

Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion

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Background: Nonspecific lipid transfer proteins (nsLTPs) have been identified as major fruit allergens in patients from the Mediterranean area. Sensitization to nsLTPs is accompanied by severe reactions, possibly because of specific biophysical and biochemical properties of this allergen family.

Objective: To assess the protein stability and allergenic potency of nsLTP from fruits in comparison with birch pollen-related allergens from the same allergenic source.

Methods: Stability of natural and recombinant cherry allergens Pru av 3 (nsLTP), Pru av 1 (Bet v 1 homologue), and Pru av 4 (profilin) to pepsin digestion and to thermal processing and stability of allergens in skin prick test reagents was investigated by immunoblotting and/or circular dichroism spectroscopy. Moreover, allergenicity of processed and fresh fruits in regard to Pru av 1 and Pru av 3 was analyzed by histamine release assays. **Results:** Lipid transfer proteins showed the highest resistance to digestion by pepsin (rPru av 3 > rPru av 1 > rPru av 4). Immunologically active Pru av 3 was detectable after 2 hours of digestion by pepsin, whereas IgE reactivity of Pru av 1 and Pru av 4 was abolished within less than 60 minutes. In contrast with Pru av 1, IgE reactivity to nsLTPs was not diminished in thermally processed fruits, and secondary structures of purified Pru av 3 were more resistant to heating. Moreover, nsLTPs were stable components in skin prick test reagents. Histamine release assays confirmed the strong allergenicity of nsLTPs, which was not affected by protease treatment or thermal processing of fruits.

Conclusion: In contrast with birch pollen-related allergens, nsLTPs are highly stable to pepsin treatment and thermal processing and show higher allergenic potency. Therefore, nsLTPs have the potential to act as true food allergens, probably eliciting severe systemic reactions by reaching the intestinal mucosa in an intact and fully active form. (*J Allergy Clin Immunol* 2004;114:900-7.)

Keywords: Lipid transfer protein, nsLTP, food allergy, Pru av 1, Pru av 3, Pru av 4, stability, pepsin digestion, thermal treatment, histamine release

Nonspecific lipid transfer proteins (nsLTPs) have been described as predominant *Rosaceae* fruit allergens in the Mediterranean population. Sensitization to nsLTPs occurs independently from birch pollinosis, and symptoms of LTP-sensitized subjects are frequently systemic, including anaphylactic reactions in addition to oral allergy syndrome (OAS). In contrast, pollen-related food allergens in fruits most typically cause only OAS. IgE reactivity to pollen-related food allergens is the consequence of primary sensitization to inhalant allergens and an IgE cross-reactivity with homologous food allergens. In contrast, nsLTPs are probably capable of sensitizing by the ingestion route. The high stability to proteolytic digestion of nsLTPs is discussed as a possible reason for more severe and systemic reactions compared with other allergens that are digestible in gastrointestinal fluid.^{1,2} Until now, only limited information on the stability of nsLTPs to protease and thermal treatment is available, and the properties of nsLTPs have not been compared directly with Bet v 1-related allergens from the same food source.

Pru p 3,³ a nsLTP, the major allergen from peach (*Prunus persica*), has been shown to be resistant to digestion of peach extract by proteases.^{4,5} A mAb-based ELISA revealed no reduction of a reactivity of Pru p 3 in peach extracts after as long as 2 hours of pepsin treatment.⁵ In addition to the peach LTP, IgE binding to recombinant carrot nsLTP was shown to be resistant to pepsin digestion.^{6,7} Four α -helices forming a compact barrel-like structure stabilized by 4 disulfide bonds are probably responsible for the high stability of nsLTPs. The stability of nsLTPs is in strong contrast with birch pollen-related food allergens, which have been found to be digested rapidly, thereby not surviving in the gastrointestinal tract.^{8,9}

The possibility that heating procedures reduce the allergenicity of particular foods is of major interest for patients allergic to food. In general, IgE reactivity to birch pollen-related food allergens is remarkably reduced by heating—for example, by roasting of hazelnuts⁹⁻¹¹ or

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Supported by Deutsche Forschungsgemeinschaft DFG SCHE 637/2-1 and VI 165/5-1.

Received for publication April 7, 2004; revised June 2, 2004; accepted for publication June 4, 2004.

Available online August 9, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.06.017

Abbreviations used

CCD:	Cross-reactive carbohydrate determinant
CD:	Circular dichroism
EAST:	Enzyme allergosorbent test
HR:	Histamine release
LTP:	Lipid transfer protein
nsLTP:	Nonspecific lipid transfer protein
OAS:	Oral allergy syndrome
r:	Recombinant
SPT:	Skin prick test

heating of celery¹² and fruit-containing foods.¹³ In contrast, it is more likely that patients allergic to food sensitized to nsLTP develop allergic reactions to heat-processed foods, because nsLTPs were initially described as heat-stable allergens. The first reports on an association of adverse reaction to thermally processed food with a sensitization to nsLTPs were from patients with allergy to peach⁴ and maize.^{14,15} Shortly thereafter, persistent IgE reactivity of barley LTP after the malting and brewing process was shown in some patients allergic to beer.^{16,17} In addition, barley LTP displayed high stability to denaturant guanidine hydrochloride, heat, and acidic pH.¹⁸ IgE reactivity of Cor a 8, the LTP from hazelnut, was not reduced in roasted hazelnuts when tested with sera from patients allergic to hazelnut with anaphylaxis.¹⁰ In addition, heat stability of LTPs was shown by preabsorption of Pru p 3-sensitized patient sera with heat-processed apple extract.¹⁹ In the same study, skin reactivity and open oral challenge tests of 2 LTP-monosensitized patients were positive with heat-processed apple.

Recently, the nsLTP from cherry, Pru av 3, was identified as major allergen in Mediterranean patients allergic to cherry,^{20,21} but showed minor clinical relevance in Central and Northern Europe where birch trees are abundant. Patients with birch pollen-related cherry allergy are predominantly sensitized to the Bet v 1-homologous allergen, Pru av 1.²² Furthermore, the thaumatin-like protein Pru av 2²³ and profilin Pru av 4,²⁰ a birch pollen homologue, have been described as allergenic proteins in cherry. In this study, both birch pollen-related cherry allergens were compared with the nsLTP from cherry with regard to their stability and allergenicity. Stability of 3 recombinant (r) cherry allergens and a freshly prepared extract were compared by a time-dependent digestion with pepsin. Thermal denaturation of rPru av 3 and rPru av 1 was analyzed by circular dichroism (CD) spectroscopy and by IgE reactivity of extracts derived from cherry-containing processed foods. Further, the allergenic potency of Pru av 3 was compared with Pru av 1 by histamine release assays from passively sensitized basophils.

METHODS

Patients

Sera were selected from patients allergic to cherry recruited in Central Europe (n = 11) and Spain (n = 4). The study was approved

by local ethics committees, and informed consent was obtained from the patients. Subjects were regarded as being allergic to cherry if they had a clear history of immediate reaction after ingestion of cherries and a positive skin prick test (SPT) to fresh fruit or cherry extract as well as serum IgE specific for cherry. Sensitization to cherry allergens was confirmed by enzyme allergosorbent test (EAST; Allergopharma, Reinbek, Germany) and by PharmaciaCAP (Pharmacia Diagnostics, Uppsala, Sweden; Table 1). A CAP class ≥ 2 was considered positive. In addition, IgE reactivity to purified recombinant allergens and cherry extract was confirmed by immunoblotting (not shown). All patients allergic to cherry exclusively sensitized to birch pollen-related allergens Pru av 1 and/or Pru av 4 (n = 9) had birch pollinosis and reported mild oral allergy syndrome, except 1 patient (patient 3) showing additional respiratory symptoms. In contrast, 3 of 6 patients sensitized to nsLTPs (CAP classes ≥ 2) were affected by systemic reactions, eg, gastrointestinal symptoms (patient 5); angioedema, anaphylaxis, diarrhea, and urticaria (patient 13) in addition to OAS; and angioedema (patient 15).

Preparation of recombinant allergens and allergenic extracts

Extracts from ripe cherry fruit and processed food were prepared by a low-temperature method as previously described.²⁴ Recombinant cherry allergens Pru av 1, Pru av 3, and Pru av 4 were prepared as described elsewhere.^{20,22} Commercial SPT solutions for diagnosis of cherry allergy were obtained from ALK-Scherax (Hamburg, Germany) and Lofarma (Milan, Italy).

Protease treatment

The self-prepared protein extract (50 μ L; 1 mg/mL) from fresh cherries and purified recombinant allergens (20 μ L; 100 μ g/mL) were adjusted with HCl (0.1 mol/L) to pH 2.5 and subsequently digested with equal volumes of pepsin immobilized to agarose (1:1; vol/vol; ≥ 6 U/ μ L; Pierce, Rockford, Ill) according to the manufacturer's instructions. Pepsin treatment was performed at 37°C under agitation, and the reaction was stopped after 30 seconds, 1 minute, 15 minutes, 30 minutes, 1 hour, or 2 hours, respectively, by adding an equal volume of 1 mol/L Tris-HCl (pH 8). The digestion mixes were analyzed by SDS-PAGE with silver staining and for IgE reactivity. Buffer controls without pepsin were performed with H₂O or HCl, and BSA was used as positive control (2 hours digestion).

SDS-PAGE and immunoblotting

SDS-PAGE was performed under nonreducing conditions (cross-linker C = 5%; total bis/acrylamid T = 15%) according to the method described by Laemmli. For silver staining, 5 μ g of the self-prepared cherry extract protein/cm slot, and for immunoblotting, 10 μ g of the self-prepared cherry extract protein/cm slot and 1.2 μ g purified allergens/cm slot, were applied. Protein transfer to Protean nitrocellulose (0.2 μ m; Schleicher & Schuell, Dassel, Germany) was performed by tank blotting (150 V, 45 minutes).

IgE binding capacity of recombinant allergens and the allergen extract prepared from fresh cherries was tested with a serum pool from patients allergic to cherry (n = 6; 1:6 dilution; overnight) sensitized to Pru av 1, Pru av 3, or Pru av 4, respectively. IgE binding was detected by a rabbit antihuman IgE antiserum (1:4000; 1 hour; DakoCytomation, Hamburg, Germany), followed by a biotinylated goat antirabbit IgG (1:6000; 1 hour; DakoCytomation). Visualization was performed by alkaline phosphatase conjugated to streptavidine (1:3000; 30 minutes; Medac, Hamburg, Germany) and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad Laboratories, Munich, Germany), as substrate. Western blot analysis of the protein extract prepared from thermally processed cherries was performed with individual sera from patients with sensitization to Pru av 1 and Pru av 3, respectively. IgE reactivity

TABLE 1. Clinical and serologic data from patients allergic to cherry (1-11 from Central Europe, 12-15 from Spain)*

Patient	Symptoms	Cherry	rPru av 1		rPru av 3		rPru av 4		HR		
		EAST			CAP				<i>In vitro</i> HR 25% values† (ng/mL)		
		Class	U/mL	Class	U/mL	Class	U/mL	Class	Cherry	Pru av 1	Pru av 3
1	OAS	2	51.2	5	0.5	1	<0.35	0			
2	OAS	2	43.9	4	<0.35	0	0.46	1			
3	OAS, RS	2	6.16	3	<0.35	0	<0.35	0			
4	OAS	2	8.82	3	17.1	3	0.81	2			
5	OAS, GS	2	4.98	3	22.6	4	11.7	3			
6	OAS	2	9.78	3	<0.35	0	10.5	3			
7	OAS	2	<0.35	0‡	<0.35	0‡	5.2	3‡			
8	OAS	2	<3.5	2‡	<0.35	0‡	<3.5	2‡			
9	OAS	3	31.3	4	<0.35	0	7.58	3	300	<1	ND
10	OAS	ND	18.1	4	0.58	1	<0.35	0	3000	5	ND
11	QAS	0	18.0	4	<0.35	0	<0.35	0	9000	5	ND
12	OAS	3	<0.35	0	>100	6	<0.35	0	2.5	ND	0.1
13	OAS, AE, A, D, U	3	<0.35	0	26.0	4	<0.35	0	5	ND	0.25
14	OAS	2	<0.35	0	18.10	4	<0.35	0	250	ND	10
15	AE	2	<0.35	0	4.43	3	<0.35	0			

A, Anaphylaxis; AE, angioedema; D, diarrhea; GS, gastrointestinal symptoms; RS, respiratory symptoms; U, urticaria.

*Patients' sera 9-14 were applied in the *in vitro* histamine release assay.

†For HR curves, see Fig E1 in the Online Repository.

‡Specific IgE determination performed by EAST.

of patient sera was determined as described, except that 20 µg/cm total protein from extracts and a biotinylated goat anti-huIgE antiserum (1:750; 1 hour; KPL, Gaithersburg, Md) were applied. Both commercial SPT reagents of cherry were dialyzed against 0.01-mol/L PBS pH 7.4, separated by SDS-PAGE and subsequently applied for silver staining (10 µg protein/cm) or immunoblotting (3.25 µg protein/cm) with a mAb raised against Pru av 1 (1:10 supernatant) and a rabbit anti-Pru p 3 serum that cross-reacted with Pru av 3 (1:20,000; ALK-Abelló, Madrid, Spain). Dilutions of antibodies and were performed in TBS; Tween 20, 0.05%; and skim milk, 0.5%.

CD spectroscopy

Circular dichroism spectra of rPru av 3 and rPru av 1 were recorded at 0.2 mg/mL (13.8 µmol/L for Pru av 3 and 10.8 µmol/L for Pru av 1) purified protein in 10 mmol/L KH₂PO₄ buffer (pH 7.4) on a Jasco J-810 S spectropolarimeter (Gross-Umstadt, Germany) with a step width of 0.2 nm and a band width of 1 nm at different temperatures: 20°C, 40°C, 70°C, 100°C, and 20°C after cooling down. The spectral range was 185 to 255 nm recorded at 50 nm/min. Ten scans were accumulated.

Histamine release assay

Histamine release assay was performed as described by Kleine Budde et al,²⁵ with several modifications according to Foetisch et al.²⁶

RESULTS

Lipid transfer proteins are highly resistant to gastric enzymes

Freshly prepared cherry extract was digested for as long as 2 hours with pepsin immobilized to agarose. After 30 seconds, 1 minute, 15 minutes, 30 minutes, 1 hour, and 2 hours, the proteolytic digestion of cherry proteins was analyzed by SDS-PAGE, followed by silver staining

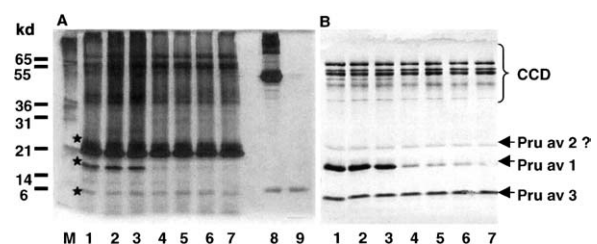


FIG 1. Cherry extract (50 µg total protein) was digested with pepsin, separated by SDS-PAGE, silver stained (**A**), and tested for IgE reactivity with a pool of cherry allergic patients' sera (**B**). Two hours without pepsin (1); 30 seconds (2); 1 minute (3); 15 minutes (4); 30 minutes (5); 1 hour (6); 2 hours (7); BSA without pepsin (8); BSA, 2 hours (9). *Cherry allergens.

(Fig 1, A) and by IgE reactivity with a pool of sera from patients allergic to cherry (n = 6; Fig 1, B). IgE reactivity to the Bet v 1-related fruit allergen Pru av 1, with a molecular weight of 18 kD, was nearly completely abolished after 15 minutes of incubation. No reduction of IgE reactivity was observed for the 9-kD nsLTP and high-molecular-weight glycoproteins containing cross-reactive carbohydrate determinants (CCDs). The IgE reactivity of 3 of 6 donors of the serum pool was confirmed in independent experiments with purified glycopeptides (not shown). Moreover, the serum pool showed weak IgE binding to a stable protein with a molecular weight of 24 kD, probably Pru av 2. The reactivity to this allergen resulted from a single serum (patient 4) component of the serum pool (not shown). Profilin was not detectable either in the treated extract or in the untreated extracts. BSA, used as positive control, was completely digested within 2 hours.

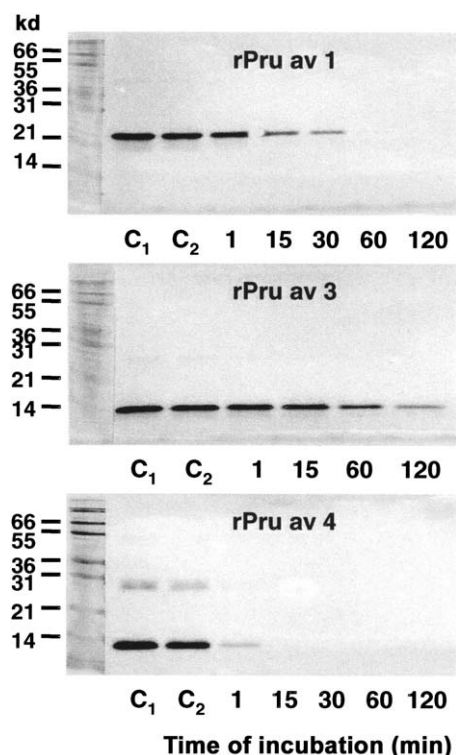


FIG 2. Purified recombinant cherry allergens (Pru av 1–HisTag, Pru av 3–HisTag, Pru av 4; 2 μ g each) were digested with pepsin (20 μ L; as long as 2 hours). Analysis of IgE reactivity was performed with a pool of cherry allergic patients' sera (1:6 dilution) sensitized to Pru av 1, Pru av 3, or Pru av 4, respectively. Buffer control (C1, H₂O; C2, HCl).

In addition, the effects of pepsin on the IgE reactivity of 2 recombinant birch pollen–related allergens (rPru av 1, rPru av 4) and the recombinant, birch pollen–unrelated, nsLTP Pru av 3 were compared (Fig 2). Pru av 3 showed the strongest stability to proteolytic digestion by pepsin under acidic conditions (rPru av 3 > rPru av 1 > rPru av 4). Immunologically active Pru av 3 was still detectable after 2 hours of digestion. In contrast, IgE reactivity of Pru av 1 and Pru av 4 was abolished after 60 minutes and 15 minutes, respectively.

CD spectroscopy of cherry LTP and Pru av 1

The CD spectra of recombinant Pru av 3 (Fig 3, B) clearly indicated the presence of α -helical structures on the basis of 2 theoretical minima at 208 nm and 222 nm, instead of a typical minimum at 215 nm for β -sheeted proteins. The temperature-induced loss of structure was progressive. Changes in the ellipticity at 222/208 nm started at 70°C and were completed at 100°C. After heating to 100°C, the signature ellipticities of the native protein were irreversibly lost. In contrast, the secondary structures of Pru av 1 (Fig 3, A) were already nearly completely lost at 70°C, but were partially refolded at room temperature after the heating process.

LTPs retain IgE reactivity in thermally processed foods

IgE reactivity to the 2 most important cherry allergens, Pru av 1 and Pru av 3, was assigned by immunoblotting of thermally processed foods containing cherry with individual sera from patients sensitized to Pru av 1 (patient 2 and patient 5) and nsLTP Pru av 3 (patient 5; Fig 4). IgE binding to nsLTP was retained in all extracts derived from heat-processed cherry-containing foods and was similar to that of extract prepared from fresh cherries. The identity of the protein as nsLTP was confirmed with a polyclonal antibody raised against Pru p 3, the homologous nsLTP from peach (not shown). In contrast, IgE reactivity to Pru av 1 was nearly completely abolished in all 3 processed foods, except for a weak but strongly reduced reactivity of patient 2 to Pru av 1 when the protein extract from preserved cherries was tested.

Commercial SPT reagents contain nsLTP, but no Bet v 1 homologue

Both commercial SPT reagents showed reactivity with the antiserum raised against Pru p 3 and a serum sample from a patient (patient 13) sensitized to Pru av 3 (Fig 5). Although both SPT reagents revealed a similar protein pattern, LTPs were more abundant in the extract from supplier A. By application of a mAb raised against Pru av 1 and a serum sample from a patient (patient 3) reactive exclusively to Pru av 1, the Bet v 1 homologue was not detected in either of the SPT reagents.

Allergenic potency of LTPs is not impaired by thermal and pepsin treatment of allergen extracts

Histamine release assays were performed with passively sensitized basophils to compare the allergenic potency of nsLTP rPru av 3, rPru av 1, and a self-prepared extract from fresh cherries (see Fig E1 in the Journal's Online Repository at www.mosby.com/jaci). Six sera (patients 9–14) from patients sensitized to Pru av 1 (patients 9–11) or Pru av 3 (patients 12–14) were selected on the basis of a high specific IgE titer to the respective allergens (Table 1). Induction of mediator release was performed in a dose-dependent manner with rPru av 1 (10^{-3} to 1 μ g/mL), rPru av 3 (5×10^{-6} to 5×10^{-2} μ g/mL), and cherry extract (10^{-1} to 10^1 μ g/mL total protein) for the Pru av 1–sensitized patients, or 10^{-4} to 1 μ g/mL for the nsLTP-sensitized donors (see Fig E1 in the Online Repository). For all patients, a significant and dose-dependent mediator release was induced by all of rPru av 1, rPru av 3, and cherry extract. To obtain maximum histamine levels, Pru av 1 concentrations of 10^{-1} μ g/mL or higher were required. In contrast, a Pru av 3 concentration less than 10^{-2} μ g/mL was sufficient to induce a maximum mediator release for 2 sera from LTP-sensitized subjects (patients 12 and 13). Approximately 300-fold to 1800-fold higher concentrations of cherry extract were required to obtain histamine release values (HR 25%) compared with Pru av 1. In contrast, only

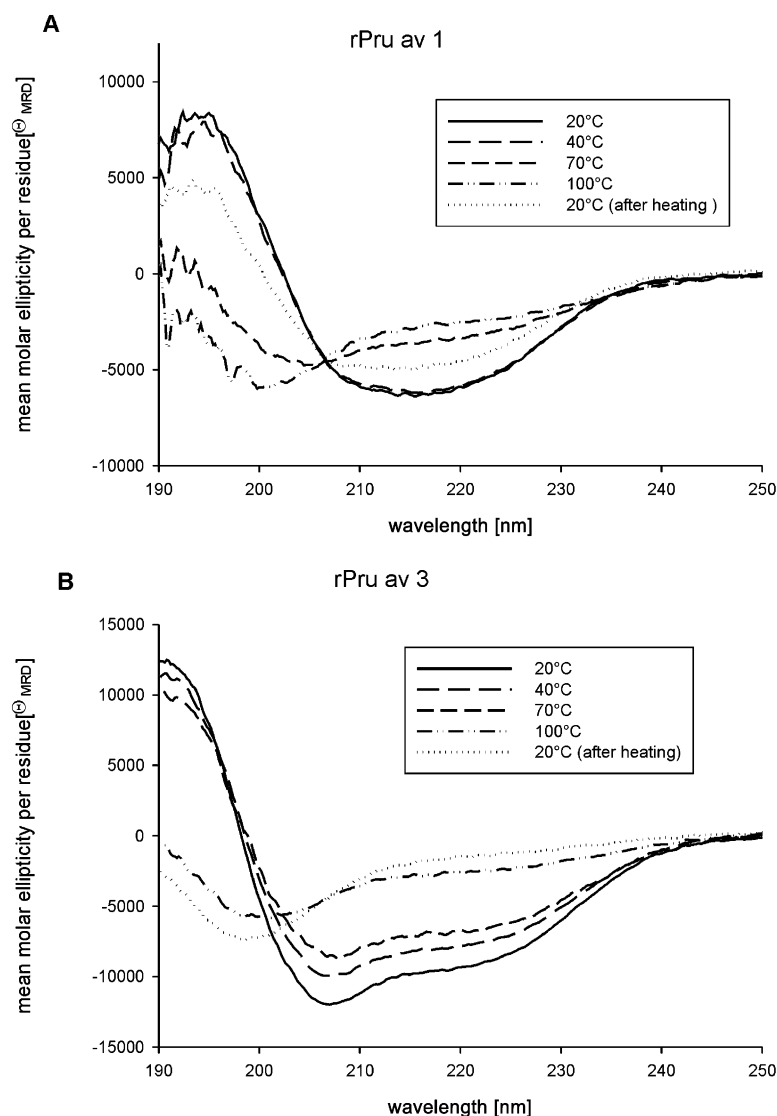


FIG 3. CD spectra of rPru av 1 (**A**) and rPru av 3 (**B**) were recorded at different temperatures: 20°C, 40°C, 70°C, 100°C, and after cooling down to 20°C. The spectral range was 185 to 255 nm, and the scanning rate was 50 nm/min. Ten scans were accumulated. MRD, Magnetic relaxation dispersion.

20-fold to 25-fold higher concentrations of extract were needed to induce a mediator release of 25% (Table 1; Fig E1) compared with Pru av 3.

Extracts from the 3 heat-processed foods containing cherry, a pepsin-treated cherry extract, an extract prepared from fresh cherries, and both recombinant allergens Pru av 1 and Pru av 3 were used in additional histamine release studies (Fig 6). All processed extracts were capable of causing histamine release from basophils passively sensitized with a serum from a nsLTP-monosensitized patient, patient 12 (Fig 6, A). Histamine release values derived from these extracts were in the same order of magnitude as for the untreated extract. Recombinant Pru av 3 was applied as positive control. As expected, no specific mediator release was induced by rPru av 1. Divergent allergenic potency was determined for the processed and

untreated extracts by using sera from patients (eg, patient 9) with sensitization to Pru av 1 and no reactivity to Pru av 3 (Fig 6, B). All 3 heat-treated extracts revealed none or a nearly abolished biological potency. In addition, cherry extract treated for 1 hour with pepsin had a strongly reduced histamine release capacity in comparison with the untreated extract. Recombinant Pru av 1 was applied as positive control. As expected for this patient (patient 9), no mediator release was induced by rPru av 3.

DISCUSSION

It is well known that nsLTPs are clinically relevant allergens in the Mediterranean area. Sensitization to this allergen family is frequently associated with systemic and

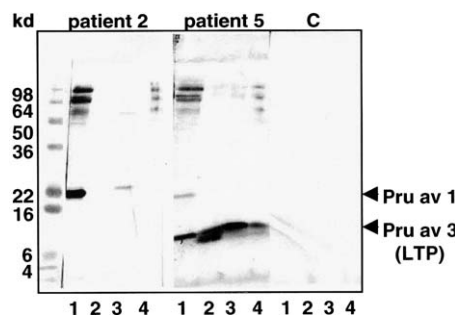


FIG 4. Analysis of IgE reactivity of heat-processed foods containing cherry by using sera from 2 patients allergic to cherry with sensitization to Pru av 1 (patient 2) and additional sensitization to Pru av 3 (patient 5). Fresh sweet cherries from the market (1); cherry fruit juice, 35% fruit content (Rapp's, Karben, Germany) (2); preserved cherries, sweetened (Kaiserkirschen mit Stein, Erlenhof, REWE-Erlenhof, Germany) (3); cherry jam, 45% fruit content (Erlenhof) (4).

severe reactions. This clinical pattern is thought to be related to the high stability of these molecules. Even though nsLTPs are generally reported to be resistant to heat and protease treatment, only a limited number of reports confirming such properties of this allergen family are available. Few studies have addressed the stability of nsLTPs in relation to their IgE reactivity, and in particular the allergenic potency. Until now, no study was performed directly comparing stability of birch pollen-related food allergens with nsLTP from the same food. Here, we investigated the stability of the fruit allergen Pru av 3 (nsLTP) in comparison with birch pollen homologous allergens Pru av 1 (Bet v 1 family) and Pru av 4 (profilin) from cherry. We studied the effects of both pepsin treatment and heat processing on the allergenic activity, not only in IgE binding tests but also with a functional histamine release assay.

Cherry allergens were digested by pepsin coupled to agarose to almost simulate gastrointestinal fluid. In contrast with Pru av 1, cherry nsLTP, CCDs, and a 24-kD protein retained their IgE reactivity after the treatment. In particular, Pru av 2, a 24-kD thaumatin-like protein, has been recently described as an allergenic structure with a potentially high stability.^{23,27} Pru av 4 was not detected in the self-repaired cherry extract by the applied serum pool, probably because of an insufficient quantity in the extract. The stability data obtained with the extract were confirmed by comparison of the 3 purified recombinant cherry allergens in the same assay, resulting in a pepsin stability ranking rPru av 3 > rPru av 1 > rPru av 4. These results are in accordance with recent findings showing the IgE reactivity of peach LTP (Pru p 3) after 2 hours of pepsin digestion.⁵ We can not ensure that the activity of purified proteases reflects the physiological conditions *in vivo*, but the approach clearly showed the relative stability of nsLTPs in comparison with birch pollen-related food allergens. Many food allergens are known to be resistant to pepsin,²⁸ a feature of a classical or true food allergen,

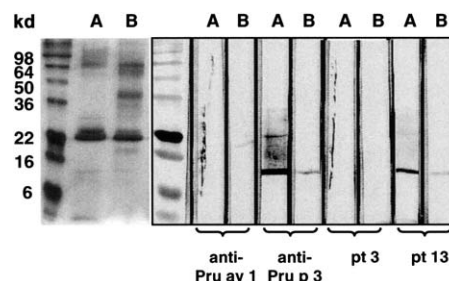


FIG 5. SDS-PAGE and silver staining (left) and immunoblotting (right) of 2 commercial cherry SPT extracts (A and B). Right, Detection of Pru av 1 and Pru av 3 by specific IgG (mouse anti-Pru av 1, rabbit anti-Pru p 3) and specific IgE from patients allergic to cherry (patient [pt] 3 sensitized to Pru av 1; pt 13 sensitized to Pru av 3).

without relation to pollen allergens, to which sensitization takes place by the oral route.

In addition to their stability to proteases, nsLTPs are characterized by their resistance to thermal processing. By comparison of both purified allergens by CD spectroscopy, we found the secondary structure of Pru av 3 more resistant to heating than that of Pru av 1, which was nearly completely lost at 70°C. The data are in accordance with results of Pastorello et al,¹⁵ who described an irreversible alteration of the secondary structure of isolated maize LTP after progressive heating. In the case that heated samples were still IgE-reactive, they postulated the presence of linear IgE epitopes on LTPs. In contrast, studies on 2S albumins have shown that disulfide bridges stabilize the secondary structure and prevent irreversible denaturation of the allergen during heat treatment.²⁹ Because Pru av 3 was expressed in *Escherichia coli* BL21(DE3), we can not rule out the possibility that not all disulfide bridges were correctly formed, and thus, the natural protein would retain its secondary structure under the same experimental conditions.

The effect of thermal processing on food allergens is of major interest for patients who need to avoid all food preparations containing active allergens. In addition to purified allergens, we therefore investigated the stability of allergens in industrially prepared foods. Frequently, thermally treated foods, such as juices, jams cakes, and sweets are prepared from *Rosaceae* fruits, and other foods like maize, hazelnut, wheat, and barley are processed by thermal procedures. Heat-stable allergenic LTPs are a constituent of all of these foods, even if quantification of the peach nsLTP Pru p 3 showed strong variability in juices.⁵ Although the allergenic potency of foods can be modified by the Maillard reaction during heating processes—for example, an irreversible reduction of IgE reactivity of Pru av 1 undergoing a reaction with specific carbohydrates—has been observed,³⁰ Pru av 3 was still IgE-reactive in heat-processed products containing high amounts of sugars. Therefore, thermally processed foods still pose an allergenic risk to patients with sensitization to nsLTPs.

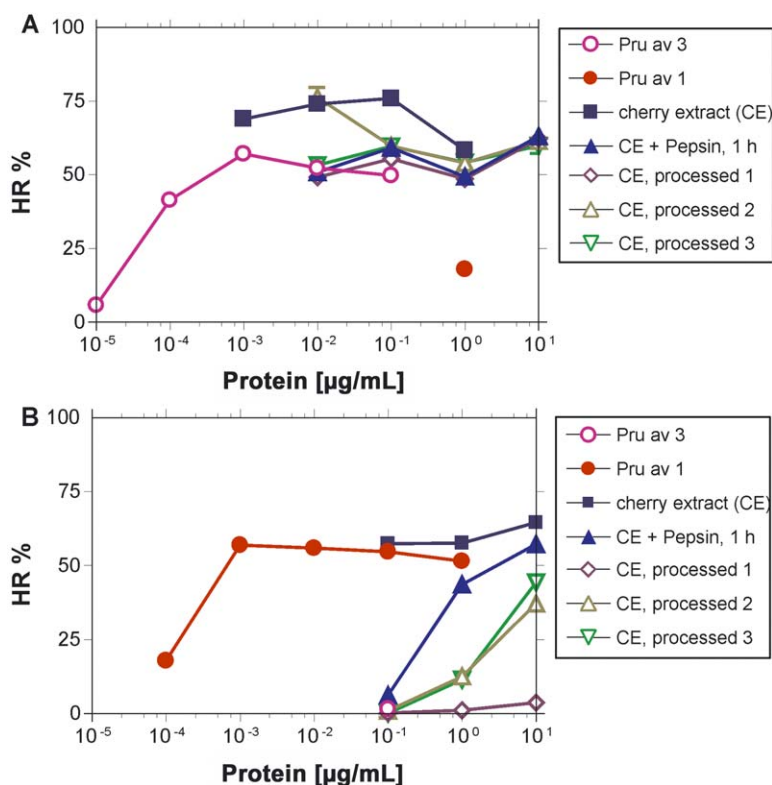


FIG 6. Histamine release from passively sensitized basophils from a cherry LTP-sensitized patient (patient 12) (**A**) and a Pru av 1-sensitized patient (patient 9) (**B**), induced by cherry extract (CE) prepared from fresh fruits, CE digested by pepsin, and CE prepared from 3 processed food products containing cherries (1, cherry fruit juice; 2, preserved cherries; 3, cherry jam).

Stability of allergens is crucial for the quality and allergen composition of commercial SPT reagents for an accurate allergy diagnosis. In this study, we found different levels of Pru av 3 in the investigated cherry SPT reagents, but no detectable Bet v 1 homologue. In keeping with our observation, strong variation of the concentration of both nsLTP and Bet v 1 homologue was recently reported by Akkerdaas et al³¹ for hazelnut SPT reagents. This phenomenon is probably caused by different extraction methods and the instability of Bet v 1-homologous food allergens in the presence of polyphenol oxidases. Hence, both of the tested SPT reagents from cherry may be sufficient for an accurate diagnosis of nsLTP-sensitized patients, but the application of such extracts will tend to give false-negative results for patients allergic to fruit with birch pollen-related cherry allergy.

The allergenic potency of nsLTP Pru av 3 was recently demonstrated *in vivo* by SPT.²¹ Here, we compared the allergenic potency of Pru av 3 and Pru av 1 by an *in vitro* mediator release assay with passively sensitized basophils. We showed that very low concentrations of Pru av 3 are sufficient to induce a significant histamine release in LTP-mono-sensitized patients, reflecting the allergenic potency of this allergen family. In addition, the results clearly showed that the allergenic potency of nsLTPs is retained after pepsin digestion of Pru av 3 and even after thermal processing of commercially available foods containing cherry, whereas Pru av 1 was found to be more

labile under the same conditions. Stability to enzymatic digestion is to be used as 1 criterion in a decision tree proposed by Food and Agriculture Organization/World Health Organization³² to assess the allergenic potential of novel plant food proteins. It is noteworthy that patients with sensitization to LTPs probably will not reduce their risk of adverse reaction by cooking or boiling of the food and should be advised to peel fruits even after thermal processing. Unfortunately, we have no information whether the LTP-sensitized patient who showed strong *in vitro* histamine release with the treated cherry extracts revealed allergic symptoms after ingestion of processed food, because he avoided fruit in general.

The overall stability of nsLTPs is consistent with their compact structure, stabilized by 4 disulfide bounds, and allows the allergen to be presented to the immune system of the gastrointestinal tract in an allergenic conformation. Their resistance to degradation is also in accordance with the frequently observed severe reactions that occur in patients sensitized to this allergen family. Similarly, mild symptoms, which are frequent in patients with pollen-related allergy to fruits, correlated well with the stability data of Pru av 1 obtained in our study. In addition to allergen stability, we have to take into consideration that properties that determine allergen uptake by the mucosa of the gastrointestinal tract may also have an effect on the presentation to the cells of the immune system and therefore to the allergenic response.

In summary, stability of allergens to processed foods is important for the quality considerations for allergenic extracts for therapy and diagnosis, and for assessment of the allergenic potency of novel foods. Nonspecific LTPs are extremely heat-stable and protease-resistant. Therefore, they have the potential to initiate allergic sensitization to foods after ingestion and are relevant allergenic components in thermally processed foods.

We thank Domingo Barber and Manuel Lombardero (ALK-Abelló) for providing the anti-Pru p 3 serum.

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