was also derived from one butterhead lettuce (data not shown). However, we can only speculate whether both isoforms were expressed simultaneously as proteins. The heterogeneity of allergenic LTPs has been previously described in *Parietaria judaica* (Costa et al., 1994; Duro et al., 1996, 1997) and in certain foods, e.g., Cit s 3 of orange (Ahrazem et al., 2005), Aspa o 1 of asparagus (Diaz-Perales et al., 2002b), Fra a 3 of strawberry (Zuidmeer et al., 2006), and Mal d 3 of apple (Diaz-Perales et al., 2002a; Gao et al., 2005).

Since the identified isoforms were not identified as predominant molecules in the natural Lac s 1 preparation, and more likely have been selectively amplified by the applied PCR, we decided to use purified natural LTPs for the purpose of comparison of Lac s 1 with the major peach allergen Pru p 3 with respect to species specific IgE reactivity, cross-reactivity and allergenic potency. This strategy was supported by the fact that immunological comparison of recombinant Lac s 1 isoforms was hampered by loss of structural integrity due to intermolecular disulfide bonds using *E. coli* as heterologous expression system. Expression of rLac s 1 isoforms in *E. coli* resulted in low expression yields, oligomerisation, and partially unfolded protein structures as determined by CD-spectrometry (data not shown). Moreover, both Lac s 1 isoforms were found to lack IgE binding after expression in *P. pastoris* which was due to a proteolytic cleavage of the N-terminal ends following the conserved sequence YLRNGG₂₂ (data not shown). Due to these difficulties, an effort was instead to purify natural Lac s 1 from butterhead lettuce leaves.

As determined using an experimental nLac s 1 ImmunoCAP test, 93% of the lettuce allergic patients reacted to nLac s 1 of butterhead lettuce. These results are similar to the data obtained by immunoblotting of butterhead lettuce extract (90%). In contrast, the commercial lettuce ImmunoCAPTM (prepared from the same lettuce cultivar) showed a lower sensitivity (76%). Here, 6/7 lettuce allergic patients with a negative lettuce ImmunoCAPTM result tested positive when purified nLac s 1 was used as antibody-capturing reagent. Notably, IgE sensitisation was analysed by skin testing with lettuce allergens as well as with nLac s 1 in experimental ImmunoCAP tests in patients allergic only to peach and not to lettuce. This finding is in agreement with a report showing that sensitisation to mugwort LTP is frequently without clinical expression (Pastorello et al., 2002a). We observed a significant correlation of IgE binding to Lac s 1 and Pru p 3 in all study groups, but also cases of species specific

reactivities to LTPs in patients with a manifestation of lettuce or peach allergy (groups B and C). No IgE binding to Lac s 1 was found in 8/44 subjects sensitised to Pru p 3 and *vice versa* in 1/37 subjects sensitised to Lac s 1. Therefore, we conclude (1) that species specific IgE epitopes exist and (2) that reactivity of patients to non-Rosaceae LTPs from foods without IgE antibodies against Pru p 3 does not occur, except in very rare cases (Zuidmeer et al., 2005).

The postulated epitope heterogeneity between LTPs from different species was confirmed by dose-dependent inhibition assays. Pru p 3 showed a stronger inhibitory potency than Lac s 1, indicating a higher IgE binding capacity of Pru p 3 by an enhanced antibody affinity and/or additional epitopes not present on Lac s 1. Moreover, for single patients we found only a limited association between Lac s 1 and Pru p 3 specific IgE concentrations and no correlation between specific IgE values and the severity of symptoms. Allergenic potency of Lac s 1 was shown by positive skin reactivity using sera from LTP mono-sensitised patients and by *in vitro* tests. We showed the capability of Lac s 1 of triggering mediator release by histamine release assays performed with sera from three selected lettuce allergic patients. Although patients SSG and SGR had similar levels of IgE specific for the respective LTPs, the *in vitro* assay showed an individual response to the respective allergens. In contrast to patient LMC, the allergenic potency for Pru p 3 was higher than for Lac s 1 in peach allergic patients. Notably, lettuce allergic patient LMC showed higher specific IgE to Lac s 1 than to Pru p 3, and tolerated peach after ingestion. This indicates that besides cross-reactive epitopes also at least one Pru p 3 independent IgE epitope must exist on Lac s 1.

In summary, individual isoform- and species specific IgE reactivities against certain LTPs are important factors which may need to be considered in allergy diagnosis. Thus, including purified nLac s 1 in the *in vitro* test systems will improve the diagnosis of lettuce allergic patients. However, it has to be taken into account that IgE sensitisation to LTPs is frequently observed although no clinical manifestations are associated. We conclude that primary sensitisation to Pru p 3 is mostly required for the subsequent development of lettuce allergy in patients allergic to both, lettuce and peach.

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