

Severe Immediate Allergic Reactions to Grapes: Part of a Lipid Transfer Protein-Associated Clinical Syndrome

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Key Words

Carbohydrate determinants • Cross-reactivity • Digestion • Expansin • Grape allergens • IgE • Lipid transfer protein • Polygalacturonase-inhibiting protein • β -1,3-Glucanase

Abstract

Background: Grape allergy is considered rare; grape lipid transfer protein (LTP; Vit v 1), an endochitinase and a thaumatin-like protein (TLP) have been reported as grape allergens. A considerable number of patients have referred to our department for severe reactions to grapes, and several IgE binding proteins were detected. **Objectives:** The aim of this study was to identify and characterise the allergens involved in severe allergic reactions to grapes and describe the population in which they occur. **Methods:** Patients with reported severe allergic reactions to grapes ($n = 37$) are described. Grape allergens were purified/fractionated by a combination of chromatographic techniques, identified by proteomic analysis and biochemically characterised. Immunoreactivity was assessed by blot (inhibitions) and RAST (inhibitions), and skin prick tests were performed with the isolated allergens. **Results:** All subjects were polyallergic, sensitised and reactive to several additional foods and pollen. All patients were

sensitised to grape LTP. A 28-kDa expansin, a 37.5-kDa polygalacturonase-inhibiting protein, a 39-kDa β -1,3-glucanase and a 60-kDa protein were identified as minor grape allergens. Endochitinase and TLP did not play a role. Inhibition experiments revealed the possible cross-reactive role of LTP for clinical sensitivities to other LTP-containing plant foods, but also the involvement of cross-reactive carbohydrate determinants of minor allergens in IgE cross-reactivity. **Conclusions:** LTP is the major grape allergen, while additional minor allergens may contribute to clinical reactivity. Severe grape allergy presents in atopic patients who frequently react to other LTP-containing, plant-derived foods. The 'LTP syndrome' is the appropriate term to describe this condition.

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Introduction

Grapes (species *Vitis vinifera*) belong to the Vitaceae family and are widely cultivated and consumed all over the world as fresh fruits and processed foods, such as juices and wine. In Greece, grape cultivation takes part during February and June when the first cultivars are mature

for consumption. Although the majority of grapes are consumed between June and September, imported grapes are consumed during the whole year [1, 2].

Occasionally, allergy to grapes has been reported in the literature [3–5, and references therein]. A lipid transfer protein (LTP) and an endochitinase have been characterised as major grape allergens in a Mediterranean adult population, while a thaumatin-like protein (TLP) was designated a minor allergen [3]. However, in the only available study in a paediatric population, it was suggested that nine allergens not further identified may also be involved in grape allergy. Their molecular weights denoted from immunoblotting experiments were 9, 17, 24, 28, 31, 34, 60, 94 and 100 kDa [4].

In the Mediterranean region, non-specific LTPs (nsLTPs) have been described as predominant Rosaceae fruit allergens. Sensitisation to nsLTPs occurs independently from birch pollinosis, and symptoms of LTP-sensitised subjects are frequently systemic, including severe anaphylactic reactions [6, 7].

Patients showing severe reactions after the consumption of grapes were referred to our department the last few years, including a considerable number of children and adolescents [8]. The purpose of this study was to describe this population, and identify and characterise the allergens involved.

Patients and Methods

Patients

Thirty-seven patients were recruited into the study. Of these, 18 were prospectively included after referral to the Allergy Department, Second Paediatric Clinic, University of Athens, Greece, between 2000 and 2003 for one or more acute or sub-acute (≤ 2 h) reactions after grape consumption. The other 19 patients had a previously established diagnosis of acute grape allergy and were recruited during a follow-up visit. The study was approved by the local ethics committee, and informed consent was obtained from all patients or their parents. A thorough medical history was recorded, and specific IgE for grapes, other fruits, nut mixes, latex and pollen allergens were determined using the CAP System IgE FEIA (Phadia, Uppsala, Sweden). Skin prick tests (SPTs) were performed on the flexor aspect of the forearm with a standardised prick needle (ALK Lancet) according to previously published methods [9, 10]. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT extracts was used as a negative control (ALK-ABELLÓ, Horsholm, Denmark). Patients underwent SPT with the grape extract used for protein fractionation and purified fractions thereof freshly diluted in water (0.1, 1 and 10 $\mu\text{g/ml}$) and with fresh white grapes (cv stafida; prick-to-prick method). Reactions were measured after 15 min by copying the wheal reaction with transparent adhesive tape onto a record

sheet. SPTs were regarded positive when the area was at least 7 mm^2 .

Oral challenges were not performed due to parents' refusal to subject their children to such a procedure.

Extracts and Allergen Fractionation

A grape extract was prepared from the skin of fresh white grapes (*Vitis vinifera*) [11]. Protein fractionation was performed with a combination of ion exchange and size exclusion chromatography. The first grape extract was separated on an SP Sepharose 4 FAST Flow anionic exchange column, desalted on a PD10 column against water and further separated on a Superose 6 high-resolution anion exchange column (POROS HQ; internal diameter: 100 $\times$ 4.60 mm, and particle size: 20 μm). Ion exchange chromatography columns were equilibrated and eluted with 0.1 mM 4-morpholineethanesulfonic acid monohydrate (MES; pH 5.5) using a linear gradient from 20 MES:80 H_2O (v:v) to 20 MES:33 NaCl:47 H_2O (v:v:v) in 120 min. Finally, samples were fractionated with a gel filtration Superdex 75 column (internal diameter: 300 $\times$ 10.00 mm, and flow rate 1 ml/min) equilibrated and eluted with a buffer (pH 7) containing 50 mM Tris, 150 mM NaCl and 0.01% (w:v) NaN_3 . In all cases, columns were attached on a Biocad Sprint (PerSeptive Biosystems, Framingham, Mass., USA), and UV absorbance was detected at 280 and 220 nm.

The purity of the fractions was checked by gel electrophoresis. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, Ill., USA) as described by the manufacturer.

Pepsin Digestion of Grape Extract

The digestibility of the grape extract in simulated gastric fluid was examined according to Astwood et al. [12], but with a lower final enzyme:substrate ratio (1:20), aiming to be closer to the physiological ratio [11]. Samples were incubated at 37°C for 120 min and aliquots drawn at 0, 2, 5, 15, 30, 60 and 120 min. The progress of digestion was followed by SDS-PAGE. BSA was used as a control.

SDS-PAGE/Immunoblotting

Proteins (0.5 $\mu\text{g/well}$) were separated by SDS-PAGE [NuPAGE® 4–12% Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel] according to the protocol of the manufacturer, under reduced conditions (Invitrogen, Carlsbad, Calif., USA). Gels were stained using colloidal Coomassie brilliant blue or silver staining kits (Invitrogen). Molecular weights were calculated using pre-stained See-blue2 standards (Invitrogen) or unstained Precision standards (BioRad). Immunoblot (inhibition) was done as described previously [13], with the inhibitor added in a concentration of 50 $\mu\text{g/ml}$ for purified protein and fractions or 250 $\mu\text{g/ml}$ for extracts in 150 μl PBS-AT (PBS-albumin and 0.1% Tween-20) together with the patient's serum.

Radioallergosorbent Test (RAST) and RAST Inhibition

Two milligrams of extract or 200 μg of purified allergen were coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham-Pharmacia-Biotech, Uppsala, Sweden) [13]. 50 μl of serum and 0.5 mg Sepharose were used per test. RAST values >0.3 IU/ml were regarded positive [14]. The same protocol was used for RAST inhibitions, with a 2-hour pre-incubation of 50 μl of serum with 50 μl of inhibitor diluted in PBS/AT (0.3% BSA and 0.2%

Table 1. Clinical history and concurrent sensitisations of grape-allergic patients

Pa- tient No.	Age/ sex	Grape allergy				Other food allergy		Other atopic disease	
		symptoms	first reaction years	Pt P mm ²	sIgE kU/l	sensitisation	symptoms	sensitisation	symptoms
1*	25/M	u, gi, a	13	38.5	1.6	Fx1, f17	banana, hazelnut, wine, peach, sunflower, cabbage	t9, w19, gx2, m2, t23	a, r
3	13/F	u, an, h	4	7.1	6.0	Fx1, Rfx22, f92, f10, f13	cabbage, banana, peanut, walnut, hazelnut, almond, cherries	t9, w19, gx2	r
4*	21/M	u, an, a	9	28.3	3.4	Fx1, f95	walnut	t9, w19, gx2, d1, d2	a, r
5*	17/M	u, an, h	7	19.6	4.7	Rfx22, f49, f92, f10, f13	apple, hazelnut, walnut, peanut, cabbage	t9, w19, gx2, m6	a, r
6*	12/M	u, an, a, r, h	3	28.3	12.4	Fx1, Rfx22, f95	hazelnut, peanut, almond, orange, peach, apple	t9, gx2	r
7	18/M	u, gi, a	13	28.3	12	Fx1, Rfx22	hazelnut, walnut, almond	t9, i71	–
8*	16/M	oas, an, u, a	11	12.6	0.4	F92	peach	t9, gx2, m6	a, r
9*	10/M	u, an, gi	4	12.6	11.4	Fx1, Rfx22, f95	peach, apricot, banana, fish	t9, w19, gx2, d1, m2, m6	ad, a, r
10	15/F	u, an, a, r	4	14.8	1.2	Fx1	walnut	t9, w19, gx2, d1	a, r
11	12/F	oas, u, gi	8	44.2	1.6	Fx1	pomegranate, peanut, hazelnut, almond	–	a, r
12*	8/F	u, an, a, r	6	8.4	0.9	Fx1, Rfx22	tomato, hazelnut, walnut	–	a
13*	5/F	u, an, a	2	12.6	5.3	Fx1	apple	–	r
15*	20/F	u, an, a, h	7	7.1	1.4	Rfx22, f95, f44, f12	strawberry	t9, w19	ad, a
16*	24/M	u, an, hy, gi	9	10.8	9.4	f49	apple	t9, w19, gx2	ad, a, r
18*	17/M	u, an, a	11	9.8	5.3	F49, Rfx22		t9, w19, gx2	ad, r
19*	14/F	u, an, gi, ar, h	19	17.1	13.0	Fx1, f92, f4, f49, f8	banana, pistachio, latex	t9, t16, w19, gx2, d1, d2, i71	ad, a, r
20	14/M	an	10	15.9	12.6	Fx1, f49, f95, f13, f44, f84, k84	hazelnut, peanut, walnut, pistachio, almond, cashew nut	t9, w19, gx2	
22*	16/M	u, an, ar, h	13	12.8	12.4	Fx1, Rfx22	pomegranate, peach	t9, w19, gx2, d1	ad, r
23*	11/M	an, gi, ar	4	7.1		f95	fish	t9, w19, d1	ad, a
24	10/F	u, an, a, h	3		7.1	f3	peach, fish, hazelnut, sunflower, egg	–	ad, a
25*	13/M	u, an	8	8	5.2	Fx1, Rfx22, f95, f10		t9, w19, gx2	r, a
27*	13/M	u, a, h	8	15.9	2.1	Fx1, Rfx22, f92, f13	walnut, peanut, almond, hazelnut, banana, chestnut, apple, cabbage	t9, w19, gx2	ad, r
32	14/M	gi	8	12.6	24.7	Fx1, Rfx22, f1, f10, f14, f8	egg, walnut, pistachio, peanut, almond, hazelnut, fish, hazelnut	t9, w19, gx2, d1	
33	11/M	u, a, h	16	17.1	14.4	Fx1, f13, f14, f17, f20, f235	spinach, hazelnut	t9, w19, gx2, d1	ad, r, a
34*	12/F	u, gi, h	19	28.3	15.5	Fx1, Rfx22, f235	walnut, pistachio, peanut, almond, hazelnut	t9, w19, gx2, d1	a, r
35	18/M	u, an, gi, r	15	19.6	11.4	Fx1, Rfx22, k84	sunflower, watermelon, hazelnut	t9, w19, gx2	ad, a
36	11/M	gi	17	19.6	10.6	Fx1, Rfx22, f92, f235, f95	almond, lentils, walnut, peach, watermelon, banana	t9, w19, gx2, d1	R
38*	24/M	oas, u, an, gi, a, r, h	17	44.2	4.3	Fx1, f95, f256, f328, k84	peach, peanut, fig, sunflower, sesame	t9, w19, gx2	ad, a, r
40	15/M	u, an, a, r	8	9.6	1.9	Fx1, Rfx22, f10, k84, f95, f25	sunflower, sesame	t9, w19, gx2	R
41	17/M	an, a, r	13	7.1	0.9	Fx1, Rfx22, f48, f203, f202	cashew nut, pistachio, peppermint, onion	–	ad, a, r
42	20/M	u, an, a	6	12.6	1.0	Fx1, f44, f3, f13, f203	strawberry, fish, peanut, pistachio	t9, w19, gx2, m6	–
43*	25/F	u, r, gi, a	7	10.6	3.9	Fx1, Rfx22, f13, f216	peanut, hazelnut, pistachio, lentils, cabbage	t9, w19, gx2, d1	ad, a, r
44*	13/M	u, an, h, a	11	12.6	17.8	Fx1, Rfx22, f95, f49, f13	peach	t9, w19, gx2	Ad
45*	10/F	u, gi, a, r, h	18		12.1	Fx1, Rfx22, f95, f237	peach, apricot, strawberry, sesame	t9, w19, gx2	ad, a, r
46	15/M	u, an, gi, a, r, h	3	9.6	7.6	Fx1, Rfx22, f95, 13, f256, f20	walnut, peach, almond	–	ad, r
48*	18/F	u, an, a, gi	7	12.6	20.6	Fx1, Rfx22, f95	peach	t9, w19	Ad
49*	19/F	oas, u, an, r	13	7.1	1.1	Fx1		t9, d1, w7	–

Tween, pH 7.4) before the addition of Sepharose-coupled allergen. For grape LTP inhibition, 8-fold serial dilutions of extracts (initial concentrations 5 mg/ml) from grape, peach, apple, hazelnut, olive pollen, *Parietaria* and peanut or purified natural (n) or recombinant (r) grape (nVit v 1), strawberry (rFra a 3) [15], hazelnut (nCor a 8) [unpubl. data], maize (nZea m 14) [16], sunflower (nHel a 3) [17] or wheat rLTP [18] used at a concentration of 50 µg/ml.

Proteomic Analysis

For the identification of grape proteins, in-gel trypsin digestion was performed as described [19], and the resulting tryptic peptide profiles were analysed on a Reflex III MALDI-TOF-Mass Spectrometer (Bruker, Coventry, UK) equipped with a SCOUT 384 ion source. When required, further analysis was performed with a nano-electrospray ion source of a quadrupole-time-of-flight mass spectrometer (Q-TOF2 Micromass, Manchester, UK) in positive ion mode. Peptide profiles were searched (on the basis of mass) against the NCBI non-redundant protein database, using the Mascot program search tool (Matrix Science, London, UK, <http://www.matrixscience.com/>) and selecting Viridiplantae green plants as taxonomy [19].

The probability of identification was shown by the MOWSE score [20] and the sequence alignment was achieved by the T-COFFEE program [21].

Detection of Glycoproteins

Concanavalin A (ConA) was used to detect glycosylation of grape proteins. The glycan-ConA interaction was visualised using horseradish peroxidase [22]. A complete digestion of grape proteins in the extract (4 mg/ml) was performed using proteinase K (source: *Tritirachium album*; Boehringer Mannheim, Mannheim, Germany; enzyme:substrate = 1:200, water buffer, pH 8) for 8 h at 50°C. Proteinase K was subsequently inactivated for 5 min at 100°C [23]. Efficiency of digestion was confirmed by SDS-PAGE.

IgE-binding to cross-reactive carbohydrate determinants (CCDs) was inhibited with the glycopeptide from pineapple stem, bromelain (100 µg/ml) [24], grape carbohydrates (from proteinase K digestion) and grape extract (4 mg/ml).

Furthermore, strips were treated with periodate (100 mM sodium periodate in 100 mM sodium acetate, pH 5), which oxidises protein-glycan bonds [25]. The efficacy of periodate oxidation was tested by ConA staining. Periodate-treated strips were further used to establish the role of CCDs in recognition of grape allergens by immunoblotting.

Results

Patient Profile

The patients' clinical characteristics are presented in detail in table 1. Mean age ($\pm$ SD) at initial reaction was 7.9 years ($\pm$ 3.5), with a male predominance (24 males and 13 females). Total IgE ranged from 25 to >2,000 kU/l. Specific IgE to grapes, determined by the CAP FEIA System, was positive in all patients (ranging from 0.4 to 24.7 kU/l). Prick-to-prick tests with fresh grapes were positive in 33 of the 35 patients tested and negative in all control subjects (n = 14).

The majority of patients (33/37, 89.2%) were referred because of systemic anaphylaxis to grapes, occurring 5–120 min after the consumption of grapes. Eighteen patients reported more than 1 and up to a maximum of 6 episodes. Clinical manifestations of acute grape allergy included urticaria (84%), angio-oedema (75.6%), lower and upper respiratory symptoms (67.5 and 54%, respectively), and cardiovascular and/or gastrointestinal symptoms (37.8 and 43.2%, respectively). One patient reacted only to a specific grape variety (black grape, cv sultana). Two patients reported anaphylactic reactions to wine, and 1 had exercise-induced anaphylaxis after eating grapes. Four children could tolerate small quantities of grapes several years after experiencing a severe reaction and 2 tolerated raisins.

All patients had multiple IgE sensitisations (confirmed by CAP and SPT) and were polyallergic, with a positive history of asthma (57%), allergic rhinitis (65%), atopic dermatitis (48.5%) and/or other food allergies (89%). Grapes were the cause of the first-ever food allergic reaction in 20/37 patients.

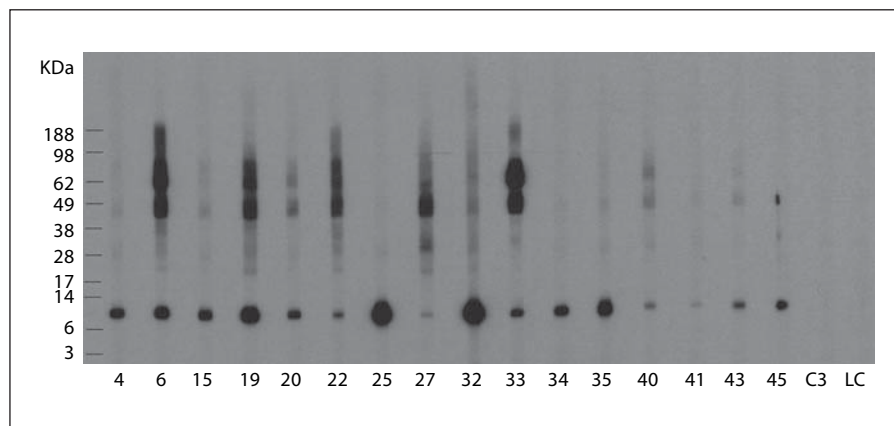
IgE Reactivity to Grape Extract in RAST and Immunoblots

In-house RASTs with grape extract were positive in all cases tested (n = 21), with a mean IgE reactivity of 5.6 IU/

u = Generalized urticaria; a = asthma; ad = atopic dermatitis; an = angio-oedema; gi = gastro-intestinal symptoms; h = hypotension; r = rhinitis; oas = oral allergy symptoms. SPTs <7 mm² and CAP values <0.3 IU/ml are considered negative (–). Patients whose first allergic food reaction was to grapes are marked with an asterisk. The clinical history of other food allergy symptom reactions are given in chronological order. Patients with a grey background were retrospectively included in the study. CAP codes: Fx1 = nut mix (peanut, hazelnut, brazilnut, almond and coconut); Rfx22 = nut mix (pecan, cashew, pistachio and walnut); f49 = apple; f92 = banana; f95 = peach; k84 = sunflower seed; f203 = pistachio; f20 = almond; f256 =

walnut; f202 = cashew nut; f235 = lentil; f44 = strawberry; f3 = fish; f8 = maize; f10 = sesame seed; f12 = pea; f13 = peanut; f14 = soya bean; f17 = hazelnut; f25 = tomato; f48 = onion; f84 = kiwi; f216 = cabbage; f237 = apricot; f328 = fig; t9 = olive pollen; w19 = *Parietaria*; t16 = white pine; gx2 = grass mix (*Cynodon dactylon*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, *Sorghum halepense* and *Paspalum notatum*); m6 = *Alternaria fumigatus*; m2 = *Cladosporium herbarum*; w7 = margarine; d1 = *Dermatophagoides pteronyssinus*; d2 = *Dermatophagoides farinae*; t23 = *Cupressus sempervirens*; i71 = mosquito. M = Male; F = female.

Fig. 1. Immunoblot of grape extract with sera from patients with severe grape allergy. Representative results from 17 patients are shown. Serum codes are shown under each lane. C3 = Healthy control serum; LC = label control. Marker sizes are indicated on the left.



ml (range 0.7–22.5 IU/ml), significantly correlating with the corresponding CAP values ($R_{\text{Spearman}} 0.6$, $p < 0.005$). Immunoblots with grape extract revealed that all sera specifically reacted to a band of approximately 9 kDa in the extract, while most also recognised bands of higher molecular weight (28–98 kDa; fig. 1).

Grape Allergen Identification and Purification

Subsequently, grape proteins were separated in fractions, according to their IgE binding capacity on blot (fig. 1), with a molecular weight of 6–14.400, 21.5–31.000, 36–55.000 and 55–66.000, using anion exchange and gel filtration chromatography and identified using proteomic analysis (table 2). In addition to grape LTP, we purified grape TLP, endochitinase and β -1,3-glucanase. A 60-kDa protein and polygalacturonase-inhibiting protein (PIP) were semi-purified and contained in a pool (pool 1, P1). Another semi-purified preparation (pool 1a, P1a) additionally contained a 39.8-kDa protein and expansin.

IgE Binding to Purified/Fractionated and Digested Allergens

The IgE binding capacity of purified LTP was confirmed in immunoblots ($n = 6$) and RAST ($n = 21$, range 0.3–10.4 IU/ml, mean 2.62 IU/ml). Among eight sera who recognised a ~40-kDa band in the crude extract blot, four (50%) detected purified β -1,3-glucanase on immunoblot.

Finally, among 13 sera recognizing high-molecular-weight bands in the crude extract, 12 (92.3%) reacted with proteins contained in P1 and P1a on immunoblot, whereas 8/11 (73%, range 0.5–9.7 IU/ml; mean 2.67 IU/ml) or 11/12 (91.6%, 0.4–11.4 IU/ml; mean 3.08 IU/ml) gave a positive RAST for these pools, respectively (data not shown).

Endochitinase and TLP were negative for all sera tested using blot and/or RAST ($n = 10$), whereas a control serum with known sIgE against apple TLP also reacted to grape TLP, proving the IgE binding capacity of the molecule (not shown).

Pepsin digestion of grape extract revealed that LTP, TLP, expansin and the 60-kDa protein retained stability when a time-dependent digestion of profilin, protein homologous to Bet v 1, β -1,3 glucanase and PIP was observed (fig. 2a). Similarly, the IgE binding activity of LTP, expansin and the 60-kDa protein remained unaltered after pepsinolysis, in contrast to the β -1,3-glucanase and PIP which disappeared after 5 and 15 min of digestion, respectively (fig. 2b).

Grape LTP Cross-Reactivities

RAST inhibition using Sepharose-coupled grape LTP was performed in order to assess potential cross-reactivities between LTP-containing allergenic sources as well as between different LTP species. Complete inhibition of binding was obtained with concentrations of grape extract $>23 \mu\text{g/ml}$. A significant decrease in binding (up to 77%) was observed with peach, apple and hazelnut extracts, but no significant inhibition with olive pollen, *Parietaria* or peanut extracts (fig. 3a). Other plant-derived LTPs were also partially inhibitive, to a different extent in RAST (wheat LTP 18%; maize LTP 27.5%; hazelnut LTP 35%; strawberry LTP 50.6%, and sunflower LTP 58.6%), as well as blot inhibition experiments (fig. 3b).

Role of CCDs in IgE Recognition

IgE binding to the 60-kDa protein, PIP, 39.8-kDa protein and expansin was inhibited with bromelain in 3/6 patients, with a range of 22–35% (not shown), and al-

Table 2. Proteomic identification of purified or fractionated grape proteins by MALDI-TOF or Q-TOF mass spectrometer

Protein identification	MALDI-TOF or Q-TOF estimated molecular weight, kDa	MALDI-TOF or Q-TOF accession No. (MOWSE score)	
Non-specific LTP isoform 1 (<i>Vitis vinifera</i>)	11.672 (pI 8.88)	Q850K8 (score 225)	200 116 97.8 66 55 36 31 21.5 14.4 6 3.5 2.5
TLP (<i>Vitis riparia</i>) TLP (VVTL1)	27.819 (pI 7.82) 24.866 (pI 5.09)	Q9SPE0 (score 121) OO4708 (score 121)	
Endochitinase A precursor (maize) Endochitinase B precursor (maize)	30.018 28.436 (pI 8.31)	P29022 (score 130) P29023 (score 130)	
β -1,3-Glucanase (<i>Vitis vinifera</i>)	39.512	Q9M563 (score 155)	
Putative gag-pol polypeptide (<i>Oryza sativa</i> ; japonica cultivar group)	59.018	Q6UUA4 (score 34)	
PIP (<i>Vitis vinifera</i>)	37.522 (pI 8.75)	Q9AXP4 (score 164)	
Expansin (Glycine max)	28.119 (pI 9.12)	Q8GZD3 (score 224)	
Hypothetical protein (<i>Arabidopsis thaliana</i>)	39.873 (pI 7.57)	Q8LCM9	

The last column presents the analyzed protein molecules on SDS-PAGE stained with colloidal brilliant blue. Marker sizes are indicated on the first row.

though being low could indicate the presence of carbohydrate moieties. Furthermore, proteinase K digestion-derived grape carbohydrates significantly inhibited IgE binding in both RAST (n = 5, mean inhibition 62%) and blot (fig. 4a). Finally, periodate treatment completely abolished carbohydrate moieties from these molecules, as shown with ConA staining (fig. 4b) and their IgE binding in immunoblot, suggesting that CCDs may be involved in IgE reactivity (fig. 4c).

Biological Reactivity (SPT) with Grape Extract and Purified Fractions

Representative numbers of patients were tested in SPT with fresh grapes, grape extracts and purified and fractionated allergens. SPT with self-prepared grape extract was positive in 10/12 (83%) subjects tested, in contrast to commercial diagnostic extracts, which were positive in 2/8 (25%) and 3/6 (50%) patients tested, for two different products, respectively. Grape LTP SPT was positive in all patients tested (n = 13), with a mean wheal diameter of

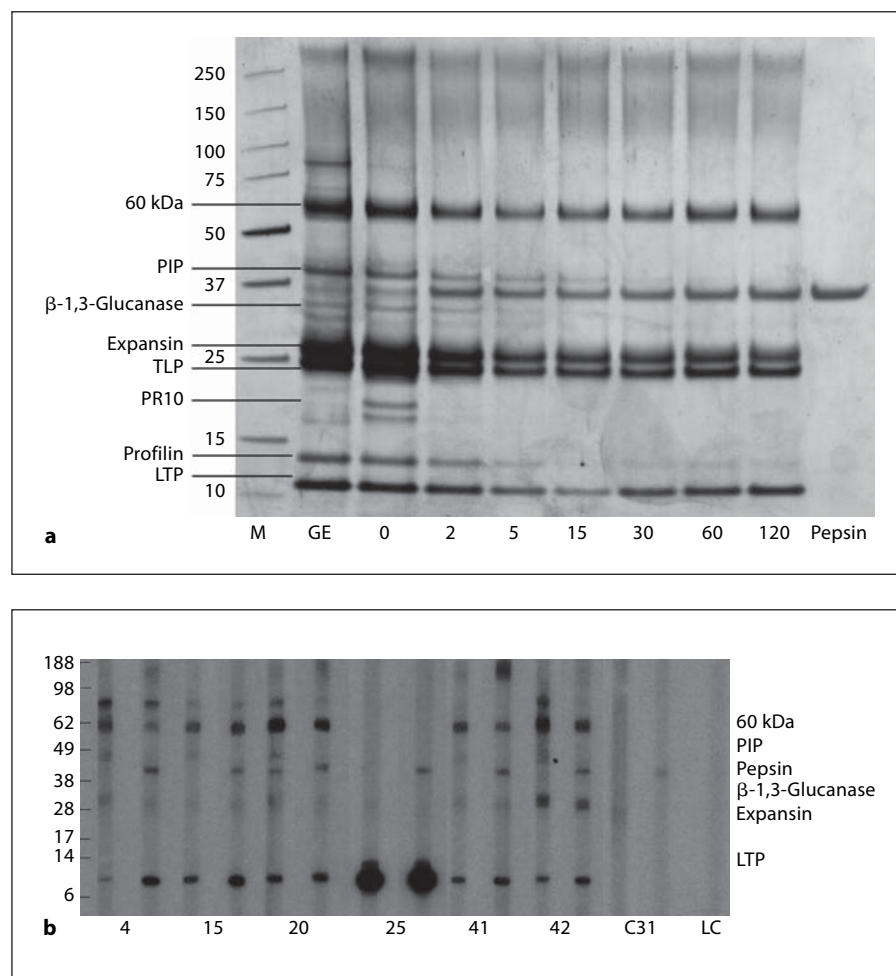
5.9 mm (range 3–10 mm); 4/13 developed very strong reactions with pseudopodia.

Three of 11 and 3/9 patients reacted to the pooled fractions P1 and P1a, respectively. One patient reacted only to P1a but not to P1, suggesting that expansin and/or the 39.8-kDa protein was the relevant allergen. Furthermore, 1 patient was positive to β -1,3-glucanase. SPTs for purified endochitinase and TLP were negative in all 14 patients tested, as well as in 8 control subjects.

Discussion

Although IgE-mediated hypersensitivity to grapes has been considered an uncommon problem [3], often associated with oral allergy syndrome reactions [26–28], we describe a group of 37 children and young adults, who had faced one or more severe reactions after the consumption of grapes. This cohort suggests that grape allergy may not be a trivial problem requiring epidemio-

Fig. 2. Effect of gastric digestion on grape allergen stability. **a** Time course in a silver-stained gel. Proteins, identified by homologous inhibition or antibody blocking, are marked. A time-dependent digestion of profilin, protein homologous to Bet v 1, β -1,3-glucanase and PIP is observed, in contrast to LTP, TLP, expansin and the 60-kDa protein which are stable to digestion. **b** Immunoblot showing IgE reactivity of a selected serum panel of grape-allergic patients to unprocessed grape extract (odd lanes) and digested grape extract (even lanes). The IgE binding capacity of LTP, expansin and the 60-kDa protein remained unaltered, in contrast to that of β -1,3-glucanase and PIP. C31 = Healthy control serum; LC = label control. Marker sizes are indicated on the left.



logical evaluation as well as elucidation of the mechanisms involved in terms of primary sensitisation, occurring symptoms, molecular structure and cross-reactions as well as clinical relevance of allergens involved in allergic reactions.

The described population was polyallergic with extensive sensitisation to food, pollen and other allergens, but also clinical expression of a variety of atopic diseases and food allergies. Our data suggest that grapes were likely to be responsible for primary food sensitisation in the majority of patients. Interestingly, most patients first reacted to grapes, before (if at all) reacting to peach, which is the fruit most frequently implicated in LTP sensitisation [29]. Different consumption patterns may be one explanation for this observation. The remaining subjects were primarily sensitised to tree nuts and/or Rosaceae fruit, or had multiple independent sensitisations.

The major allergen to which all our patients reacted, in serological and SPTs, was confirmed to be grape LTP isoform P1 [11], homologous to Vit v 1 (isoform P4), identified by Pastorello et al. [3] as a major allergen in an Italian adult grape-allergic population. Grape LTP isoform P1 was accepted by the International Union of Immunological Society Allergen Nomenclature Subcommittee and termed Vit v 1.0101.

nsLTPs have been characterised as major food allergens especially in the Mediterranean area [7]. However, 2/3 sera from Dutch patients used as controls in this study due to their positive RAST to grape also bound grape LTP (data not shown), indicating that LTP sensitisation may also occur outside the Mediterranean region, as suggested by a recent report [30].

Our case series supports the pan-allergenic nature of LTPs, which are well conserved among the Prunoideae [31], but also in botanically unrelated foods such as tree

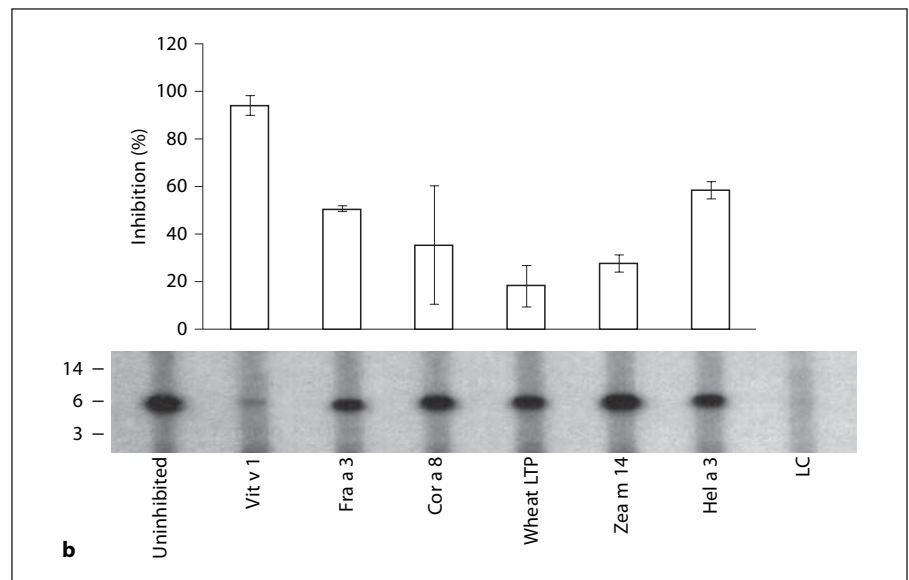
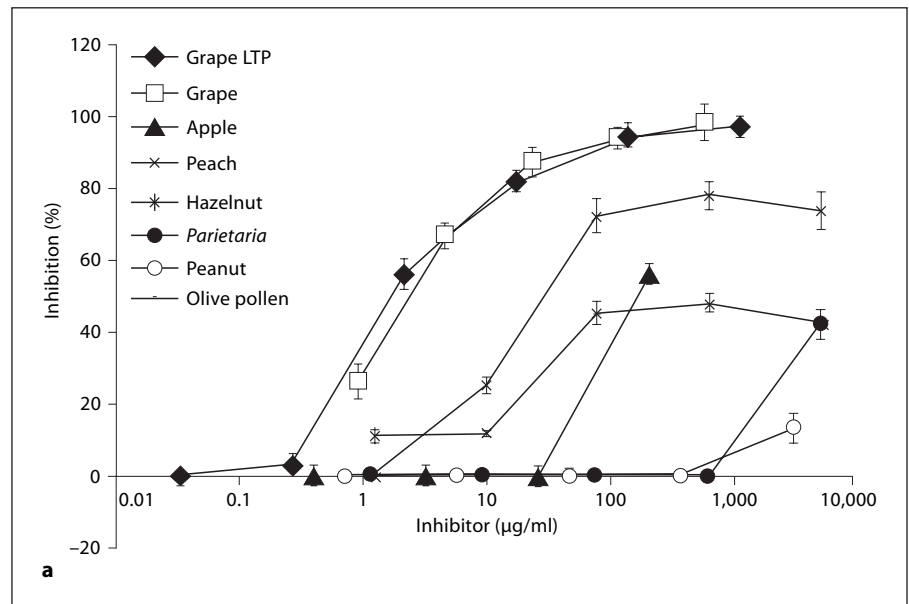


Fig. 3. Grape LTP inhibitions in RAST with crude extracts from grape, peach, apple, hazelnut, olive pollen, *Parietaria* and peanut (**a**, food extracts) and in RAST and blot with purified (natural or recombinant) LTPs from grape (Vit v 1), strawberry (rFra a 3), hazelnut (Cor a 8), wheat LTP, maize (Zea m 14) and sunflower (Hel a 3; **b**, plant LTPs). Inhibition rate with LTPs was similar in blot: 100% Vit v 1, 50% rFra a 3; 30% Cor a 8; 28% wheat LTP, 18% Zea m 14 and 43% Hel a 3.

nuts and maize [32]. Thus, LTPs are potentially responsible for several of the reported immediate reactions to phylogenetically unrelated plant-derived foods. We suggest that the term 'LTP syndrome' to be more extensively used to describe such a clinical presentation [33, 34]. This population represents an important clinical challenge, as it is currently impossible to safely predict the possibility of a reaction to an LTP-containing food, once a reaction to another has occurred.

In contrast to the findings of Pastorello et al. [3], endochitinase and TLP were not allergenic in our popula-

tion. Age, location and clinical presentation are among the differences between the cohorts that may account for this discrepancy. In contrast to the Italians, Greek patients had an atopic background and in addition faced anaphylactic reactions to grapes that were not preceded by the oral allergy syndrome.

In addition, we identified expansin, PIP and a β -1,3-glucanase homologous to β -1,3-glucanase from latex (*Hev b 2*) as minor allergens in this population, all belonging to protein groups with members previously characterised as allergenic [35–38]. In cases where, in addi-

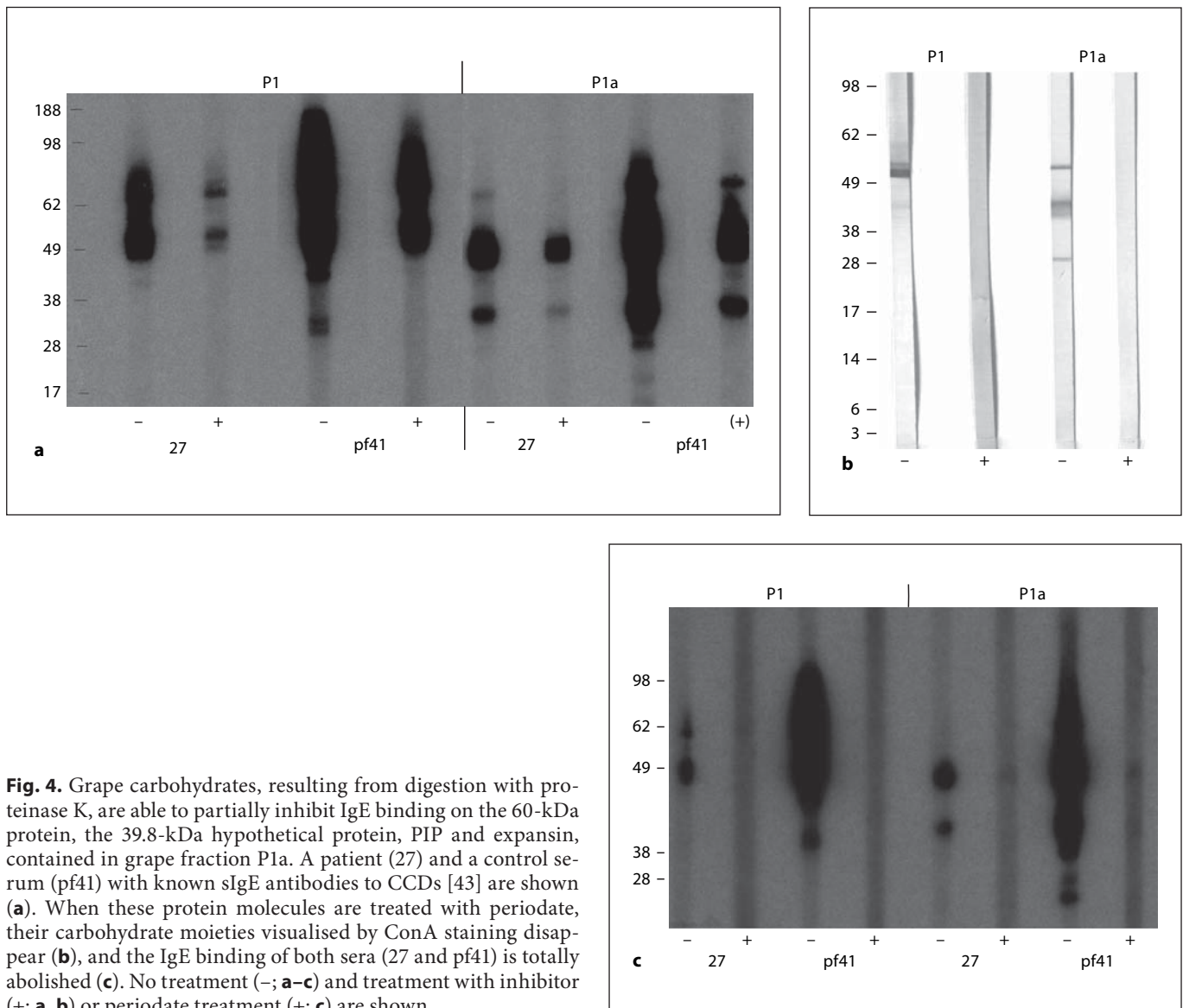


Fig. 4. Grape carbohydrates, resulting from digestion with proteinase K, are able to partially inhibit IgE binding on the 60-kDa protein, the 39.8-kDa hypothetical protein, PIP and expansin, contained in grape fraction P1a. A patient (27) and a control serum (pf41) with known sIgE antibodies to CCDs [43] are shown (a). When these protein molecules are treated with periodate, their carbohydrate moieties visualised by ConA staining disappear (b), and the IgE binding of both sera (27 and pf41) is totally abolished (c). No treatment (–; a–c) and treatment with inhibitor (+; a, b) or periodate treatment (+; c) are shown.

tion to LTP, minor allergens were involved in immune reactivity, the intensity of symptoms was higher; however, this was not significant, possibly due to lack of power of statistics. The positive SPTs to intact protein fractions containing the 60-kDa protein, PIP and expansin in some patients, in addition to the loss of in vitro IgE reactivity after the oxidation of CCDs, indicated a possible contribution of such structures in allergenicity. This is in accordance with previous studies where their significance in allergenicity has been discussed, adding to the need of answering the question about their clinical relevance [6, 36, 39, 40].

In line with the view that proteins responsible for anaphylactic symptoms are stable to digestion [11, 41, 42], grape LTP [11], expansin and the 60-kDa protein remained intact and with unaltered IgE-binding capacity after pepsinolysis.

In conclusion, grape allergy is described in patients characteristically suffering from a clinical syndrome of multiple allergies, e.g. severe reactions to LTP-containing foods. In addition to LTP, three additional minor grape allergens were characterised, which may contribute to the severity of the reactions and/or the observed extensive cross-reactivity.

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