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Identification and characterization of the major allergen of green bean (*Phaseolus vulgaris*) as a non-specific lipid transfer protein (Pha v 3)

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ABSTRACT

Background: Green bean (GB) has been reported to cause allergic reactions after ingestion, contact or inhalation of particles deriving from processing or cooking. Up-to-date no food allergens have been fully characterized in GB.

Objective: To characterize the GB major allergen(s) on a molecular level and to verify the involvement of non-specific lipid transfer proteins (nsLTPs) in GB allergy.

Methods: We recruited 10 Spanish patients reporting adverse reactions to GB. Skin prick tests, specific IgE detection and oral provocation were performed. Two nsLTP cDNAs were cloned from GB and over-expressed in *Pichia pastoris*. The recombinant LTPs (rLTPs) were characterized by circular dichroism spectroscopy and IgE-binding assays (immunoblotting and ELISA) with the patients' sera. Three natural LTPs (nLTPs) were further purified from GB fruit by chromatography. *In vitro* histamine release test was applied to compare the allergenic potency of rLTPs and nLTPs.

Results: Oral provocation test confirmed GB allergy. A 10 kDa protein in GB extract was recognized by 80% of the sera and identified as nsLTP. The two rLTPs (named LTP1a and LTP1b), share 61.3% aa identity and present the typical nsLTP-like secondary structure. The IgE-binding and histamine release assays provided evidence that rLTPs and nLTPs possess different allergenic potency.

Conclusions: nsLTP (Pha v 3) is the major allergen in GB and constitute a potential risk for patients affected by LTP-syndrome. GB encodes for several LTPs with different immune reactivity.

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1. Introduction

Green bean (GB), also known as French bean, snap bean or string bean is a leguminous plant belonging to Fabaceae family, genus *Phaseolus*, species *vulgaris*. The fruit is constituted by a slender green pod with small seeds inside. Differently from other bean species, the entire pod is edible. In various case reports GB has been indicated as responsible for allergic reactions after ingestion (Asero et al., 2001), contact or mere inhalation of particles deriving from processing or cooking of the fruit (Igea et al., 1994; Daroca et al., 2000).

A number of GB IgE-binding proteins with molecular mass ranging from 14 to 80 kDa have been detected by immunoblotting (Bernhisel-Broadbent et al., 1989; Igea et al., 1994; Daroca et al., 2000; Asero et al., 2001). In some cases the effect of heat treatment on IgE-binding capacity was analyzed and this turned out to be completely abolished for certain proteins (Daroca et al., 2000), while in other cases it persisted (Asero et al., 2001), suggesting the involvement of these proteins in the onset of symptoms after ingestion or inhalation. Up-to-date, no GB allergen has been included in the official allergen database of the allergen nomenclature subcommittee of the International Union of Immunological Societies (IUIS).

Recent clinical findings (Dr. Joan Bartra, unpublished) indicate that Spanish patients reporting adverse reactions after ingestion of GB manifest allergic reactions also to peach, lettuce and hazelnut. All these plants are described to express non-specific lipid transfer proteins (nsLTPs) capable of eliciting severe allergic reactions. These data suggest that nsLTPs might be involved also in the pathogenesis of GB allergy. nsLTPs represent a class of small polypeptides ubiquitously expressed in the plant kingdom which

Abbreviations: BCIP/NBT, 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium; GB, green bean; HR, histamine release; nLTP, natural lipid transfer protein; nsLTP, non-specific lipid transfer protein; OAS, oral allergy syndrome; PTB, PBS, 0.05% Tween 20, 1% BSA; PVDF, polyvinylidene fluoride; rLTP, recombinant lipid transfer protein; SPT, skin prick test.

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are known for their capability of binding and transferring different types of lipids between biological membranes (Kader, 1996). On the basis of molecular mass they are subdivided in 2 groups, nsLTP1 (9–10 kDa) and nsLTP2 (6–7 kDa) even though they are all characterized by a conserved pattern of 8 cysteine residues (Douliez et al., 2000). In scientific literature nsLTP1s are often defined as nsLTPs (or LTPs) since the majority of characterized nsLTPs belong to the nsLTP1 group. Besides their biological activity in plants nsLTPs are also reported to cause severe allergic symptoms in many patients, especially in the Mediterranean area (Asero et al., 2000; Pastorello and Robino, 2004; Fernandez-Rivas et al., 2006). In particular, Pru p 3, the peach nsLTP, is considered the panallergen involved in the primary sensitization of the subjects allergic to nsLTPs (Asero et al., 2000; Pastorello and Robino, 2004). The severity of the symptoms seems to be correlated with the high resistance of these polypeptides to heat treatment and proteolytic digestion (Asero et al., 2000; Scheurer et al., 2004). The multiple sensitizations to various nsLTP-containing foods experienced by LTP-allergic subjects is a consequence of the high degree of IgE cross-reactivity among different nsLTPs even taxonomically distantly related (Asero et al., 2002; Diaz-Perales et al., 2000). This condition is also known as “LTP-syndrome” (Pastorello and Robino, 2004).

Here we demonstrate that different LTPs are expressed in GB and they constitute a major allergen.

2. Methods

2.1. Patients and sera

Ten patients reporting adverse reactions to green bean were recruited in Spain (three patients at the Allergy Department of Institut Universitari Dexeus in Barcelona and seven patients at the Unit of Respiratory Allergy and Pneumology of the Hospital Clinic of the University of Barcelona). Nine patients had a clear history of adverse reactions after ingestion of boiled GB. In addition, one patient (no. 10), who experienced contact urticaria to fresh GB but tolerated the boiled product, was also included. Written informed consent was obtained from all patients and the study was approved by the ethics committees of the hospitals. Skin prick tests (SPTs) with commercial extracts of airborne allergens (ALK-Abello, Madrid, Spain: dog and cat dander, dust mite, *Alternaria alternata*, and pollens from *Artemisia vulgaris*, *Chenopodium album*, *Cupressus arizonica*, *Olea europea*, *Parietaria judaica*, *Phleum pratense* and *Platanus acerifolia*) and of plant foods (ALK-Abello or LETI laboratories, S.L., Barcelona, Spain: green bean, peach peel, lettuce, hazelnut, lentil, walnut, peanut, kiwi, maize, mustard) were performed by standard procedures (Dreborg and Frew, 1993). Histamine dihydrochloride (10 mg/mL) and PBS/glycerol solution served as positive and negative controls, respectively. A mean wheal diameter of 3 mm or larger (15 min after puncture) compared with that of the negative control was considered a positive response. Specific IgE for GB (f315) and Pru p 3 (f420) was measured by ImmunoCAPTM (Phadia, Uppsala, Sweden). Values >0.35 kU_A/L were considered as positive. An open labial challenge with boiled green bean was performed in 5 patients in order to protect them from exposure to higher doses of ingested allergen and the risk of severe reactions. A sample of boiled GB (15 min) was wiped across the upper lip. The labial challenge test was considered positive if signs such as erythema, swelling or urticaria were observed or itching and discomfort of the lip or in the mouth were referred by the patient.

2.2. Protein extraction

Fresh, ripe GB fruits were obtained from a local market. The pod of each fruit was dissected in order to separate it from the seeds.

Seeds and pods were ground in liquid nitrogen and extracted in PBS (1:3, w/v) for 1 h adding a complete protease inhibitors cocktail (Roche, Mannheim, Germany) and 1% polyvinylpyrrolidone (PVPP). The extracts were centrifuged at 10,000 × g at 4 °C and the supernatants collected and filtered through Whatman paper. The samples, named “pod extract” and “seed extract”, respectively, were stored at –20 °C.

For protein purification 100 g of entire GB fruits (pod and seed together) were ground in liquid nitrogen and treated as described above in order to obtain the “GB-total-extract”. This was subjected to overnight dialysis against 20 mM Na-Acetate, pH 4.5 or otherwise stored at –20 °C. The proteins precipitated during dialysis were removed by centrifugation. The supernatant was subjected to a 3-step chromatographic procedure in order to purify putative nsLTPs.

- (1) A cation exchange chromatography was performed by an AKTA FPLC (GE Healthcare Life Sciences, Uppsala, Sweden) using a MonoS Column (GE Healthcare Life Sciences). The dialyzed extract was loaded on the column in 20 mM Na-Acetate buffer, pH 4.5 and eluted with a NaCl gradient from 0 to 0.25 M in the same Na-Acetate buffer. The absorbance was monitored at 280 nm.
- (2) The fractions showing the presence of 10 kDa proteins reactive with patients' sera by immunoblotting were collected and loaded onto a size exclusion High Load 16/60 Superdex-75 column (GE Healthcare Life Sciences) and run in 0.1 M ammonium-acetate by FPLC. The peak corresponding to proteins of approximately 10 kDa was collected and lyophilized.
- (3) Finally the samples were separated by RP-HPLC performed by a Beckman System Gold chromatographer (Beckman Coulter, Fullerton, CA), on an Alltima C4 column (Alltech, 4.6 mm × 250 mm, particle size 5 μm) at 40 °C. Absorbance was monitored at 214 nm by a Beckman 168 Diode Array Detector. Proteins were loaded in 0.1% trifluoroacetic acid–15% acetonitrile (ACN) and eluted by a linear ACN gradient from 15 to 35%. The flow rate was 1 mL/min. The eluted protein fractions were lyophilized.

2.3. Cloning of green bean LTP-cDNA

Seeds and pods from GB fruits were separated and finely ground with liquid nitrogen. Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to reverse transcription (RT) with First Strand cDNA kit (Pharmacia, Freiburg, Germany) using the Not I-d(T)₁₈ primer. RT-cDNA was amplified by PCR using a degenerated sense primer (primer A: 5'-GACCACCCWGACCGC-3') designed basing on a consensus sequence shared by the cDNA of a nsLTP from common bean (*Phaseolus vulgaris*) (Acc. N. U72765), named pvLTP-24, and the cDNA of Pru p 3 (Acc. N. AJ277163). The anti-sense primer (primer B: 5'-AACTGGAAGAATTCGCGGCCG-3') was complementary to the Not I-d(T)₁₈ primer used for RT-cDNA synthesis. For identification of the unknown 5'-cDNA end, a rapid amplification of the cDNA 5'-end (5'-RACE) was performed using the 5'-Race Kit (Invitrogen, Groningen, Netherlands) with the anti-sense primer (primer C: 5'-GCCAGTTGGTGGAGG-3'). By overlapping of the partial sequences of the 3'-ends and the 5'-ends the full length sequences of two LTPs named LTP1a and LTP1b were deduced. The full length cDNA of LTP1a and LTP1b was obtained by PCR using the primer B and two sense primers homologues to the signal peptide regions (primer C: 5'-CACACTGCCAGGGG-3' for LTP1a, and primer D: 5'-CACTGCACATGCCGCC-3' for LTP1b). The start of the mature protein was deduced by comparison with known nsLTP sequences. The PCR products were cloned in pCR II[®] TOPO Vector (Invitrogen) and analyzed by cDNA sequencing (MWG, Munich, Germany).

2.4. cDNA cloning and heterologous over-expression of green bean recombinant LTPs

The cloning of GB LTP-cDNA into pICZαA and integration into the yeast *Pichia pastoris* by means of electroporation was performed as previously described (Lauer et al., 2008). The expression of recombinant LTP1a and LTP1b was initiated by inoculation of a positive transformant (both Mut⁺ phenotypes) into 400 mL buffered sorbitol-complex medium (BMS), respectively. The pre-culture was incubated for 2 days at 30 °C in a shaking incubator until the culture reached an OD₆₀₀ = 2–3, and subsequently used for inoculation of 4 L BMS in a BioFlow[®] 110 fermenter (New Brunswick Scientific, NJ, USA). Process parameters were adjusted to temperature (30 °C), pH (5.0) and dissolved oxygen (30%), whereas the dissolved oxygen was controlled by the stirrer (100–500 rpm) and aeration (1–10 L/min). Within the first phase (24 h) of fed-batch fermentation 0.66 M sorbitol containing 33% methanol was used as the feeding solution, whereas in the second phase the feeding solution was changed to 100% methanol and the fermentation was continued for additional 71 h. Induction of expression of recombinant LTPs was induced after first addition of first phase feeding solution (12 h after inoculation). Culture supernatant was separated from the yeast cells by centrifugation (8000 × g for 20 min at 4 °C) and stored at –20 °C until purification.

2.5. Purification of recombinant GB LTPs

For purification of GB LTPs each culture supernatant was clarified by centrifugation and filtered through a 0.45 μm membrane and subsequently concentrated by ultrafiltration using an Ultrasette[™] Lab Tangential Flow Device (Pall GmbH, Dreieich, Germany) with 3 kDa cut-off. Purification of LTPs was performed after dialysis by a two step chromatography (ion exchange chromatography and size exclusion chromatography) according to Lauer et al. (2008).

2.6. Protein quantification

Protein concentration was measured by bicinchoninic acid assay (BCA assay, Pierce, Cheshire, UK) using bovine serum albumin (BSA) as standard. Plant extracts were previously desalted by PD10 column (GE Healthcare Life Sciences) in order to remove low molecular mass compounds (e.g. polyphenols) that might interfere with the assay.

2.7. SDS-PAGE and immunoblotting

SDS-PAGE was performed by Mini-Protein III apparatus (Bio-Rad, Munich, Germany) as described by Laemmli (1970) in 16% polyacrylamide gels. Samples of extracts (50 μg/cm) and of purified proteins (0.5 μg/cm) were diluted in 4× sample buffer (0.2 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS) and separated at constant 20 mA current under non-reducing conditions. Gels were stained by Coomassie R-250 Blue. Alternatively, proteins were electroblotted onto PVDF membrane (Millipore, Schwalbach, Germany) at 50 V × 150 min. The membrane was blocked with PTB (1% BSA, 0.05% Tween 20 in PBS) and probed with anti-Pru p 3 polyclonal antibodies (kindly provided by Dr. D. Barber) at 1:10,000 dilution or with patients' sera at 1:10 dilution. Rabbit IgG was detected with a biotin-conjugated anti-IgG antibody (1:10,000, Dako, Hamburg, Germany) and an alkaline phosphatase-conjugated streptavidin (1:5000, Caltag Laboratories, Burlingame, CA, USA). The detection was achieved by BCIP/NBT solution (Bio-Rad). Human IgE was detected by a monoclonal horseradish peroxidase-conjugated anti-IgE antibody (Southern Biotech, Birmingham, Alabama, USA) at 1:1500 dilution, followed by chemiluminescence reaction with ECL

(GE Healthcare Life Sciences). A Lumi-Imager F1 (Roche) apparatus was used for image acquiring. For IgE inhibition sera were preincubated with 10 μg of nPru p 3 per lane. nPru p 3 was purified in a previous study according to Pastorello et al. (1999).

2.8. N-terminal amino acid sequencing

The N-terminal amino acid sequencing of the purified natural GB LTPs was performed on a 492 Procise sequencer (Applied Biosystems, Foster City, CA, USA) in pulse-liquid mode by Edman degradation (Proteome Factory, Berlin, Germany).

2.9. Circular dichroism (CD) spectroscopy

CD spectra of purified recombinant GB LTPs and of nPru p 3 were recorded at a 20.0 μM concentration in 10 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.4) on a J-810 S spectropolarimeter (Jasco, Gross-Umstadt, Germany) as previously described (Lauer et al., 2008).

2.10. Histamine release assay

Peripheral blood mononuclear cells isolated from a non-allergic donor were stripped of their unspecific IgE by lactic acid treatment as described previously (Foetisch et al., 2003). The basophils were resensitized with IgE of patients' sera, followed by stimulation with GB-total-extract (0.01–10 μg/mL), natural and recombinant purified proteins (0.0001–1 μg/mL), or with BSA (1 μg/mL) as negative control. Antigen induced histamine release was quantified by 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. The results were considered positive when a release >10% of total histamine was obtained, after subtraction of the spontaneous release.

2.11. ELISA

ELISA inhibition was performed on Maxisorb 96 well-microplates (Nunc A/S, Roskilde, Denmark). LTP1b (50 ng per well) was non-covalently coated in PBS for 30 min at RT. After washing with PBS, 0.05% Tween 20 the wells were blocked with PTB for 30 min at RT. Sera were diluted at 1:10 in PTB and incubated overnight in the presence of inhibitors (nPru p 3, LTP1a, LTP1b) in a 0.001–10 μg/mL dilution range. A buffer control was considered (no serum incubation), while the 100% signal was given by the omission of inhibitor. Bound IgE was detected by incubation with a goat anti-human IgE antibody (1:1000, Abcam, Biozol, Eching, Germany) for 1 h followed by an horseradish peroxidase-conjugated anti-goat antibody (1:10,000, Jackson, Dianova, Hamburg, Germany) for 1 h, both diluted in PTB. Tetramethylbenzidine (TMB, BD Biosciences, Heidelberg, Germany) was used as chromogenic substrate. Absorbance was monitored at 450 nm by a SpectraMAX 340PC microplate reader (Molecular Devices, Munich, Germany). All analyses were carried out in triplicate.

3. Results

3.1. Green bean allergy is frequently linked to peach allergy

Ten adult patients (9 females and 1 male) were recruited. Nine of them presented different symptoms after ingestion of boiled GB, whereas one patient (no. 10) presented contact urticaria after exposure to fresh green bean but tolerated the ingestion of boiled green bean (see Table 1). Two patients reported GB-induced anaphylactic reactions. Interestingly, some patients had also experienced anaphylactic episodes after consumption of other plant foods, such as

Table 1

Clinical data of the GB allergic patients.

No.	Sex	Age	Symptoms to GB ^a	CAP (KU _A /L)		Positive SPT		Open challenge with GB
				GB	Pru p 3	Food allergens ^b	Aero-allergens ^c	
1	f	30	A	3.28	0.80	p, l	Pl	n.p.
2	f	35	U	4.59	4.83	p, mz, l, w	Pl, M	n.p.
3	f	28	U	9.03	15.1	p, h, l, w, pn	Pl, G	n.p.
4	f	31	OAS	7.24	6.28	p, mz, h, l, k	G, M, Pl, Dd, Cd, D	Positive
5	f	26	OAS	0.37	4.26	p, mz, l, h, w, pn,	G, Pe, Pl, M	Positive
6	m	19	A	12.6	17.6	p, mz, ms, l, ln, k	Pl, Pe	n.p.
7	f	23	GI	2.11	5.63	p, mz, h,	G, Pl, A, Dd, Cd	Positive
8	f	35	OAS, GI	0.28	5.97	p, mz, h, w, l, pn	Pl, Pe	Positive
9	f	24	OAS, GI	0.57	2.43	p, h,	G, M, Pl, Dd, Cd, D	Positive
10	f	40	CU	6.2	11.6	p, mz, l	G, Pl, Cd, D	Positive

n.p.: not performed.

^a A, anaphylaxis; GI, gastrointestinal symptoms; OAS, oral allergy syndrome; U, urticaria; CU, contact urticaria.^b p, peach; l, lettuce; h, hazelnut; ln, lentil; w, walnut; pn, peanut; k, kiwi; mz, maize; ms, mustard.^c A, *Alternaria alternata*; Cd, cat dander; Dd, dog dander; D, dust mite; G, timothy grass (*Phleum pratense*); M, mugwort (*Artemisia vulgaris*); Pl, plane tree (*Platanus acerifolia*); Pe: pellitory (*Parietaria judaica*).

peach and/or hazelnut and/or walnut (no. 4, 6, 7, 9) and lettuce (no. 5, 10) (not shown). Accordingly with the clinical symptoms, all patients showed skin reactivity to green bean and peach. Moreover, some of them were SPT positive to lettuce ($n = 8$), maize ($n = 7$), hazelnut ($n = 6$), and walnut ($n = 4$). Regarding pollen sensitization, all subjects showed positive SPT responses to pollen from *P. acerifolia* (plane tree). Nine patients out of 10 showed detectable IgE specific for GB by ImmunoCAPTM, while all the patients had IgE specific for Pru p 3. We performed a labial challenge in 5 patients (no. 4, 5, 7, 8, 9) who had given their written informed consent. All challenged patients presented OAS symptoms. The patient who referred contact urticaria with fresh GB (no. 10) was asked to manipulate fresh GB fruits with her hands in our presence. The onset of urticaria was confirmed.

3.2. LTP is the major allergen in green bean and cross-reacts with Pru p 3

Individual patients' sera were used for IgE-immunoblotting experiments to test the reactivity of GB-total-extract (Fig. 1A). A 10 kDa band, highly suggestive of nsLTPs, was detected by 8 out of 10 patients' sera (80%). It is noteworthy that also serum no. 8, resulted negative by ImmunoCAPTM measurement, recognized the 10 kDa band. In order to identify the 10 kDa IgE-binding protein an immunoblotting inhibition experiment was carried out pre-incubating the serum of 3 patients monosensitized to the 10 kDa

protein (no. 4, 6, 10) with nPru p 3. The results (Fig. 1A, lanes 11–13) showed a complete abolishment of the signal, indicating homologous proteins belonging to LTP1 family expressed in GB presenting common epitopes with Pru p 3. The same band was detected also by rabbit anti-Pru p 3 polyclonal antibodies confirming the previous results (Fig. 1B, lane 1). A control performed omitting the polyclonal antibodies tested negative (not shown). These data strongly suggest the involvement of Pha v LTP in GB allergy.

In order to determine whether GB LTPs are expressed in the pod or in seed the of GB fruit, an immunoblotting of the respective extracts was performed using a pool of the 10 sera. Fig. 1C shows that LTPs are predominantly accumulated in the pod, the external part of the fruit (Fig. 1C, lane 3).

3.3. Identification of green bean LTP1-cDNAs by RT-PCR

Since LTPs are localized mostly in the pod, we decided to use this tissue for LTP-cDNA cloning. The cDNAs belonging to two different nsLTP1s have been cloned (Fig. 2). LTP1a-cDNA (GenBank Acc. N. GQ434007) is 345 bp length, coding for a mature 91 amino acid protein and a predicted leader sequence of 24 amino acids. LTP1b-cDNA (GenBank Acc. N. GQ434008) is 354 bp length, coding for a 93 aa protein and a predicted leader sequence of 25 aa. The amino acid identity degree among the mature sequences is only 61.3%. A BLAST search showed higher aa identities with other plant nsLTP1s, in particular 76% identity among LTP1a and a nsLTP1 expressed in

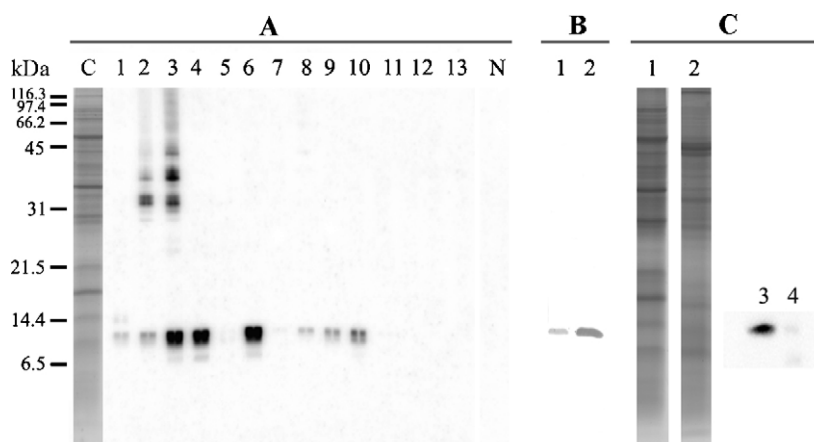


Fig. 1. (A) IgE-immunoblotting of GB-total-extract: lane C, Coomassie staining; lanes 1–10, patients' sera no. 1–10; lanes 11–13, pre-incubation of sera no. 4, 6, 10 with nPru p 3; lane N: non-allergic control. (B) Immunoblotting of GB-total-extract with anti-Pru p 3: lane 1, GB-total-extract; lane 2, nPru p 3. (C) IgE-immunoblotting of pod and seed extracts: Coomassie staining: pod (lane 1), seed (lane 2); immunoblotting (pool of all sera): pod (lane 3), seed (lane 4).

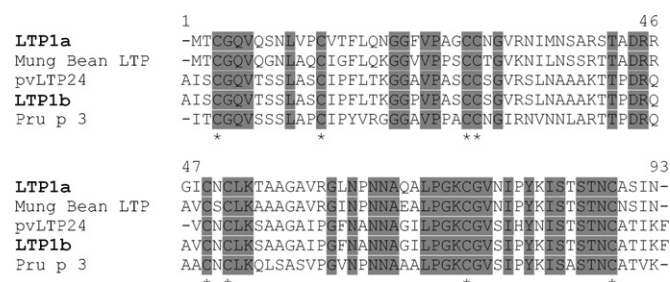


Fig. 2. Alignment of the mature protein sequences encoded by LTP1a and LTP1b cDNAs in comparison with the LTPs from peach, Pru p 3 (GenBank Acc. N. CAB96876) from common bean, pvLTP-24 (GenBank Acc. N. U72765) and from mung bean (UniProtKB Acc. N. P83434). The 8 conserved cysteines are marked (*).

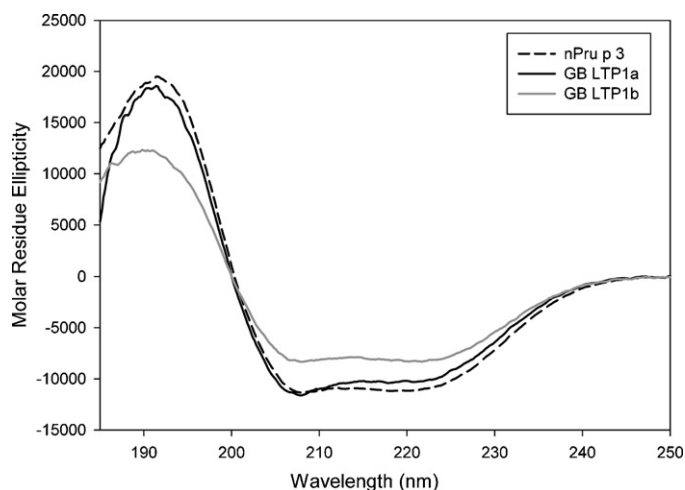


Fig. 3. CD spectrum of purified GB LTP1a and LTP1b compared with nPru p 3.

mung bean (*Vigna radiata*), and 95% among LTP1b and pvLTP-24 from common bean. The % identity of LTP1a and LTP1b in respect with Pru p 3 is 78% and 66%, respectively.

3.4. Expression and purification of recombinant *Pha v* LTPs

Although the same conditions were applied for expression of both LTP1a and LTP1b, the growth of *P. pastoris* showed different results. After 24 h of expression of the GB LTP1a the culture reached an $OD_{600} = 18$ and after 95 h an $OD_{600} = 39$. In contrast,

the expression of GB LTP1b reached an $OD_{600} = 11$ after 24 h and $OD_{600} = 16$ after 95 h. Even though the cell density of the culture for GB LTP1b expression was not high and the supernatant analyzed by SDS-PAGE and Coomassie staining showed no accumulation of LTP, the supernatant (4.0 L) was concentrated (33-fold) to 120 mL by ultrafiltration. The analysis of the concentrate showed the presence of GB LTP1b and therefore the purification was continued. Subsequent dialysis, ion exchange chromatography and size exclusion chromatography according to Lauer et al. (2008), resulted in 21.8 mg purified GB LTP1b equilibrated in MOPS-buffer (20 mM MOPS, 500 mM NaCl, pH 7.4) with a concentration of 0.8 mg/mL. GB LTP1a was concentrated from 3.6 L culture supernatant to a volume of 90 mL (40-fold). Further purification steps were performed as described above and yielded a final product of 19.4 mg purified GB LTP1a in MOPS-buffer with a concentration of 1.8 mg/mL. Purity was calculated >90% by Coomassie staining (see Fig. 4).

3.5. CD spectroscopy analysis

The results of CD spectroscopy analysis are depicted in Fig. 3. Both recombinant proteins showed the expected alpha-helix structure typical of nsLTPs characterized by the two minima at 208 and 222 nm and an intersection of the baseline at 200 nm. This is also confirmed by the similarity of the spectra with that obtained for nPru p 3. The difference in the amplitude of the curves might be due to different responsiveness of the two proteins to bichinchonic acid used for the quantification assay.

3.6. GB rLTPs show different IgE reactivity

The IgE-binding capacity of LTP1a and LTP1b was first assayed by IgE-immunoblotting using the patients' sera (Fig. 4). Sera no. 1 and 2 showed a generally weak reactivity towards both rLTPs. On the other hand, sera no. 3, 4 and 6 were characterized by a markedly higher reactivity for LTP1b than for LTP1a. Interestingly, the serum from patient no. 10, suffering from contact urticaria, recognized only LTP1b.

The cross-reactivity between LTP1a and LTP1b was analyzed by IgE ELISA inhibition. LTP1b was coated onto the immobilized phase and probed with 2 of the most reactive sera, sera no. 4 and 6. Vice versa, LTP1a was not used on the solid phase due to its low IgE reactivity. The results, depicted in Fig. 5, are expressed as % inhibition of IgE-binding. For both patients' sera nPru p 3 turned out to be the strongest inhibitor, while LTP1a resulted to be partially cross-reacting with LTP1b for serum no. 6, giving an inhibition of

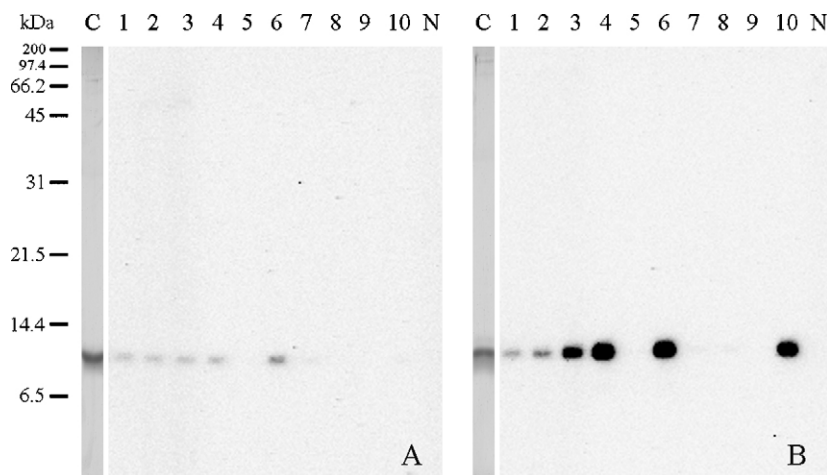


Fig. 4. IgE reactivity of GB LTP1a (panel A) and LTP1b (panel B) analyzed by IgE-immunoblotting. Lane C: Coomassie staining; lanes 1–10, patients' sera no. 1–10; lane N: non-allergic control.

approximately 60% at 10 $\mu\text{g/mL}$. No cross-reactivity was observed in the concentration range 0.001–10 $\mu\text{g/mL}$ for serum no. 4.

3.7. Purification of natural green bean nLTPs

In order to study the accumulation of GB LTPs we sought to purify the natural LTPs present in the fruit. Small amounts of five proteins (called P1–P5), almost comigrating by SDS-PAGE, could be separated by HPLC as demonstrated by the Ponceau S stained blot (Fig. 6A, lanes 1–5). Three of these proteins were detected by the pool of GB allergic patients' sera (Fig. 6B, lanes 1–3). By N-terminal sequencing these proteins were identified as putative LTPs (Fig. 6C). Besides 2 unidentifiable aa, the N-terminus of P1 is shared by both the sequences of the pvLTP-24 and LTP1b. P2 shares the same N-terminal sequence with P1, but the apparent molecular mass is definitely smaller (about 6 kDa). P3 is characterized by a slightly different N-terminal sequence lacking the first alanine present in P1 and P2 and encoding for Thr₂ instead of Ser₃.

3.8. GB LTPs are capable of inducing histamine release from basophils

The biological activity of the recombinant and the purified natural LTPs was confirmed by histamine release test. Basophils from a non-allergic donor were sensitized with IgEs present in sera no.

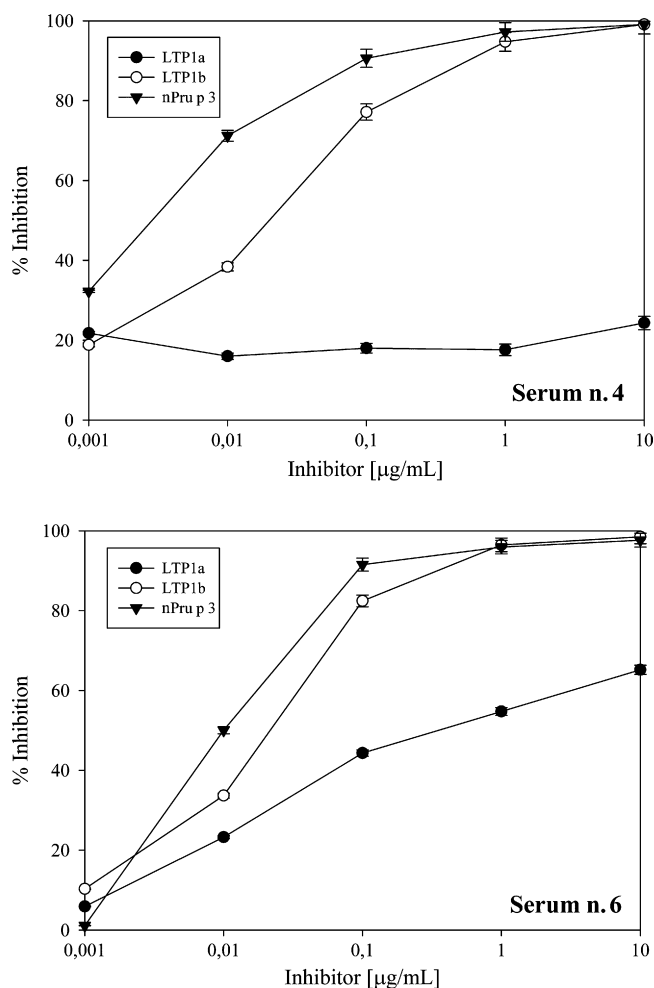
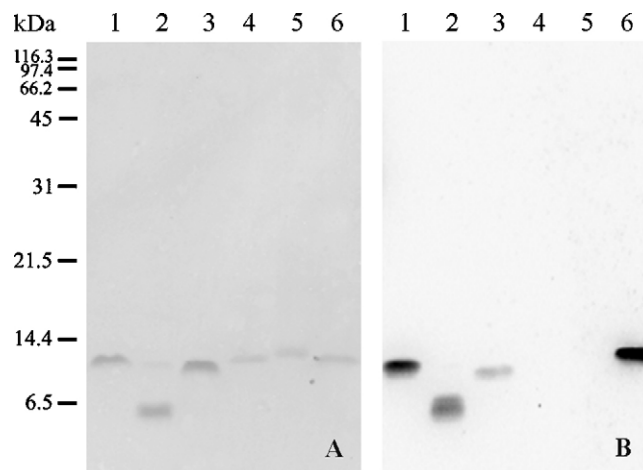


Fig. 5. Analysis of cross-reactivity between GB LTP1a and LTP1b performed by ELISA inhibition with sera no. 4 and 6. LTP1b was coated on the solid phase and the IgE-binding was inhibited with increasing concentrations of LTP1a, LTP1b and nPru p 3 from 0.001 to 10 $\mu\text{g/mL}$.



N-terminal sequencing of GB nLTPs

	1	2	3	4	5	6	7	8	9
P1	A	I	S	X	G	Q	V	X	S
P2	A	I	S	X	G	Q	V		
P3		I	T	X	G	Q	V	A	S
LTP1a		M	T	C	G	Q	V	Q	S
LTP1b	A	I	S	C	G	Q	V	T	S
pvLTP-24	A	I	S	C	G	Q	V	T	S

C

Fig. 6. SDS-PAGE and immunoblotting of the proteins purified from GB-total-extract. (A) Ponceau S staining. (B) Immunodetection with pooled GB LTP reactive sera. Lanes 1–5, purified GB natural proteins (P1–P5); lane 6, nPru p 3. (C) N-terminal sequencing of P1, P2 and P3, compared with the translated sequences of LTP1a, LTP1b and pvLTP-24 (mature protein). X: unidentifiable aa.

4 and 6, respectively. The results shown in Fig. 7 indicate that LTP1b seems to be more active in inducing histamine release in respect with LTP1a, whose activity is minimal for serum no. 4 even at high concentration (1000 ng/mL). At concentration >10 ng/mL the % release induced by LTP1b is almost comparable with that induced by nPru p 3. The other proteins show at least a maximum of 40% release. Dose-dependent mediator release is shown also for GB-total-extract inducing a maximum histamine release at higher concentrations (i.e. 1 $\mu\text{g/mL}$).

4. Discussion

Clinical association among GB and other legume allergies has rarely been reported, although patients allergic to legumes such as peanut possess IgE specific for GB proteins (Bernhisel-Broadbent et al., 1989; Bernhisel-Broadbent and Sampson, 1989; Ibanez et al., 2003). Only few cases of GB allergy have been described, and the information available concerning the allergens involved and their molecular characteristics is scarce (Asero et al., 2001; Igea et al., 1994; Daroca et al., 2000). In this work we recruited 10 GB allergic patients in order to identify the major allergens of GB. A subgroup of patients was orally challenged after clinical evaluation in order to confirm the clinical relevance of GB allergy.

By IgE-immunoblotting and IgE cross-inhibition experiments we first identified the presence of at least one protein belonging to the nsLTP1 family in GB. Even though GB is not described among the main foods responsible for LTP-syndrome, the clinical data seem to confirm this possibility. In fact, (1) 80% of the patients'

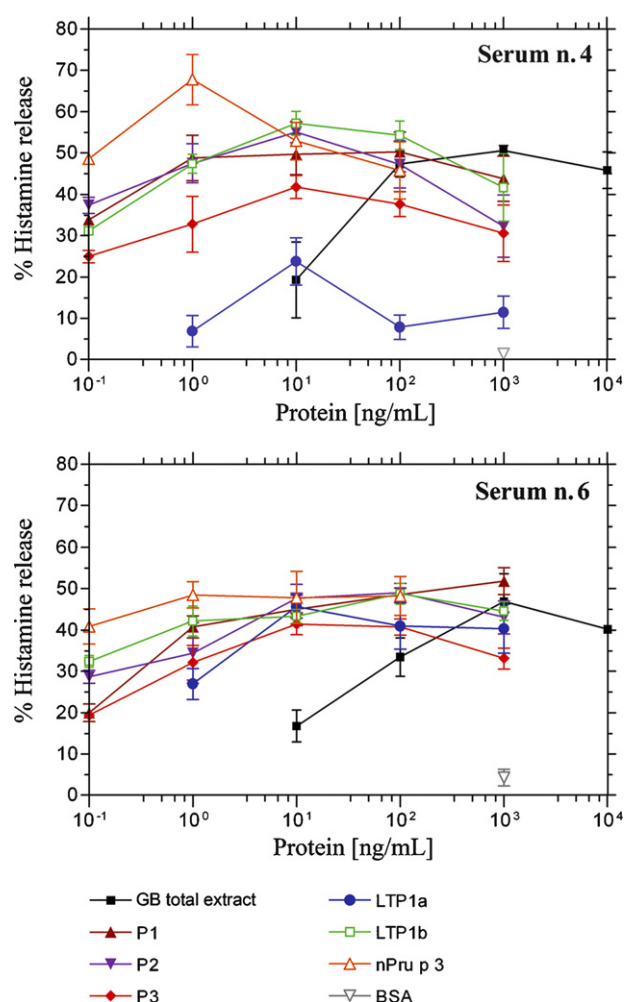


Fig. 7. Histamine release after stimulation of basophils sensitized with IgE from two GB allergic patients (no. 4 and 6). Cells were stimulated dose-dependently with GB-total-extract, LTP1a, LTP1b, P1, P2, P3 and nPru p 3. BSA (1 μ g/mL) was used as negative control.

sera recognized GB nsLTP1 and 6 patients were even monosensitized to this protein (see Fig. 1A); (2) all the patients displayed a sensitization profile typical for allergy to nsLTP: besides GB allergy, they showed in some cases clinical manifestations after ingestion of peach, hazelnut, lettuce and walnut among others. Accordingly, positive SPT responses to peach, hazelnut, lettuce, and maize were obtained in most of the patients. All these plant foods express nsLTPs reported as major allergens (Salcedo et al., 2007). The sensitization of the selected patients to pollens, and in particular to plane pollen, corroborates the hypothesis of LTP-syndrome since plane tree nsLTP (Pla a 3) is a major allergen for patients from Spain affected by peach allergy and its cross-reactivity with Pru p 3 is well known (Lauer et al., 2007). We demonstrated that IgE reactivity to LTP is limited, almost entirely, to the pod in respect with the seed. This observation is in agreement with the results of Asero et al. (2005) who found that LTP is normally not involved in allergy to common bean, taxonomically related to GB. Indeed, the pod of common bean is normally not consumed, due to previous elimination during preparation. As a consequence, patients with food allergy to LTPs would tolerate common bean, while patients affected by contact urticaria would suffer from handling the entire fruit, as patient no. 10 in our study.

By our cloning strategy two nsLTP cDNAs (LTP1a and LTP1b) were identified and subsequently over-expressed in *P. pastoris*. When probed with the patient's sera, LTP1a and LTP1b showed dif-

ferent IgE-binding capacity. Since the results of CD spectroscopy confirmed that both proteins possess the typical secondary structure of nsLTPs, and thus a correct folding could be assumed, the differences in IgE reactivities observed by immunoblotting and ELISA are reasonably explained by epitope differences between the two proteins. The frequency of sensitization and the reactivity of LTP1b were found to be generally higher than that of LTP1a. Considering the IgE reactivity pattern of LTP1b the results are mainly in line with the reactivity to GB-total-extract, showing strongest IgE-binding for patients 3, 4 and 6, and lack of IgE-binding to the 10 kDa protein for patient 5 and 7. However, the IgE-binding seems not to be related with the severity of the symptoms. The absence of reactivity of the serum of patients no. 8 and 9 to either LTP1a or LTP1b is in apparent contrast with the positive signal observed in the GB-total-extract (Fig. 1A). This result might be explained by further members of the LTP family expressed in GB whose epitopes are not represented by the recombinant proteins. The results of ELISA inhibition confirmed that LTP1a and LTP1b possess different IgE-binding capacities. It is noteworthy that in our experiments nPru p 3 turned out to be the strongest inhibitor. Even though further experiments are necessary to establish if this phenomenon is due to higher antibodies avidity or to additional epitopes of Pru p 3, the data collected suggest the involvement of this protein as a primary sensitizer in our GB allergic patients, as reported previously for other plant foods (Asero et al., 2000). All the patients were indeed sensitized to Pru p 3 as determined by ImmunoCAPTM (Table 1). A second route of sensitization, mediated by pollen inhalation, has been proposed for patients with food allergy to nsLTP, but it is still matter of debate (Lombardero et al., 2004; Lauer et al., 2007). In our case, since all patients were sensitized also to plane pollen, we cannot exclude that Pla a 3 might trigger the sensitization via respiratory route. The investigation of IgE-binding regions by techniques like epitope mapping (Garcia-Casado et al., 2003) could shed light on the different cross-reactivity between GB LTPs and Pru p 3.

In order to compare the IgE reactivity of recombinant LTPs with the natural counterparts we made efforts to purify the 9 kDa IgE-binding protein observed in Fig. 1. Three IgE-binding proteins (P1, P2 and P3) were actually isolated, and their N-terminal sequences turned out to match those of several known plant nsLTPs. It is remarkable that the N-terminal sequence of P1, besides two unidentifiable aa is identical to that of recombinant LTP1b indicating that these 2 gene products might be the same protein. Very recently Pravettoni et al. (2009) identified an IgE-binding protein in GB sharing the same N-terminal sequence with LTP1b, hence confirming the relevance of this protein as an allergen. It is noteworthy that, although P2 shares the same N-terminal sequence with P1, the molecular mass is smaller. It is unlikely that P2 is a degradation product of P1, as LTPs are known for their extraordinary stability and their IgE-binding capacity is determined by the tertiary structure of the protein. It is tempting to speculate that P2 might belong to LTP2 family. Some LTP2s have been characterized in other plants so far (Conti et al., 2001; Douliez et al., 2000), but their role as allergens has never been elucidated.

The rLTPs and nLTPs have been tested in order to evaluate their biological activity, i.e. their capacity of inducing histamine release from activated basophils. The results showed that LTP1a is not capable of inducing histamine release using serum no. 4 even at high protein concentration, whereas its biological activity is shown for serum no. 6. These results are in agreement with IgE reactivity testing (Figs. 4 and 5) and demonstrate that the two rLTPs are characterized by different allergenic potencies. LTP1a and LTP1b have been accepted in the IUIS official allergen database as Pha v 3.0101 and Pha v 3.0201, respectively. Among the purified natural LTPs, P1 and P2 were capable of inducing histamine at a level comparable with that triggered by nPru p 3. Unfortunately, the very low amount of nLTPs obtained was not sufficient to perform further

experiments (e.g. CD spectroscopy, IgE-immunoblotting) but the data collected suggest that P1 and P2 possess higher potency in comparison to P3. Previous publications dealt with the identification of different nsLTPs from the same plant source and with their immune reactivity testing (Lauer et al., 2009; Gadermaier et al., 2009). Very recently two nsLTP isoforms (Ara h 9.0101 and Ara h 9.0201) have been cloned from peanut and expressed in heterologous systems (Lauer et al., 2009). Differently from our case, the two nsLTPs shared 90% aa identity and showed an equivalent immune reactivity pattern when tested with sera of Spanish patients allergic to peanut. Similar results were obtained for Art v 3.0201 and Art v 3.0301 recombinant isoforms whose allergenic potency was shown to be comparable (Gadermaier et al., 2009).

In conclusion, our results demonstrate that nsLTP is a major allergen for Spanish patients allergic to green bean. The multi-sensitization pattern of the patients to different plant species, even taxonomically non-related such as peach, tree nuts and plane pollen, and the fact that in these species nsLTPs have been reported to be major allergens causing severe allergic reaction, correlates with the clinical picture known as LTP-syndrome. We demonstrate the co-presence of different nsLTPs in GB characterized by different allergenic potency. Further molecular characterization of these LTPs is crucial to understand the differences in the immune reactivity of the various allergenic epitopes. On this basis, the selection of certain nsLTP isoforms has to be considered to develop new formulations for component resolved diagnosis.

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