

Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein

Emilia Vassilopoulou, PhD,^a Neil Rigby, BSc,^b F. Javier Moreno, PhD,^{b,*}

Laurian Zuidmeer, PhD,^c Jaap Akkerdaas, MSc,^c Ioannis Tassios, MD,^a

Nikos G. Papadopoulos, PhD,^a Photini Saxoni-Papageorgiou, PhD,^a

Ronald van Ree, PhD,^c and Clare Mills, PhD^b Athens, Greece, Norwich,

United Kingdom, and Amsterdam, The Netherlands

Background: Severe grape allergy has been linked to lipid transfer protein (LTP) sensitization. LTPs are known to be resistant to pepsin digestion, although the effect of gastroduodenal digestion on its allergenicity has not been reported.

Objective: We sought to investigate the effect of gastric and gastroduodenal digestion on the allergenic activity of grape LTP.

Methods: The proteolytic stability of grape LTP was investigated by using an *in vitro* model of gastrointestinal digestion. The allergenicity of LTP and its digesta was assessed *in vitro* by means of IgE immunoblotting, RASTs, and *in vivo* skin prick tests in the same patients with grape allergy.

Results: Grape LTP was resistant to gastric digestion, and yielded a 6000-d relative molecular mass C-terminally trimmed fragment after duodenal digestion. This fragment retained the *in vitro* IgE reactivity of the intact protein. Inclusion of phosphatidylcholine during gastric digestion protected the LTP to a limited extent against digestion. Digestion did not affect the *in vivo* (skin prick test) biologic activity of LTP.

Conclusion: The allergenic activity of grape LTP was highly resistant to *in vitro* digestion. This property might facilitate sensitization through the gastrointestinal tract and might also

potentiate the ability of LTPs to elicit severe allergic reactions in sensitized individuals.

Clinical implications: Purified natural allergens will facilitate the development of component-resolved diagnostic approaches, including allergen chips. This study contributes to our understanding of the role digestion plays in symptom elicitation in true food allergy. (J Allergy Clin Immunol ■■■■;■■■: ■■■-■■■.)

Key words: Grape, lipid transfer protein, gastric digestion, duodenal digestion, phosphatidylcholine, basophil histamine release, skin prick testing, food allergy

Nonspecific lipid transfer proteins (LTPs) have been identified as major allergens for non-pollen-related allergy to Rosaceae fruits, and it is now evident that they form a panallergen family.^{1,2} Although allergenic LTPs have been identified in a broad variety of foods, the allergies are frequently associated with allergy to peach LTP, Pru p 3. Thus to date, all the patients reported with IgE toward LTPs from plant foods outside the Rosaceae family also have IgE to Pru p 3. This has led to the view that peach LTP is the most likely sensitizing agent in these patients, although there is evidence for cross-reactivity between plant food and pollen LTPs, which has led to an alternative hypothesis that pollen LTPs are the primary sensitizers.^{3,4}

The LTP family is an important member of the prolamin superfamily of plant food allergens,⁵ being low-molecular-weight proteins, typically around 90 to 95 amino acids, containing 8 cysteine residues forming 4 disulfide bridges. They are extremely stable, resisting thermal denaturation and pepsin digestion.⁶⁻⁸ The latter property might allow the allergen to reach the intestinal immune system in an immunogenic way (ie, with the potential to sensitize). Moreover, the stability of the IgE-binding conformation is probably the most important factor contributing to the observation that LTP-reactive patients have a higher incidence of severe systemic reactions than patients with the classical birch-related food allergy.^{3,9}

Grapes belong to the Vitaceae family and are consumed all over the world as fresh fruits, raisins, grape juice, must, and wine. There are a number of reports referring to allergic reactions after the consumption of grapes,¹⁰

From ^aResearch Laboratories of 2nd Paediatric Clinic, University of Athens;

^bthe Institute of Food Research, Norwich Research Park, Norwich; and

^cthe Academic Medical Centre, University of Amsterdam.

*Dr Moreno is currently affiliated with Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain.

E. Vassilopoulou was supported through the Institute of Food Research Marie Curie Training Site Fellowship (QLK1-CT-2000-60030) and an Iraklitos fellowship for PhD students (Ministry of Greece for Education and Religions E.P.E.A.E.K II KA 70/3/7148). F. J. Moreno was supported by an EU Marie Curie Fellowship (QLK1-CT-2001-51997). C. Mills and N. Rigby were funded by the BBSRC competitive strategic grant to the Institute of Food Research. L. Zuidmeer and R. van Ree were (partly) funded by a grant from the EC: SAFE QLK1-CT-2000-01394.

Disclosure of potential conflict of interest: N. Rigby and C. Mills have consultant arrangements with the European Union and UK BBSRC. R. van Ree has consultant arrangements with HAL Allergy BV, Stallargenes SA, and Ventria Bioscience. The rest of the authors have declared that they have no conflict of interest.

Received for publication December 1, 2005; revised April 14, 2006; accepted for publication April 18, 2006.

Reprint requests: Emilia Vassilopoulou, PhD, Allergy Research Laboratories, 2nd Pediatric Clinic, University of Athens Greece, 41, Fidiplidou str, Athens 11527, Greece. E-mail: emi@allergy.gr.

0091-6749/\$32.00

© 2006 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2006.04.057

TABLE I. Main clinical characteristics of 4 patients with grape IgE-mediated hypersensitivity included in the study

Patient no.	Age at 1st reaction (y)	Sex	No. of episodes to grape	Time of reaction after consumption (min)	Symptoms	Grape CAP FEIA	Grape LTP RAST	Other atopic disease	Other food allergies
8	11	Male	1	120	Urticaria, angioedema	0.4	0.39	Asthma, rhinitis	Peach
33	6	Male	1	45	Urticaria, rhinitis, hypotension, anaphylaxis	4.36	0.849	Asthma, rhinitis	Hazelnut, spinach, apple, peach
35	5	Male	4	60	Urticaria, asthma, gastrointestinal symptoms, rhinitis, anaphylaxis		1.458	Rhinitis	Hazelnut, watermelon
44	11	Male	1	60	Respiratory symptoms, hypotension, anaphylaxis	17.8	10.365	Atopic dermatitis, asthma	Peach

Abbreviations used

LTP: Lipid transfer protein
 M_r: Relative molecular mass
 PC: Phosphatidylcholine
 SPT: Skin prick test

wine,¹¹ and grape juice.¹² Recently, the LTP from wine grape *Vitis vinifera* (Vit v 1) has been characterized as a major allergen in 2 Mediterranean populations with grape allergy.¹¹ IgE-mediated allergy to grape LTP (Vit v 1) has also been reported outside the Mediterranean area in a German patient who had a severe anaphylactic reaction after consumption of wine, fresh grapes, and raisins.¹³

The aim of this study was to investigate how LTP stability might affect allergenicity by using the grape LTP as a model member of the prolamin superfamily. In contrast to most studies on allergen stability that exclusively assessed resistance to pepsin, we have assessed its resistance to digestion by using an *in vitro* model of gastric and duodenal digestion,¹⁴ including an assessment of the effect of the physiologic surfactant phosphatidylcholine (PC), which is secreted by the gastric mucosa and occurs in bile.

METHODS

Patient sera

Four patients were selected for immunologic evaluation of purified grape LTP digests (nos. 8, 33, 35, and 44) from a group of patients with grape LTP allergy (n = 27) who had been referred to the 2nd Paediatric Clinic of the University of Athens because of severe anaphylactic reactions after consuming grapes. Their clinical symptoms, CAP-FEIA (Pharmacia Diagnostics, Uppsala, Sweden) results to grape, and RAST results to grape LTP are summarized in Table I. The study was approved by the local ethics committee, and informed consent was obtained from the patients.

Skin prick tests

Skin prick tests (SPTs) were performed on the flexor aspect of the forearm according to standard protocols¹⁵ by using histamine dihydrochloride (10 mg/mL) as a positive control and the glycerol diluent of the SPT extracts as a negative control (Stallergènes, Antony, France). Patients underwent SPTs with purified grape LTP (1 and 10 µg/mL), LTP digests (1 and 10 µg/mL), and fresh grapes (red and white, prick-to-prick method). Reactions were measured after 15 minutes by copying the wheal reaction with transparent adhesive tape onto a record sheet. SPTs with wheal diameters of greater than 7 mm² were regarded as positive (Table II). *In vitro* digestion buffers without grape LTP resulted in negative SPT responses.

Extract preparation and protein purification

Edible green grapes (*V. vinifera*) originating from South Africa were purchased from a local store (Norwich, United Kingdom). Extracts were prepared as previously described with slight modifications.^{11,16} Grape LTP was purified by means of a combination of ion exchange chromatographic techniques and size exclusion chromatography on the basis of the method of Sancho et al.⁷ Protein concentrations were determined by using the bicinchoninic acid assay (Pierce, Rockford, Ill), as described by the manufacturer, with serial dilutions of BSA as a standard.

In vitro gastric and duodenal digestion

The digestion of grape LTP was performed as described by Moreno et al¹⁷ using an *in vitro* gastric digestion with pepsin, followed by duodenal digestion in the presence of a model bile salt mix, trypsin, and chymotrypsin, with gastric digestions being performed in the presence or absence of the physiologic surfactant egg PC (Lipid Products, Surrey, United Kingdom). Enzyme/substrate ratios were 1:20 (wt/wt) for pepsin, and for duodenal digestion (using the starting concentration of LTP for gastric digestion), they were 1:400:100 (wt/wt) with respect to trypsin and chymotrypsin, as described by Moreno et al.¹⁷ Samples were removed at various time points, and gastric digestion was terminated by increasing the pH to 6.5, with duodenal digestion being terminated by means of addition of Bowman-Birk trypsin-chymotrypsin inhibitor from soybean. LTP digestion was monitored by means of RP-HPLC by using the procedure of Moreno et al¹⁷ with a Phenomenex Jupiter 300Å column

TABLE II. Effect of digestion on the *in vivo* skin reactivity of grape LTP

Patient no.	Wheal diameter (mm)						
	Red grapes	Green grapes	Grape LTP	LTP digesta			
				Gastric –PC	Gastric +PC	Gastroduodenal –PC	Gastroduodenal +PC
8	9	7	10	4	6	6	6
33	3	3	4.5	3	3	5.5	6
35	6	5	5	3	4.5	3	5
44	Not done	4	3	3	3	3	4

(Phenomenex, Macclesfield Cheshire, United Kingdom) coupled to a Jasco PU-1585 triple pump HPLC system (Jasco, Great Dunmow, United Kingdom). SDS-PAGE and immunoblotting analysis were performed as described previously.^{17,18} For N-terminal sequencing, the final product of gastric and duodenal digestion was separated and transferred to a polyvinylidene difluoride membrane (Bio-Rad), and the protein pattern was stained with Coomassie Brilliant Blue. N-terminal amino acid sequencing of the peptide was performed with a Precise 492 protein sequencer in pulsed liquid mode (Applied Biosystems, Weiterstadt, Germany).

Molecular markers were at a relative molecular mass (M_r) of 188,000 d for myosin, 98,000 d for phosphorylase B, 62,000 d for BSA, 49,000 d for glutamic dehydrogenase, 38,000 d for alcohol dehydrogenase, 28,000 d for carbonic anhydrase, 17,000 d for myoglobin-red, 14,000 d for lysozyme, 6000 d for aprotinin, and 3000 d for insulin B chain.

RASTs

RASTs were performed as described previously.¹⁹ Calculation was performed by means of a standard curve that was obtained by using RASTs with a dilution series of a chimeric monoclonal IgE antibody against the major house dust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2.²⁰ A RAST result of greater than 0.3 IU/mL was regarded as positive. Results were expressed as international units of IgE per milliliter.

Basophil histamine release assay

White blood cells were isolated from blood of a nonallergic donor by means of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation and stripped from IgE by using lactic acid treatment, as described elsewhere.²¹ Subsequently, cells were resensitized with the serum from a single patient with grape allergy. Histamine release was performed with LTP and 120 minutes of gastric and duodenal digests (0.1 ng/mL to 10 μ g/mL). Liberated histamine was measured by using the fluorometric method, essentially as described by Siraganian.²² Stripped cells were used as a negative control and induced a histamine release of less than 3% (data not shown).

RESULTS

In vitro gastrointestinal digestion of grape LTP

Grape LTP proved to be very resistant to gastric digestion, with only one prominent band, corresponding to the intact protein, evident on SDS-PAGE, even after 2 hours of digestion (Fig 1, A). Presence of the physiologic surfactant PC did not affect the stability of LTP (Fig 1, B). Results were essentially confirmed by means of RP-HPLC

(data not shown). Subsequent duodenal digestion resulted in the partial breakdown of LTP with the appearance of a polypeptide digestion product of approximately 6000-d M_r , which became more abundant over time, as observed with SDS-PAGE (Fig 1, C and D). RP-HPLC analysis confirmed the appearance of a single breakdown product (Fig 2, A and B).^{23,24} An analysis of peak areas in the HPLC profile showed that only 9% of the LTP remained intact at the end of 2 hours of duodenal digestion. This increased to 13% when PC was included during gastric digestion, indicating it had a slight protective effect on duodenal digestion of the protein.

N-terminal sequencing of the approximately 6000-d M_r fragment yielded the N-terminal sequence Ala Ile Thr X Gly Gln Val Ala Ser Ser, which matches the first 10 residues of the full-length LTP sequence from *V. vinifera* (Q2QC17). Because the approximately 6000-d M_r fragment ran as a single species on SDS-PAGE after reduction, it can be concluded that duodenal digestion resulted in the loss of a large fragment at the C-terminus of the protein. Analysis of the predicted cleavage of the Q2QC17 sequence with the PeptideCutter and Peptide-Mass tools on ExPASy suggested that the combination of trypsin and chymotrypsin results in cleavage of the LTP at residue 46 in the intact protein (Fig 2, C), which is predicted to give rise to a series of peptides ranging in size from 550 to 937 d that would not be visible by using SDS-PAGE analysis. They were not observed in the HPLC profile (Fig 2, A and B), but the analysis was not performed with columns and elution conditions optimal for resolution of small peptides. In addition, a large fragment comprising residues 1 to 53, together with parts of the C-terminus still attached to the main protein through the intermolecular disulphide bonds between Cys⁷⁶ and Cys³⁰ and Cys⁹⁰ and Cys⁵¹, would be predicted to have a mass of approximately 5300. It seems likely that this corresponds to the approximately 6000-d M_r fragment observed by means of SDS-PAGE, which has an intact N-terminus, the differences in molecular weight probably being due to the fact that the LTP runs near the resolution limit of the gel system used and the fact that it might run slightly larger because the proteolysis might have disrupted the normal compact native structure. Only a few residues are predicted to remain flanking the N- and C-terminal sides of Cys⁷⁶ and Cys⁹⁰ after digestion and would be insufficient to make a discernable difference to the mass of

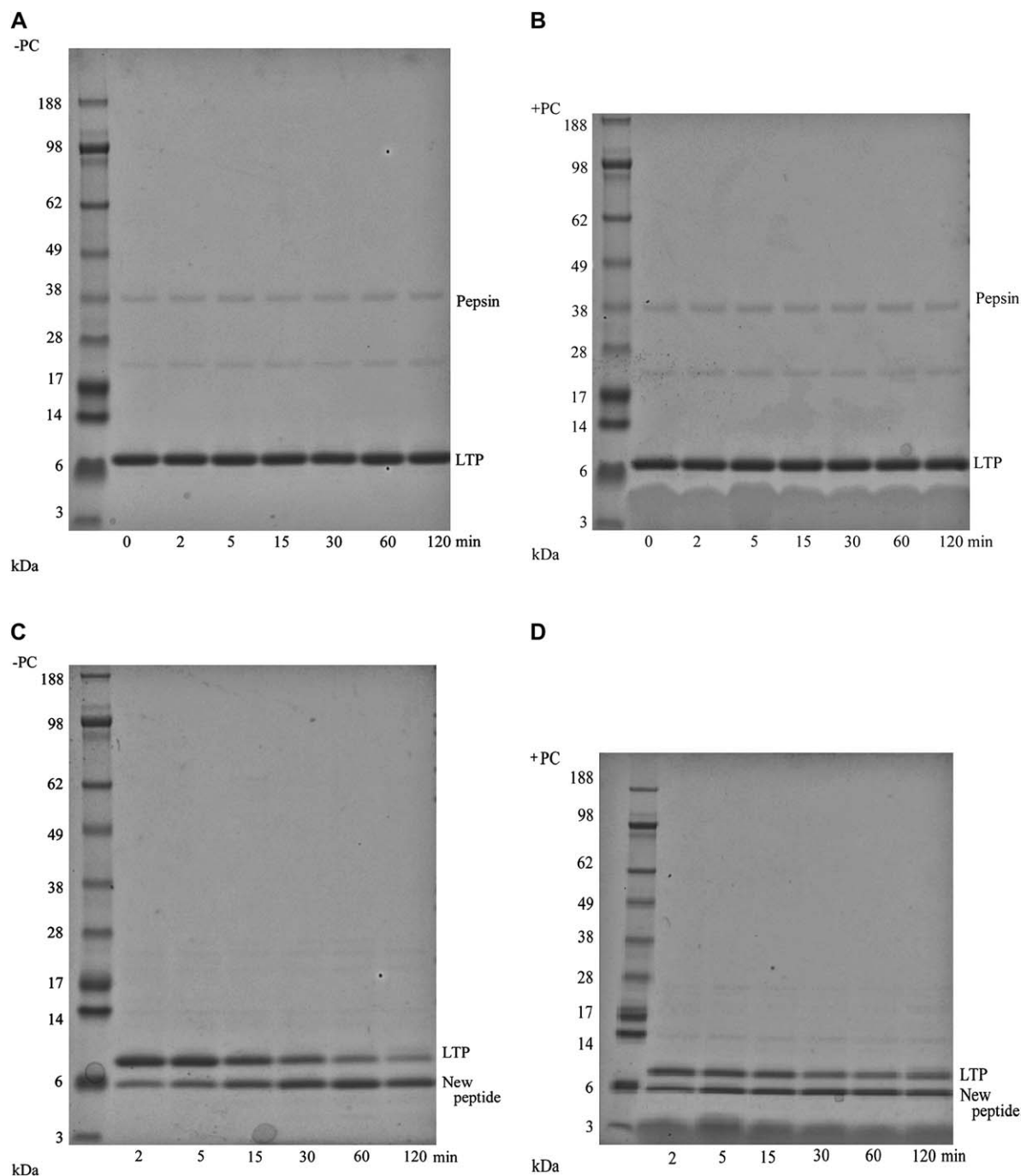


FIG 1. Effect of simulated gastrointestinal digestion on grape LTP, as determined by means of SDS-PAGE, after 2 hours of gastric digestion (**A** and **B**) or 2 hours of gastric digestion followed by 2 hours of duodenal digestion (**C** and **D**) performed in either the absence ($-PC$) or presence ($+PC$) of PC, respectively. Material staining at the dye front in Fig 1, **B** and **D**, is due to the presence of PC.

the LTP fragment when analyzed by means of SDS-PAGE under reducing conditions.

IgE reactivity of intact and digested grape LTP

IgE from the sera of individuals with grape allergy bound the approximately 9000-d M_r LTP in immunoblot strips of whole grape extract, as well as of the purified protein

(Fig 3). The IgE reactivity of the grape LTP was confirmed by means of RASTs (for selected patients 8, 33, 35, and 44, RAST values were 0.3, 0.85, 1.45, and 10.36 IU/mL, respectively). IgE binding was essentially unaltered after gastric digestion, which is consistent with the protein's stability (Fig 4, A). After gastroduodenal digestion, the IgE binding to the intact LTP decreased as the protein was degraded,

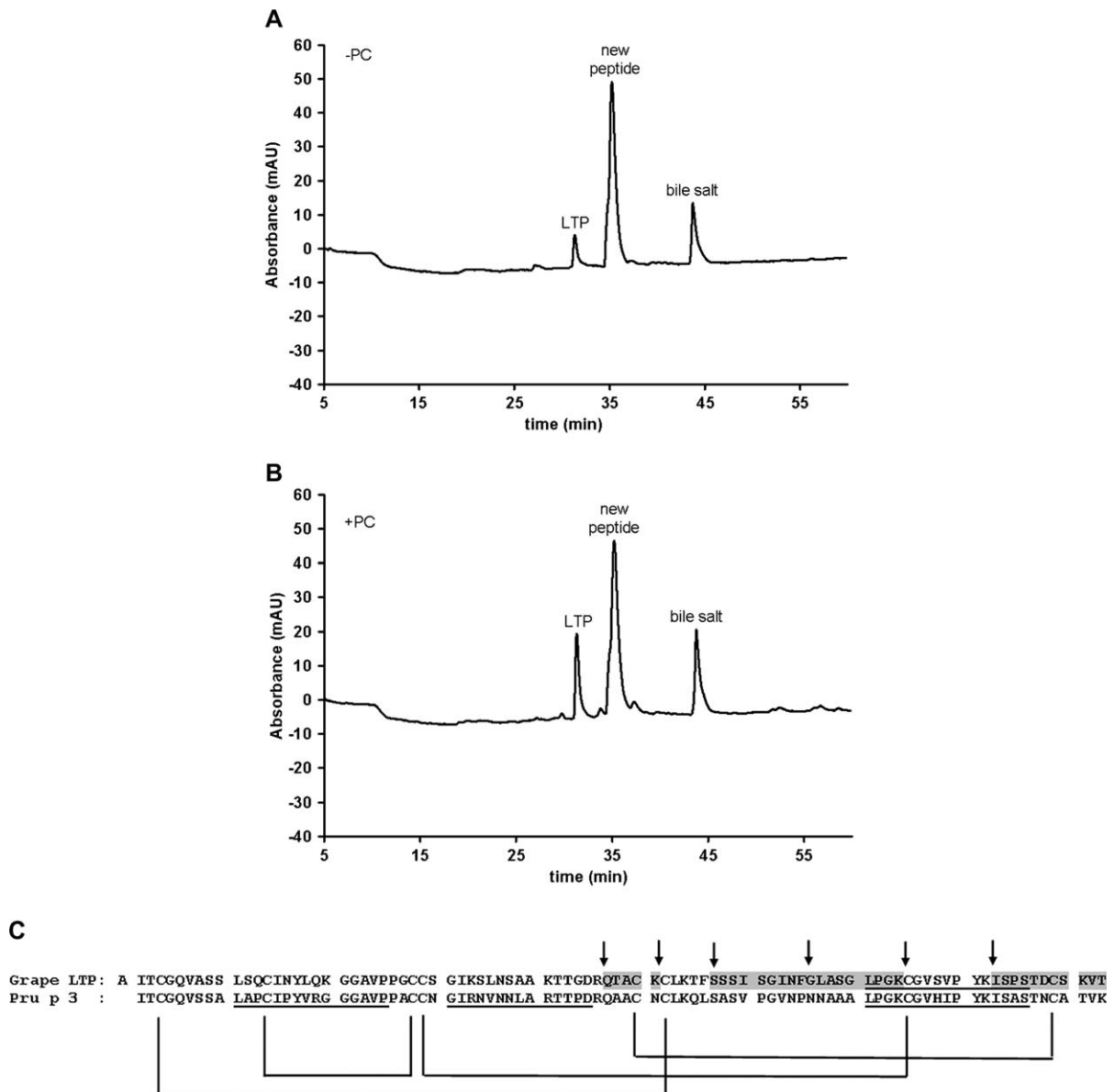


FIG 2. Effect of 2 hours of pepsin digestion followed by 2 hours of duodenal digestion on grape LTP in the absence (–PC, **A**) or presence (+PC, **B**) of PC, as determined by means of HPLC, and a diagrammatic representation of the predicted fragments resulting from duodenal digestion of grape LTP compared with the sequence of the peach LTP allergen Pru p 3 (**C**). Lines mark anticipated disulphide bond arrangements based on known LTP structures.²⁴ Arrows mark predicted tryptic/chymotryptic cleavage sites; shaded areas represent portions of the C-terminal region lost during digestion. IgE epitopes described by Garcia-Casado et al²³ are underlined.

with gastric PC having a protective effect on IgE binding to gastroduodenal digesta, which is again consistent with observations on the protein's stability to digestion. The approximately 6000-d M_r polypeptide digestion product was recognized by IgE (Fig 4, *B* and *C*). No IgE binding was observed when sera from healthy donors and atopic patients not allergic to grape were used as controls (data not shown).

Biologic activity of digested grape LTP

Basophil histamine release was carried out with stripped cells and human allergic serum from patient 44

to establish whether gastric and gastroduodenal digestion of the LTP affected the biologic activity of LTP-specific antibodies. A dose-dependent release of histamine was obtained with grape LTP. Even at the lowest dose of grape LTP (100 pg/mL), significant mediator release was observed (up to 18%), illustrating the allergenicity of LTP (Fig 5). In line with the immunoblotting results, both gastric and gastroduodenal digestion of grape LTP resulted in similar biologic activity to the native allergen. These data justified studying the ability of the LTP to elicit reactions *in vivo* by using skin testing. This was undertaken with the

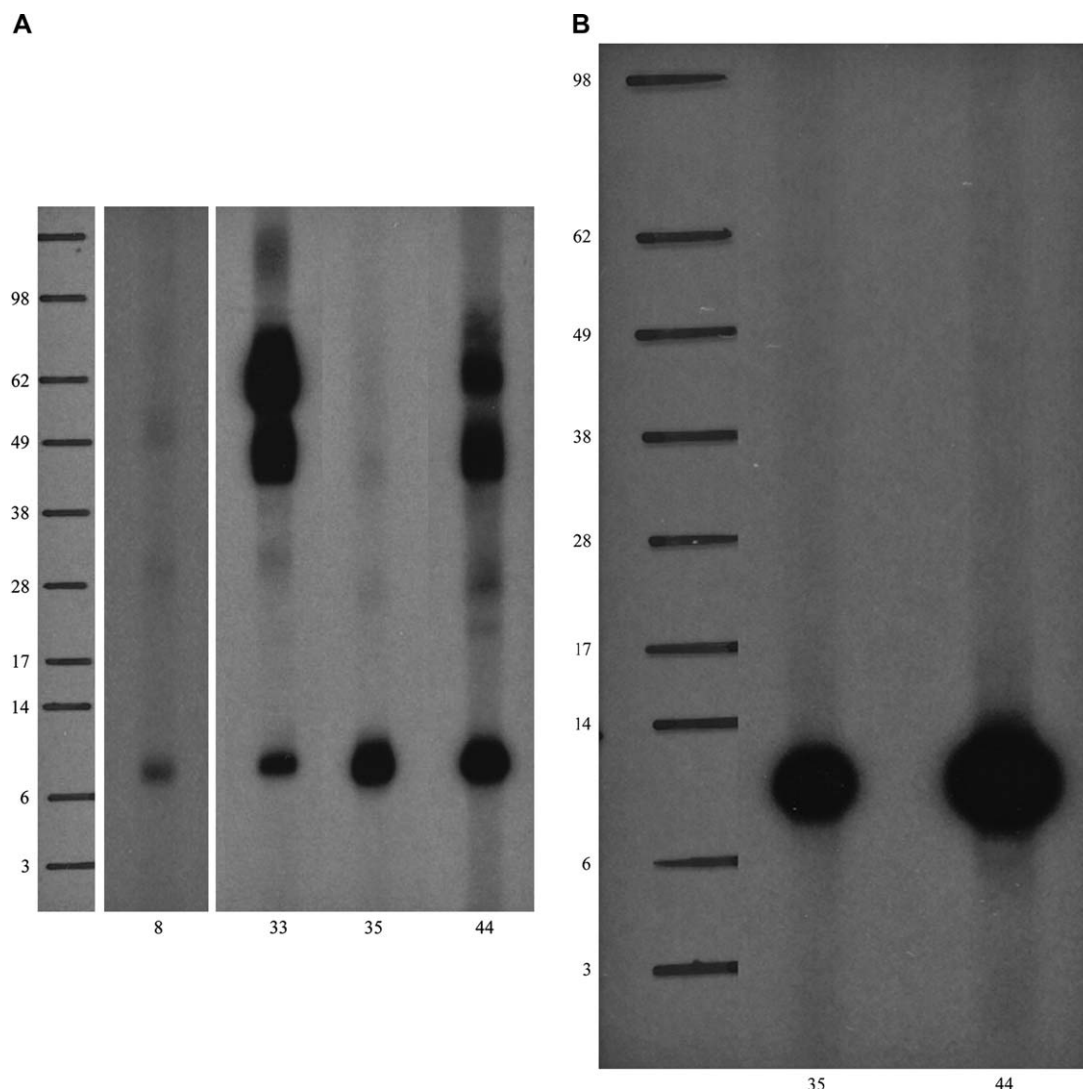


FIG 3. IgE reactivity of grape LTP determined by means of immunoblotting with sera (nos. 8, 33, 35, and 44) from LTP-reactive patients with grape allergy. **A**, Grape extract (sera 8, 33, 35, and 44); **B**, purified grape LTP (sera 35 and 44). Molecular weight markers are shown on the left.

purified protein and the products of digestion in parallel with fresh grapes (prick-to-prick testing) to confirm their allergic reaction to grapes. The SPT confirmed the allergenic potency of the LTP before and after gastric and duodenal digestion. All 4 patients had a positive reaction to all fractions tested, and in the case of patient 8, a pseudopodium was revealed with all samples. Control testing with PC and bile salts was performed to exclude potential nonspecific reactivity, which did not induce skin reactivity (Table II).

DISCUSSION

The prolamin superfamily is a dominant plant food allergen family.⁵ Such observations lead to the following question: Are there structural features or properties of

the prolamin superfamily that can potentiate their allergenicity? One common property identified for many, although not all, food allergens has been their stability to proteolytic attack.

As has been reported for several purified LTPs, including those from the Rosaceae fruits,^{9,25,26} grape LTP was also very stable during gastric digestion and showed no significant change in IgE-binding capacity on immunoblotting or in the ability to trigger histamine release. Digests also retained their capacity to induce a positive SPT response. Gastrointestinal digestion with ratios of enzymes relatively close to the physiologic conditions in the human gastrointestinal tract rapidly gave rise to a stable approximately 6000-d M_r fragment that had lost the C-terminal portion of the LTP. This fragment then remained essentially intact during the remainder of the 2-hour digestion and retained its IgE-binding potency and is probably

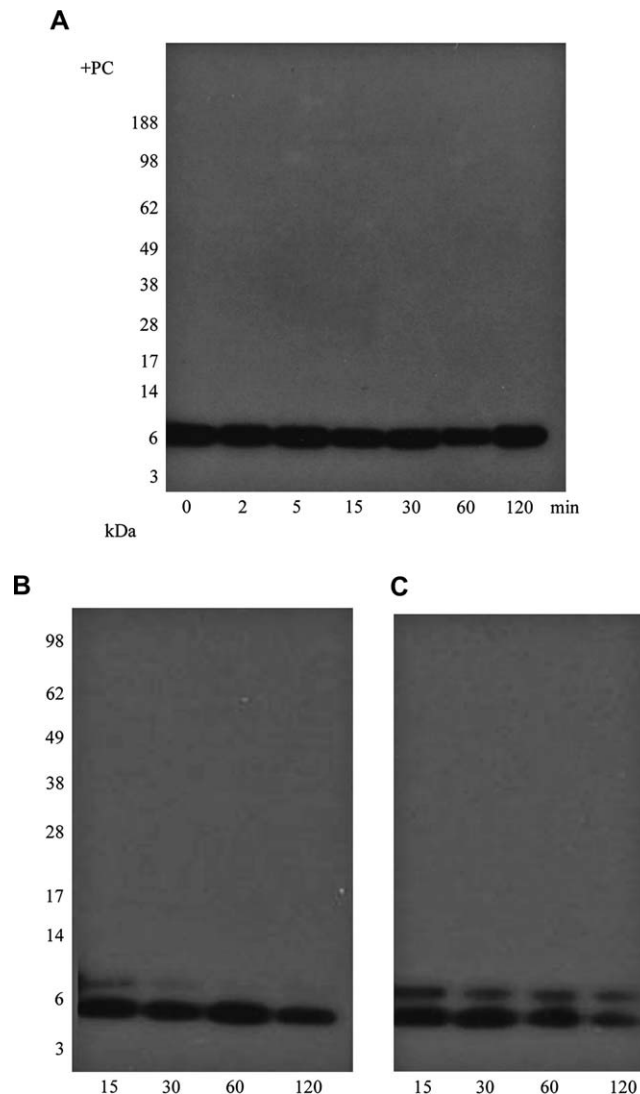


FIG 4. IgE reactivity of grape LTP digestion products determined by means of immunoblotting with sera from individuals with grape allergy. **A**, Gastric digestion (+PC) with serum from patient 44. IgE binding was identical in the absence of PC (not shown). **B** and **C**, Gastroduodenal digestion with or without PC with serum from patient 44, respectively.

why the LTP digests retained their biologic activity *in vitro* and *in vivo*. Given that the N-terminal region of the approximately 6000-d M_r fragment was intact, some of the potential tryptic and chymotryptic cleavage sites were not accessed by these proteases. It might be that the disulphide bonds restrict the polypeptide mobility more in this region, making this portion of the protein a poor substrate for proteases. Intriguingly, the bioinformatic analysis suggests that the approximately 6000-d M_r fragment probably contains 2 of the major IgE epitopes described for the peach LTP allergen Pru p 3 at residues 11 to 25 and 31 to 45.²³ A third more weakly reacting epitope, being located at residues 71 to 85, was degraded and remained attached to the main fragment through the Cys⁷⁶-Cys³⁰ disulphide bond. The resistance of IgE epitopes in the LTP was similar to the resistance of epitopes

in the 2S albumin of Brazil nut, another member of the prolamin superfamily.¹⁷

Most LTPs described to date possess a lipid-binding tunnel that is highly plastic and can bind a wide range of lipophilic molecules. The presence of the ligand can increase stability by reducing polypeptide backbone mobility, and many proteases require flexibility in substrate proteins for optimal activity.²⁷

Thus we also studied the influence of physiologic phospholipids, the natural ligands of LTPs, on the stability of the protein to digestion. This demonstrated that gastric PC had a slight protective effect on both the protein and its IgE reactivity, slowing LTP breakdown during duodenal digestion, probably as a consequence of PC binding to the LTP. The significance for the ability of LTP to sensitize or elicit reactions through the gastrointestinal tract is not

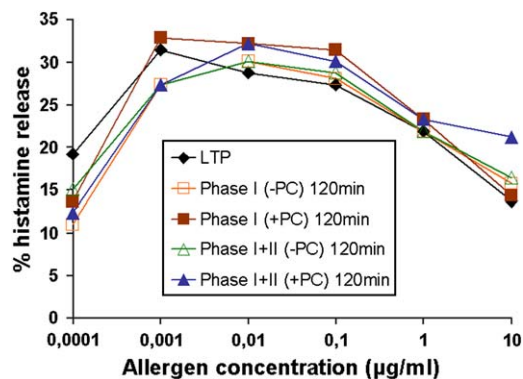


FIG 5. Effect of digestion on the ability of grape LTP to stimulate basophil histamine release. The assay was performed with serum from patient 44. *Diamonds*, Intact LTP; *open squares*, gastric digestion without PC; *solid squares*, gastric digestion with PC; *open triangles*, gastroduodenal digestion without PC; *solid triangles*, gastroduodenal digestion with PC.

clear but does point to there being lipid-protein interactions, which might have the potential to modulate allergenic activity.

The observation that grape LTP, even after duodenal digestion, retained its allergenic activity *in vivo* contributes further to our knowledge of this important group of pan-allergens. Building on such observations of allergen stability, future work will focus on investigating the role such properties might play in both sensitization processes in the gastrointestinal tract and the elicitation of reactions in sensitized individuals, which appear to be associated with such severe reactions in individuals with LTP allergy.

We thank Fridolin van der Lecq and coworkers for their excellent work on the protein sequences (Sequentie Centrum, Utrecht, The Netherlands).

REFERENCES

- Sanchez-Monge R, Lombardero M, Garcia-Selles FJ, Barber D, Salcedo G. Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* 1999;103:514-9.
- Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Spano M, Monza M, et al. The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* 1999;103:520-6.
- Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, et al. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol* 2002;109:563-70.
- Lombardero M, Garcia-Selles FJ, Polo F, Jimeno L, Chamorro MJ, Garcia-Casado G, et al. Prevalence of sensitization to *Artemisia* allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid-transfer protein allergens. *Clin Exp Allergy* 2004;34:1415-21.
- Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H, Mills EN. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an *in silico* analysis. *J Allergy Clin Immunol* 2005;115:163-70.
- Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier MF, Ciurana CL, et al. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000;122:20-32.
- Sancho AI, Rigby NM, Zuidmeer L, Asero R, Mistrello G, Amato S, et al. The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* 2005;60:1262-8.
- Scheurer S, Lauer I, Foetisch K, Moncin MS, Retzek M, Hartz C, et al. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J Allergy Clin Immunol* 2004;114:900-7.
- Schocker F, Luttkopf D, Scheurer S, Petersen A, Cistero-Bahima A, Enrique E, et al. Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for *in vitro* diagnosis of potentially severe hazelnut allergy. *J Allergy Clin Immunol* 2004;113:141-7.
- Rodriguez A, Matheu V, Trujillo MJ, Martinez MI, Baeza ML, Barranco R, et al. Grape allergy in paediatric population. *Allergy* 2004;59:364.
- Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Fortunato D, Giuffrida MG, et al. Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin. *J Allergy Clin Immunol* 2003;111:350-9.
- Vignec AJ, Weinberg S. Grape juice in infant feeding: comparative sensitivity to orange and grape. *J Pediatr* 1954;44:294-8.
- Schad SG, Trcka J, Vieths S, Scheurer S, Conti A, Brocker EB, et al. Wine anaphylaxis in a German patient: IgE-mediated allergy against a lipid transfer protein of grapes. *Int Arch Allergy Immunol* 2005;136:159-64.
- Moreno FJ, Jenkins JA, Mellon FA, Rigby NM, Robertson JA, Wellner N, et al. Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (*Bertholletia excelsa*). *Biochim Biophys Acta* 2004;1698:175-86.
- Heinzerling L, Frew AJ, Bindeslev-Jensen C, Bonini S, Bousquet J, Bresciani M, et al. Standard skin prick testing and sensitization to inhalant allergens across Europe—a survey from the GALEN network. *Allergy* 2005;60:1287-300.
- Bjorksten F, Halmepuro L, Hannuksela M, Lahti A. Extraction and properties of apple allergens. *Allergy* 1980;35:671-7.
- Moreno FJ, Mellon FA, Wickham MS, Bottrill AR, Mills EN. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant *in vitro* gastrointestinal digestion. *FEBS J* 2005;272:341-52.
- van Ree R, Fernandez-Rivas M, Cuevas M, van Wijngaarden M, Aalberse RC. Pollen-related allergy to peach and apple: an important role for profilin. *J Allergy Clin Immunol* 1995;95:726-34.
- van der Zee JS, de Groot H, van Swieten P, Jansen HM, Aalberse RC. Discrepancies between the skin test and IgE antibody assays: study of histamine release, complement activation *in vitro*, and occurrence of allergen-specific IgG. *J Allergy Clin Immunol* 1988;82:270-81.
- Schuurman J, Perdok GJ, Aalberse RC. Mouse/human chimeric antibodies directed to two nonoverlapping epitopes of the house dust mite allergen, Der p 2. *Int Arch Allergy Immunol* 1997;113:262-3.
- Budde IK, Aalbers M, Aalberse RC, van der Zee JS, Knol EF. Reactivity to IgE-dependent histamine-releasing factor is due to monomeric IgE. *Allergy* 2000;55:653-7.
- Siraganian RP. Refinements in the automated fluorometric histamine analysis system. *J Immunol Methods* 1975;7:283-90.
- Garcia-Casado G, Pacios LF, Diaz-Perales A, Sanchez-Monge R, Lombardero M, Garcia-Selles FJ, et al. Identification of IgE-binding epitopes of the major peach allergen Pru p 3. *J Allergy Clin Immunol* 2003;112:599-605.
- Mills EN, Jenkins JA, Alcocer MJ, Shewry PR. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit Rev Food Sci Nutr* 2004;44:379-407.
- Asero R. Detection and clinical characterization of patients with oral allergy syndrome caused by stable allergens in Rosaceae and nuts. *Ann Allergy Asthma Immunol* 1999;83:377-83.
- Duffort OA, Polo F, Lombardero M, Diaz-Perales A, Sanchez-Monge R, Garcia-Casado G, et al. Immunoassay to quantify the major peach allergen Pru p 3 in foodstuffs. Differential allergen release and stability under physiological conditions. *J Agric Food Chem* 2002;50:7738-41.
- Breiteneder H, Mills EN. Molecular properties of food allergens. *J Allergy Clin Immunol* 2005;115:14-24.