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RESEARCH ARTICLE

Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: Impact on structure and allergenic activity

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Scope: Four Bet v 1 homologous food allergens from celeriac (rApi g 1), apple (rMal d 1), peach (rPru p 1) and hazelnut (rCor a 1), were used to probe the structural responsiveness of the Bet v 1 scaffold to gastric digestion conditions and its impact on allergenicity.

Methods and results: Low pH induced conformational changes of all homologues, which was reduced at physiological ionic strength for all except rPru p 1 as observed by circular dichroism (CD)-spectroscopy. The homologues were rapidly digested by pepsin, losing their IgE binding activity, although the kinetics and patterns of digestion varied subtly between homologues, rApi g 1 being the most stable. We have demonstrated for the first time that gastric phosphatidylcholine (PC) induced conformational changes in all homologues but only rMal d 1 penetrated the PC vesicles as detected by fluorescence polarization, slowing its digestion and retaining more of its allergenic activity. PC enhanced basophil activation of all digested allergens except rApi g 1.

Conclusion: The Bet v 1 scaffold is generally susceptible to low pH and pepsinolysis and interacts with PC vesicles, properties which can explain effects of the gastric environment on their allergenicity. These data show the importance of including surfactants in model digestion systems.

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Bet v 1 homologues / Food allergy / Lipid–protein interaction / Pepsinolysis / Phosphatidylcholine

1 Introduction

One of the most widespread tree pollen allergies in Europe is to birch, Bet v 1 being the major allergen implicated in the condition. Allergenic homologues of Bet v 1 can be found in many plant species and tissues, such as alder and hazel pollen, fruits (e.g. apple, peach, cherry), vegetable tubers and stalks (e.g. carrot, celeriac) and nuts (e.g. hazelnut) [1]. Bet v 1 and its homologues are acidic proteins of between 153 and 160 amino acid residues comprising seven-strands of anti-parallel β-sheets and three α-helices forming a central tunnel. A glycine loop (P-loop), located between the second and third β-strands, represents a conserved area in this protein family [2] being a major IgE epitope on certain Bet v 1 homologues from the *Rosaceae* fruits [3] but not in the homologue of celeriac, Api g 1 [2, 4]. The physiological role of Bet v 1 and its homologues in plants is unknown but

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Abbreviations: CD, circular dichroism; **DMPC,** 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; **PC,** phosphatidylcholine; **SGF,** simulated gastric fluid

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they may protect plants from pathogen attack, which has led them to be classified as belonging to the pathogenesis-related protein family 10 (PR-10) [5]. Structural studies have shown these proteins bind a variety of lipophilic ligands including fatty acids, sterols, flavonoids and cytokines in a hydrophobic tunnel and are membrane active [4, 6], properties which may also relate to their biological role in plants.

Primary inhalant sensitisation to Bet v 1, and subsequent IgE cross-reactivity to homologous proteins might explain adverse reactions to many fresh fruits and vegetables [1]. In some instances, T-lymphocytes specific for Bet v 1 also cross-react with these Bet v 1-related proteins [7]. In addition, Bet v 1-sensitised individuals with food allergy often display only mild symptoms following ingestion of fresh product. This might be explained by the apparent lability of these proteins following pepsin digestion [8] with an associated loss of IgE reactivity, although the resulting peptides are still able to activate pollen-specific T cells [9]. However, sensitisation to Bet v 1 homologues from celeriac [10], soybean and peanuts [11, 12] can induce severe systemic reactions, ranging from urticaria to anaphylaxis. Furthermore, there is evidence that interactions with lipid vesicles can protect Bet v 1 from proteolysis [6]. Thus, we have investigated the stability to gastric digestion of a panel of recombinant allergenic Bet v 1 homologues drawn from three different botanical families, the *Rosaceae* (Mal d 1, Pru p 1), *Apiaceae* (Api g 1) and *Betulaceae* (Cor a 1) available from the EuroPrevall allergen library [13]. Recombinant allergens were used in this study to provide sufficient quantities, since preparing them in a natural form from plant foods can be difficult due to their sometimes low abundance and lability. The isoforms chosen were those most abundant in edible plant tissues. A combination of various methods, such as spectroscopy, mass spectrometry and molecular modelling, has been used to define, for the first time, the responsiveness of the Bet v 1-fold to pH and ionic strength and to gastric conditions and the kinetics and evolution of proteolysis, including the gastric surfactant, phosphatidyl choline. These structural effects have been related to allergenic activity in terms of IgE binding and basophil activation using human sera from individuals with cross-reactive allergies to birch pollen, fruit (apple, peach), vegetables (celeriac) and tree nuts (hazelnut).

2 Materials and methods

2.1 Allergens and other reagents

Recombinant Api g 1.01 (EMBL Genbank Database access no. Z48967.1), Cor a 1.04 (AF136945), Mal d 1 (AJ417551) and Pru p 1 (AM 493970) were purified as previously described [14–17]. Egg phosphatidyl-choline (PC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Sigma Diagnostics, St. Louis, MO, USA), were prepared as vesicles described by Moreno et al. [18]. Vesicles were labelled by mixing 3 mM DMPC with 22.4 μL of 0.001% (w:v) 1,6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes, Eugene, OR, USA) for 30 min at 37°C.

2.2 Patients' sera

For each allergen three different sera from patients with known allergy to the respective food (clear case history and positive serum IgE test) were used. Four patients had concomitant allergy to peach, apple and hazelnut and three additional patients with allergy to celeriac were selected. The specific IgE levels to the respective Bet v 1 homologous allergens measured by the ImmunoCAP technique (Phadia, Uppsala, Sweden) are listed in Table 1 of Supporting Information. For each allergen, a non-allergic serum was also used. Signed informed consents were obtained from all patients.

2.3 Circular dichroism (CD) spectroscopy

Far-ultraviolet (UV) CD spectra (190–260 nm) of allergens (0.25 mg/mL) in 10 mM sodium phosphate buffer (pH 7.0), 10 mM citrate buffer (pH 2.5–5.0) or simulated gastric fluid (SGF) (150 mM NaCl, pH 2.5) were collected using a Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) and converted into molar residue ellipticity according to Sancho et al. [19]. Secondary structure was deduced from the CD spectra using the SELCON3 package (http://lamar.colostate.edu/~sreeram/SELCON3).

2.4 Fluorescence polarization

DPH-labelled vesicles (3 μmoles) were mixed with allergens (0.25 mg/mL in SGF) in a 1 mL final volume and the pH adjusted to either 2.5 or 7.0 before incubation. The steady-state polarization of the fluorescence emission was measured using 1 cm path length thermostatically controlled cells in an LS 55 luminescence spectrometer (Perkin-Elmer, Wellesley, MA, USA) as previously described [20]. All determinations were run in triplicate.

2.5 In vitro gastric digestion

In vitro simulated gastric digestion was performed (in triplicate) as described by Moreno et al. [20] by mixing recombinant allergens (0.25 mg/mL) with either SGF (150 mM NaCl, pH 2.5) or PC vesicles (3 mM) for 10 min at 37°C. A solution of pepsin (EC 3.4.23.1) 0.32% w/v in SGF, pH 2.5 (Sigma, Poole, Dorset, UK; activity: 3300 U/mg of protein calculated using haemoglobin as the substrate), was added at an

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approximately physiological ratio of enzyme/substrate (1:20, w/w). The digestion was stopped by raising the pH to 7.5 by the addition of 40 mM ammonium bicarbonate (BDH, Poole, Dorset, UK) and aliquots were taken at 0, 20 s (1/3), 1, 2, 5, 10, 20, 40 and 60 min for further analysis.

2.6 MS analysis

Digested allergens (10 μg/mL) were subjected to LC-MS using a Phenomenex Jupiter 5 μm C4 150 × 2 mm i.d. column attached to an Accela HPLC (Thermo Scientific, Waltham, USA) coupled to an Orbitrap XL mass spectrometer (Thermo Scientific). LC employed a flow rate of 100 μl/min in 0.1% v/v formic acid with an 0–5% v/v ACN:water isocratic gradient (10 min), followed by 5–50% v/v acetonitrile linear gradient (45 min) and a 50–90% v/v acetonitrile linear gradient (5 min). Mass spectra were obtained in positive-ion electrospray mode using the Orbitrap Fourier Transform mass spectrometer stage with a mass range of m/z 300–2000 Da, a resolution 60 000, a fill target of 700 000 ions and a maximum fill time of 1000 ms. Monoisotopic mass accuracy was better than 10 ppm. MS/MS spectra were obtained using a 35 V collision voltage and product ion m/z values determined by the Linear Ion Trap stage. A maximum of four MS/MS spectra per survey scan were obtained by defaulting to the most abundant ions, with product ion m/z values determined to better than ~0.4 Da. Charge state rejection was not enabled. Dynamic exclusion was set to 1 count and 60 s exclusion with an exclusion mass window of −0.5 to +1.5 Da. MS data files were initially processed using msInspect (version 2, build 221) and a list of peptides satisfying $m > 700$ Da was extracted. De novo sequencing was performed using PEAKS (Bioinformatics Solutions, Waterloo, Canada) (http://www.bioinformaticssolutions.com/) with a precursor mass tolerance of 5 ppm, a fragment mass tolerance of 0.5 Da and charge correction enabled with maximum charge set to 10. Suitable peptide sequences were those that satisfied PEAKS quality measures with $m > 700$, score > 50 and relative sequence distance < 0.76. The msInspect candidate peptide masslist was populated with the resulting peptide sequences; unsequenced peptides were assigned tentative sequences using the FindPept Tool (http://www.expasy.org/tools/findpept.html) with a mass tolerance of 5 ppm.

2.7 Structure modelling

Multiple amino acid sequence alignments were carried out with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and displayed with Discovery Studio (http://accelrys.com/products/discovery-studio/). The 3D structures of Bet v 1, (1BV1), Pru av 1 (1E09) and Api g 1 (2BK0) (http://www.pdb.org/) were obtained and cleaned using PDB Reader from the CHARMM-GUI software (http://www.charmm.org/). Since the sequence identity was 28% and similarity of all structures after structure alignment (Align3D algorithm) (http://www.salilab.org/modeller/) was 49%, these structures were used as templates to build molecular models of rCor a 1 (Q9SWR4) and rMal d 1 (Q9SYW3) using Modeller (http://www.salilab.org/modeller/) and Discovery Studio. Ligands and crystallographic water molecules were excluded and residues protonated. Terminal groups and disulphide bridges were introduced with the CHARMM HBUILD command (http://www.charmm.org/), and modelled structures checked by Modeller Verify Protein and Profiles-3D Verify Protein protocols. Cartoons were drawn with Pymol (http://pymol.sourceforge.net/).

2.8 SDS-PAGE and immunoblotting

SDS-PAGE was performed under reducing conditions using a 10% Bis-Tris gel in a NuPAGE system (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Proteins were visualised by Sypro Ruby staining (Invitrogen). Proteins were transferred onto nitrocellulose membranes (0.2 μm) (PROTRAN BA83, Whatmann, Schleicher and Schuell, Germany), by semidry blotting (PHASE GmbH, Lübeck, Germany) at 0.8 mA per cm^2 membrane for 45 min using a discontinuous three-buffer system [21]. IgE immunoblot analysis was performed according to Lauer et al. [15]. Patients' sera and alkaline phosphatase-conjugated goat anti-human IgE were diluted 1:12 v/v and 1:3500 v/v, respectively. Membranes were developed using a chemoluminescent substrate CDP-Star® solution (Applied Biosystems, Foster City, CA, USA) and exposed to X-ray film (Eastman Kodak, Rochester, NY, USA). Image analysis of SDS-PAGE gels was carried out using Totallab 120 (Nonlinear Dynamics, Newcastle, UK) as for Mandalari et al. [22]. All determinations were run in triplicate.

2.9 ELISA inhibition

ELISA was performed as described by Wangorsch et al [23]. Briefly, Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with allergen (0.5 μg/mL in 50 mM sodium carbonate pH 9.6). Plates were developed using biotinylated goat anti-human IgE antibody (KPL, Gaithersburg, MD, USA) (1:4000 v/v) for 2 h followed by NeutrAvidin-HRP conjugate (Pierce, Rockford, USA) (1:8000 v/v) prior to addition of substrate based on 3,3′,5,5′-tetramethylbenzidine (TMB). All determinations were run in triplicate.

2.10 Basophil histamine release assay (BHR)

Specific adsorption of histamine to glass fibre-coated microtiter plates was carried out according to Stahl Skov et al. [24] and passive sensitization and histamine release

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detection as per Stahl Skov et al. [25] and Elberling et al. [26]. Four donors were screened to select one donor per allergen that was non-allergic and responsive (released histamine when stimulated with anti-IgE antibody). Results were expressed as the percentage of total cellular histamine content and were considered positive when >10% or as the allergen concentration inducing 50% of maximal release. Signed informed consents were obtained from all donors.

3 Results

3.1 Effect of gastric conditions on the secondary structure of the Bet v 1 homologues

Initially, the responsiveness of the Bet v 1 fold to reductions in pH (pH 7–2.5) found during gastric digestion was followed using CD spectroscopy at low ionic strength ($I = 0.001$–0.020). All allergens gave similar spectra consistent with Bet v 1 itself at neutral pH, with a maximum at 194 nm and a minimum at 215 nm (Fig. 1 and Supporting Information Fig. 1) consisting of a mixture of α-helical and β-sheet (Table 1). On reducing the pH, all allergens underwent conformational changes (Figs. 1A–E) but only rMal d 1 and rPru p 1 completely unfolded at pH 2.5 and 3, respectively (Figs. 1B, E and Supporting Information Fig. 1). In general the α-type conformation of these proteins decreased and was accompanied by an increase in random structures, rApi g 1 also showing a decrease in β-structure (Table 1). Raising the ionic strength to that of gastric fluid (SGF, pH 2.5, $I = 0.15$) increased the α-helical structure and decreased the random coil structure compared with pH 2.5 ($I = 0.001$) (Fig. 1C and D) except for rPru p 1 (Fig. 1C and D).

Gastric secretions also include PC in a vesicular form, and given the ability of Bet v 1 to interact with lipid vesicles [6], the impact of the biosurfactant (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC) in SGF (pH 2.5, $I = 0.15$) on the secondary structure of the homologues was also assessed. The intensities of the positive peak at 198 nm, and the double minimum at 222 and 208 nm increased (Figs. 1C and D and Supporting Information Fig. 1), as well as the α-helical content (Table 1), for all homologues, apart from rApi g 1. These data suggested that all the homologues, apart from rApi g 1, were able to interact with the lipid. The extent of the association was also assessed using fluorescence polarization at pH 2.5 in SGF (Fig. 2) and 7.0 (data not shown) using DMPC vesicles. A decrease in fluorescence polarization of the probe was observed with rMal d 1 at pH 2.5, but not at pH 7, indicating that it was interacting with the phospholipids, increasing the mobility of the probe in the acyl region of the bilayer. However, no shift in DMPC melting temperature at either pH 7.0 or 2.5, was observed for interaction with rApi g 1, rCor a 1 or rPru p 1, suggesting association with the DMPC vesicles was more superficial.

3.2 Simulated gastric proteolysis of the Bet v 1 homologues

We observed previously that interactions with PC vesicles affected the kinetics of digestion of pepsin-labile proteins such as α-lactalbumin [18]. Thus, pepsinolysis of all four homologues was followed in the absence and presence of physiologically relevant levels of PC. All allergens were degraded by pepsin within 5 min (Fig. 3) with the transient appearance of fragments of molecular masses 6–14 kDa after 20 s on SDS-PAGE. rApi g 1 showed a slightly slower rate of degradation compared with the others, with a first order rate constants of 1.02 ($\pm 0.19\,\mathrm{min}^{-1}$) compared with 2.51 ($\pm 0.24\,\mathrm{min}^{-1}$) for rMal d 1. Addition of PC did not alter the kinetics or products patterns of digestion for the allergens (data not shown), apart from rMal d 1 (Fig. 3E), where digestion proceeded more slowly. The rate constant for rMal d 1 was reduced to $0.19 \pm 0.02\,\mathrm{min}^{-1}$, whilst that of rApi g 1 was unaffected ($0.96 \pm 0.04\,\mathrm{min}^{-1}$).

Peptide fragments resulting from pepsinolysis after 0, 2 and 60 min in the absence or presence of PC were characterised by MS, 81% identified by de novo sequencing and 19% by mass only. Pepsinolysis of rCor a 1 produced fewer total peptides, 31, compared with 59 for rApi g 1, 49 for rPru p 1 and 48 for rMal d 1 after 60 min (Figs. 4 and Supporting Information Fig. 2). The largest mass detected of 2792.569 Da (not resolved by MS/MS) and the largest sequenced peptide of 2546.203 Da, corresponding to residues 64-84, were found in rPru p 1. Mapping all the products onto the primary structure for rApi g 1 and rMal d 1 (Fig. 4A–D) illustrates the almost instant pepsinolysis with peptides covering the entire length of the sequence, and showing the evolution of peptide fragments. For example peptide 68–81 in rApi g 1 present at 2 min gives rise to a complex mixture of ~8 peptides after 60 min digestion. Addition of PC modified pepsinolysis of rMal d 1 (Fig. 4) such that no peptides between residues 44–126 were generated after 2 min digestion (Fig. 4E) although by 60 min many peptides were identified in this region, in the absence or presence of PC (Fig. 4F). It is likely the M_r 10 kDa polypeptide evident on SDS-PAGE (Fig. 3E) corresponds to residues 44–126, although this large fragment proved non-resolvable to MS/MS sequencing. Mapping of the pepsinolysis products on to the 3D structure of rMal d 1 (Fig. 5) shows residues 46–127 form a β-sheet and that the P-loop region (residues 42–52, Fig, 5 in blue), previously identified as a major IgE-epitope in the allergenic Bet v 1 homologue of cherry, rPru av 1 [4] was digested more slowly in the presence of PC.

3.3 Effect of gastric conditions on the allergenic activity of the Bet v 1 homologues

The impact of gastric digestion on the allergenic activity of the homologues was assessed by a combination of IgE

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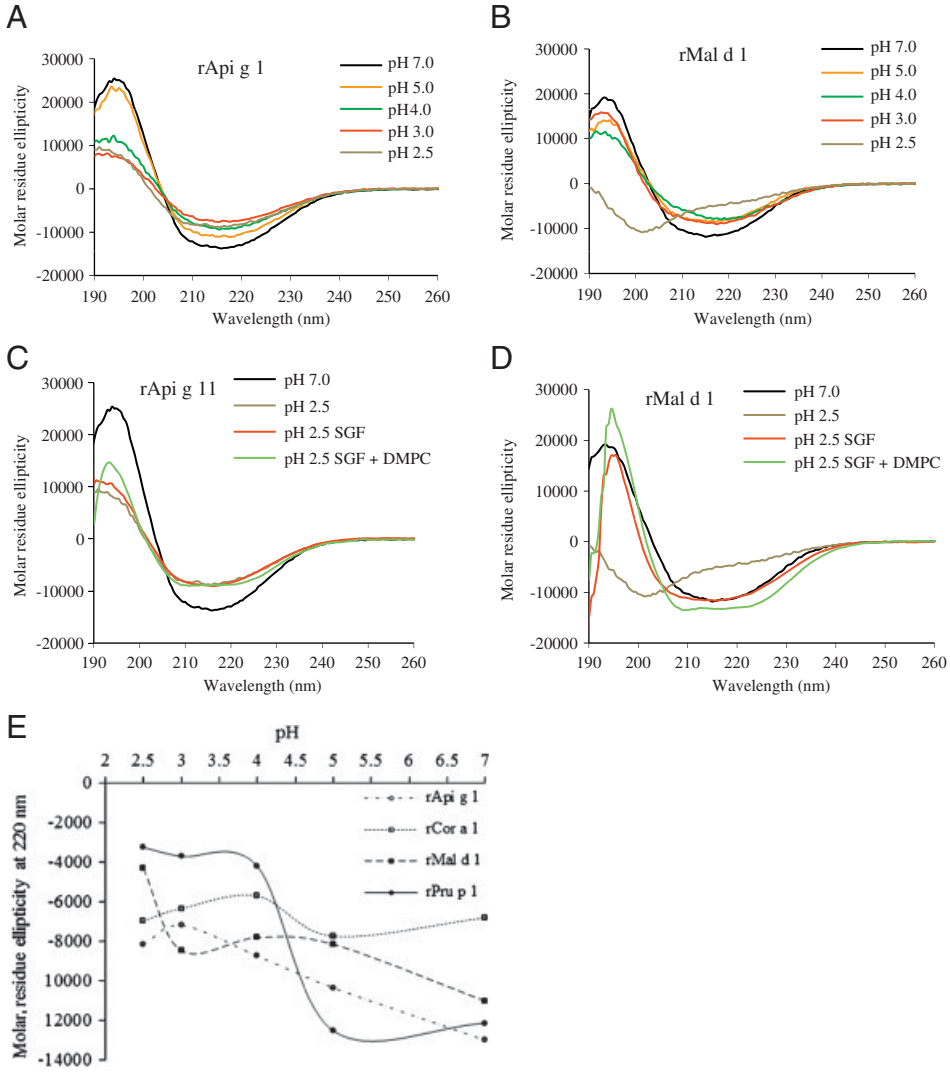

Figure 1. Far UV CD spectra of rApi g 1 (A, C) and rMal d 1 (B, D) obtained at pH 7 (black), 5 (orange), 4 (green), 3 (red) and 2.5 (grey) using either 10 mM sodium phosphate buffer (pH 7, I = 0.02) or 10 mM citrate buffer (pH 2.5–5, *I* = 0.001–0.025) (A and B) or at pH 7. I = 0.02 (black line), pH 2.5, *I* = 0.001 (grey line) or at pH 2.5, *I* = 0.15 (SGF) alone (red line) or with DMPC (green line) (C and D). Molar residue ellipticity at 220 nm of rApi g 1, rCor a 1, rMal d 1 and rPru p 1 at different pHs (E).

Table 1. Secondary structure estimation (%) predicted from Far-UV CD spectra using SELCON3.

	10 mM phosphate buffer pH 7.0			10 mM citrate buffer pH 2.5			SGF			SGF+DMPC		
Bet v 1 homologue	α-helix	β-sheet	Random	α-helix	β-sheet	Random	α-helix	β-sheet	Random	α-helix	β-sheet	Random
rApi g 1	37	40	25	21	49	29	36	41	26	37	31	30
rCor a 1	23	47	31	17	49	33	21	48	31	42	33	25
rMal d 1	31	42	27	11	49	43	32	41	28	42	34	25
rPru p 1	32	42	28	6	48	45	8	47	42	20	54	24

binding (by immunoblotting and ELISA) and biological activity, as indicated by the ability of the allergens to stimulate histamine release in appropriately sensitised cells. Human serum IgE reacted only to the intact protein by immunoblotting (Fig. 3) with some inter-individual differences which are commonly observed. A faint recognition of rCor a 1 by the non-allergic control serum was observed (Figs. 3B and 4) possibly due to non-specific binding. Intact rApi g 1 still bound IgE antibodies after 5 min of proteolysis, whereas the binding capacity of rCor a 1 and rMal d 1 was abolished after 1 min digestion (Fig. 3B 1–3 and D 1–3). rPru p 1 was the least stable allergen wich lost its IgE

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binding capacity after 20 s digestion (Fig. 3C 1–3). Addition of PC did affect only the binding to rMal d 1 which was mantained as a consequence of retaining more intact protein after 5 min digestion (Fig. 3E 1–3). Weak IgE binding to proteolytic fragments was only observed for serum number 2 for rMal d 1 both in the absence and in the presence of PC and rCor a 1 only in the presence of PC. ELISA inhibition results (using the same three allergic human sera used for immunoblotting), were broadly consistent with immunoblotting (Figs. 3 and 6 and Supporting Information Fig. 3). Intact allergens were able to inhibit IgE binding between 70 and 94% at 10 μg/mL, rCor a 1 being the most potent allergen. The non-allergic control sera were non-reactive. Following pepsinolysis, rPru p 1 lost its inhibitory redactivity between 20 s and 1 min whereas rApi g 1 exhibited residual inhibitory capacity after 10 min digestion (Supporting Information Fig. 3), rCor a 1 and rMal d 1 IgE binding capacities were almost abolished after only 5 min (Figs. 6 and Supporting Information Fig. 3). PC did not alter the inhibitory capacity of the intact allergens prior (results not shown) or after digestion apart from that of digested rMal d 1 which was still observed after 20 min digestion (Fig. 6).

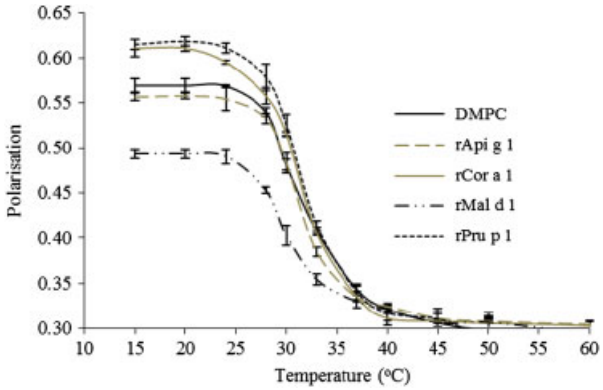

Figure 2. Fluorescence polarisation of DMPC as a function of temperature in the absence or presence of rApi g 1, rCor a 1, rMal d 1 and rPru p 1 in SGF at pH 2.5.

All intact allergens were able to induce histamine release in a dose-response manner in the absence and presence of PC, using a single well-characterised allergic human serum sample per allergen. Addition of PC only altered the capacity of the intact rCor a 1 (results not shown) and rMal d 1 (Fig. 7) with a reduction of allergen concentration able to induce 50% of maximal release of 3-fold (from 0.156 to 0.046 μg/mL) and 10-fold (from 0.208 to 0.02 μg/mL) for rCor a 1 and rMal d 1, respectively. Digestion reduced basophil activation by all homologues (Supporting Information Table 2) with slight differences. Thus, some mediator-releasing capacity of rApi g 1 was retained after 20 min digestion, but that of rMal d 1 and rPru p 1 was

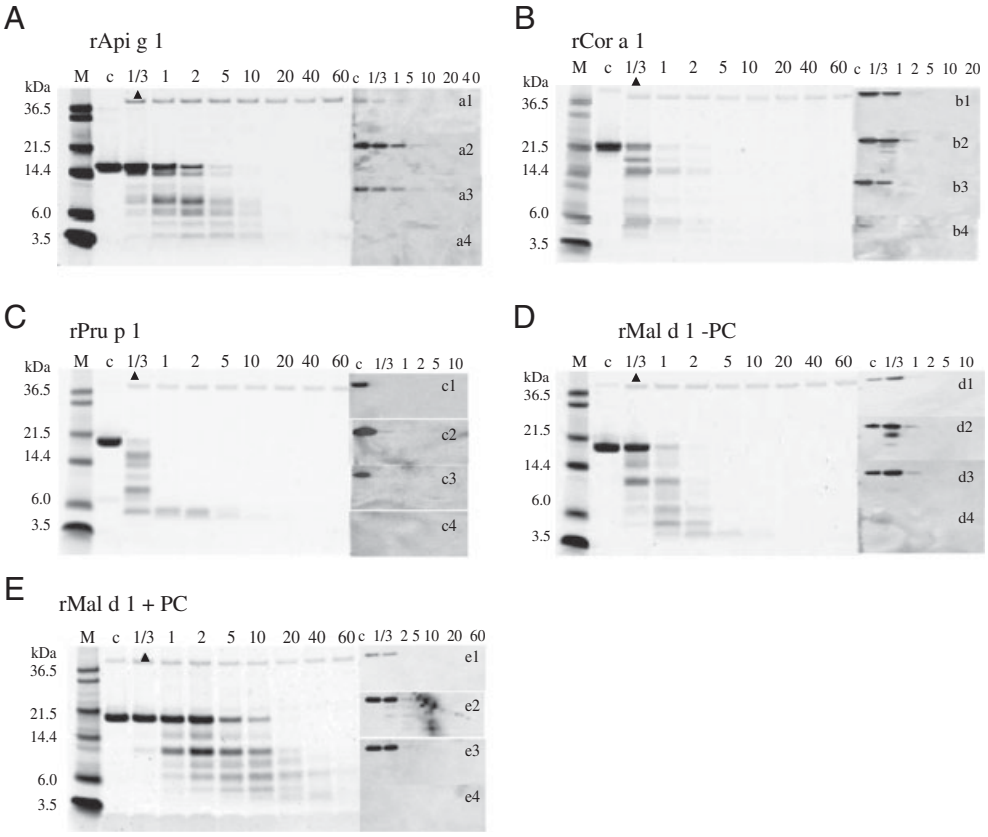

Figure 3. SDS-PAGE and immunoblots of digested rApi g 1 (A), rCor a 1 (B), rPru p 1 (C), and rMal d 1 in the absence (D) or presence of PC (E). M: marker; c: untreated allergen; lanes 2–9: allergen digested for 20 s (1/3), 1, 2, 5, 10, 20, 40, 60 min. Patients' sera used were as follows: rApi g 1: a1:5, a2:6, a3:7; rCor a 1: b1:1, b2:2, b3:3; rPru p 1: c1:1, c2:3, c3:4; rMal d 1: d1 and e1:1, d2 and e2:2, d3 and e3:3; and a4-e4:control. Polypeptides marked with (▲) correspond to pepsin.

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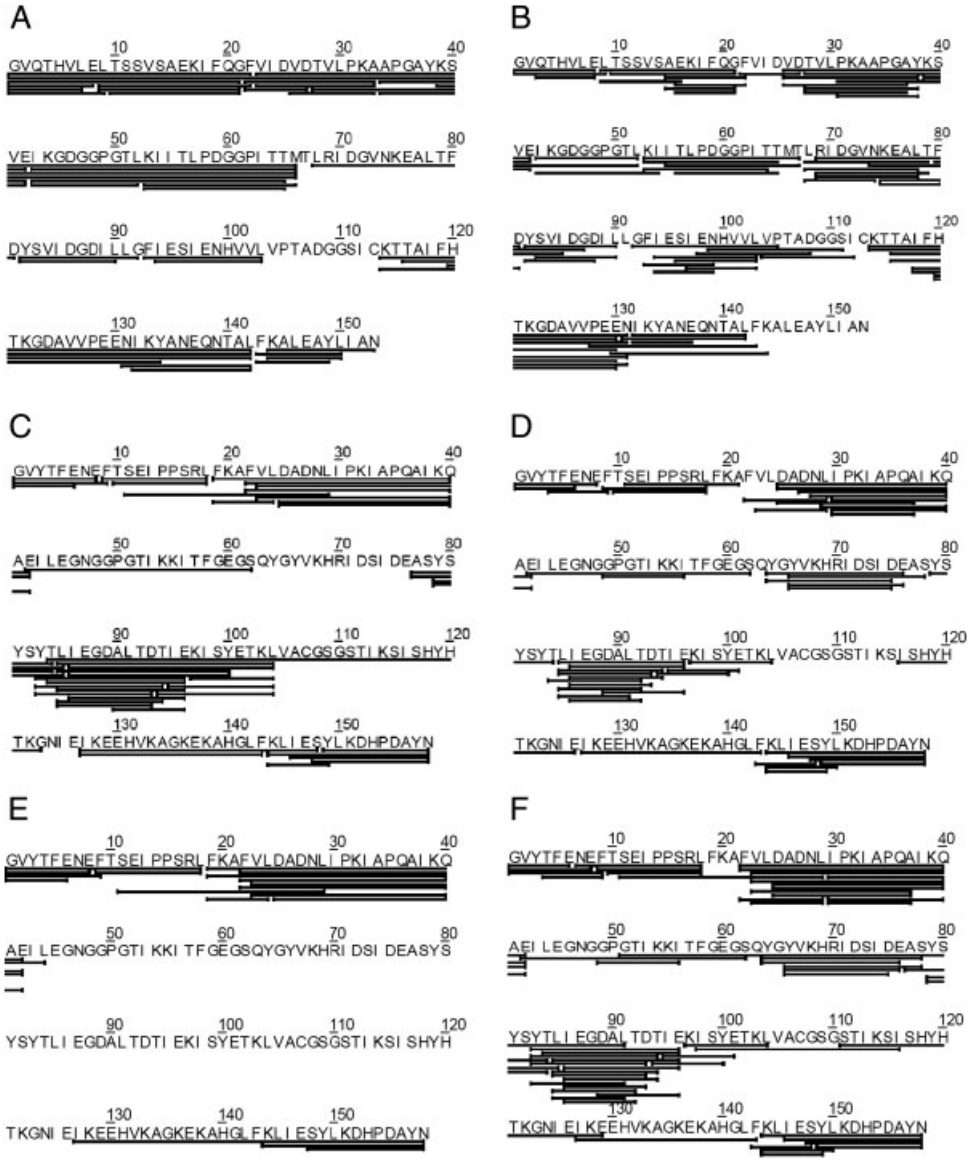

Figure 4. Location of pepsinolysis peptides mapped on to the primary structure of rApi g 1 (A, B) and rMal d 1 (C–F) after 2 (A, C, E) and 60 min (B, D, F) digestion in the absence (A–D) or presence of PC (E, F).

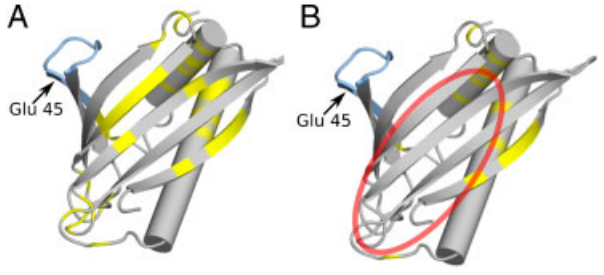

Figure 5. Mapping of experimentally-determined cleavage sites (denoted in yellow) on the modelled three-dimensional structure of rMal d 1 after 2 min digestion in the absence (A) or presence (B) of PC. Red: region protected from digestion in the presence of PC and blue: P-loop residues.

completely lost after only 5 min, and after 10 min by rCor a 1. Addition of PC to the digestion did not affect the biological activity of rApi g 1 (Supporting Information Table 2) but appeared to enhance the activity of rCor a 1 and rPru p 1 even after 60 min digestion. IC_{50} values were increased compared with intact proteins by at least 175-fold (from <0.03 to 5.3 μg/mL) and 6-fold (from <0.31 to 1.9 μg/mL), respectively (Supporting Information Table 2). PC also preserved the biological activity of rMal d 1 during digestion (Fig. 7B) showing some residual activity after 20 min but being completely abolished after 60 min digestion. The IC_{50} values increased from <0.31 μg/mL for intact protein to 2.0 after 2 min digestion (Supporting Information Table 2). PC in the absence of any of the allergens did not induce histamine release.

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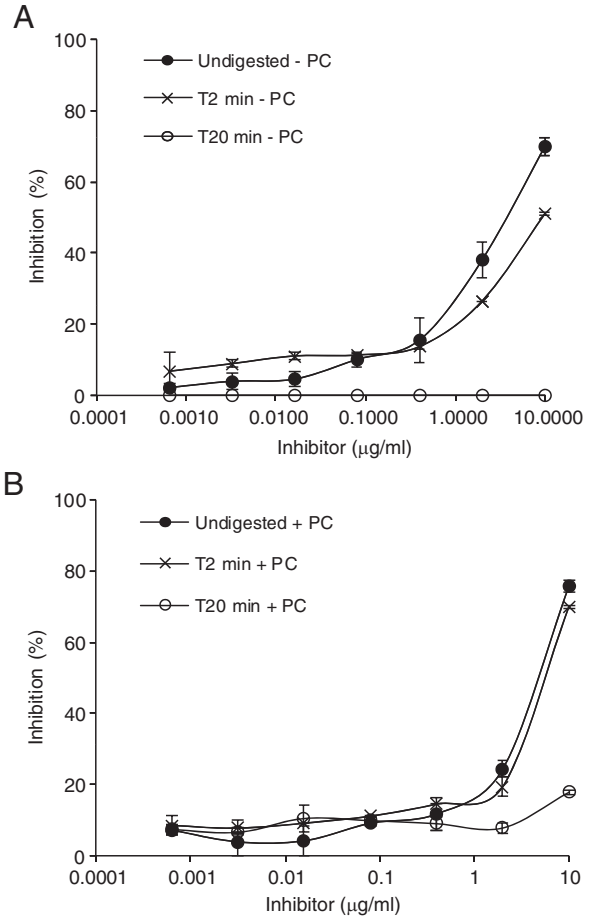

Figure 6. ELISA inhibition of IgE binding to rMal d 1 with undigested (filled circles), and allergen digested for 2 min (*X*) and 20 min (open circles) in the absence (A) or presence (B) of PC, using serum from patient number 1.

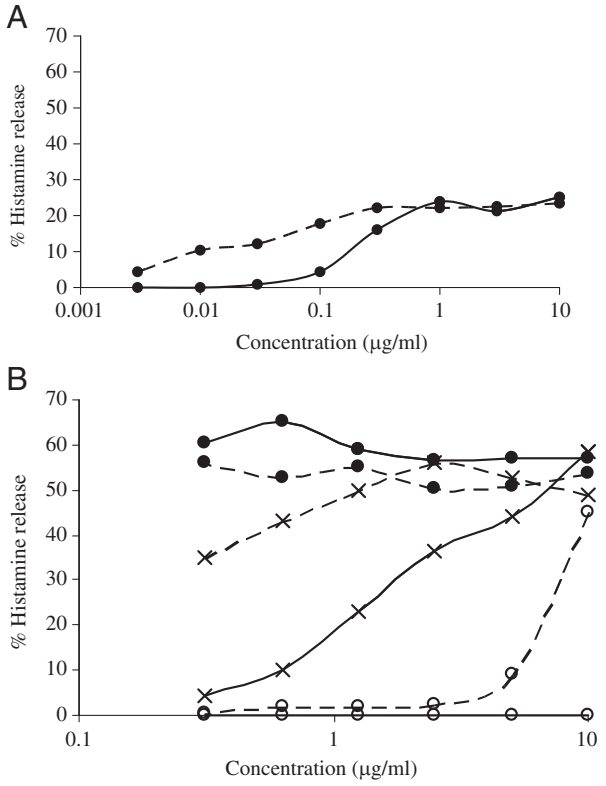

Figure 7. Histamine release induced by undigested rMal d 1 (A) in the absence (–) or presence (---) of PC, and (B) rMal d 1 before (filled circles) or after digestion for 2 min (*X*) and 40 min (open circles) in the absence (–) or presence (---) of PC, using serum from patient number 3. Different buffy coats were used for experiments (A) and (B).

4 Discussion

In general, all the homologues of Bet v 1 used in this study responded to environmental challenges found in the human gastrointestinal tract in a similar manner. Most of them underwent conformational changes induced by low pH and $I = 0.001$, although there were subtle differences in the behaviour of the individual proteins. Whereas rApi g 1 was the most stable protein followed by rCor a 1, rPru p 1 and rMal d 1 were the least stable homologues, completely unfolding at low pH. Increasing the ionic strength to that of gastric fluid ($I = 0.15$) markedly reduced the extent of low pH changes observed, except for rPru p 1. These data are consistent with observations that Bet v 1 itself unfolds at low pH with a mid-point at pH 3.6 in 20 mM sodium citrate ($I = 0.015$) [6] and previous reports that rPru p 1 is labile to low pH [17]. These data also suggest that the ability to adopt different structural forms akin to the partially unfolded "A" and "B" states described by Mogensen et al. [6] for Bet v 1, is a property of the Bet v 1 scaffold shared by several of the homologues in this study.

As shown by others [9, 27], all members of the Bet v 1 family were highly susceptible to pepsinolysis although all persisted for a longer period of time than the 1 s observed by Schimek at al [9] due to the much lower and more physiologically relevant ratios of pepsin to protein used in our study. Mass spectrometry analysis of pepsinolysis products showed some slight differences in rates of degradation, although all produced a complex mix of peptides with masses of less than 2 kDa. For other allergens, such as cows' milk whey proteins, a molecular weight greater than 3 kDa is needed to stimulate an immune reaction [28]. Molecular modelling of peptic peptides indicating cleavage sites were distributed across the surface of the molecules with no obvious correlation to specific loops and turns. The P-loop region, previously identified as a major IgE epitope in cross-reactive Bet v 1 homologues of cherry and celeriac [4], was cleaved in all homologues, consistent with their loss of IgE-reactivity as determined by immunoblotting, ELISA and histamine release following pepsinolysis. It is generally thought that the major B cell epitopes involved in birch-

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pollen fruit allergy syndrome are conformational in nature [7] which is also consistent with our observations.

In addition to the effects of pH and ionic strength, small unilamellar DMPC vesicles (~100 nm diameter), simulating those secreted by the gastric mucosa, affected the secondary structure of all homologues apart from rApi g 1. Intriguingly, Mal d 1 was the only homologue that penetrated the DMPC vesicles, consistent with the fact that Bet v 1 itself can interact with DOPC vesicles (50–100 nm diameter) [6]. The results also hint at the potential for many Bet v 1 homologues to share the properties of the parent molecule in interacting with lipid structures in a similar fashion to those described by Mogensen et al. [6]. This could be relevant to both the role of these proteins in plants and their potential to interact with mammalian cell membrane structures in terms of their allergenic activity.

The ability of rMal d 1 to penetrate into the bilayer, slowed its rate of pepsinolysis resulting in a changed pattern of pepsinolysis products, with apparent protection of one of the faces of the molecule. This region is likely to be involved in interacting with PC although there are no obvious features indicating how such an interaction might be mediated. The ability of the digested proteins to stimulate histamine release was enhanced by the presence of PC for all homologues apart from rApi g 1. These data hint that whilst the interaction with PC may be too superficial or weak to protect rCor a 1 and rPru p 1 from pepsinolysis, it was sufficient for peptide products to remain associated in some manner and hence able to elicit histamine release. Such effects would not be observed on the IgE-binding determined by ELISA since the detergent included in the assay (Tween 20) will have disrupted the vesicular structure of the PC. The relevance of such observations with regards to eliciting an allergic reaction in sensitised individuals by these allergens remains to be determined. However, it is pertinent to understanding how T-cell epitopes of these labile allergens may survive digestion. A comparison with previously reported T-cell epitopes for Bet v 1 and its homologues [7, 9, 27] showed that in Mal d 1 they were protected from digestion by PC after 2 min. However, after 60 min digestion, very few fragments of at least seven amino acids in length (a minimum size usually required to activate $CD4^{+}$T cells [9]), remained.

These data support the view that, in general terms, the Bet v 1 scaffold determines the structural properties and susceptibility to digestion of homologues from diverse plant species, ranging from Apiaceae (rApi g 1) to Rosaceae (rPru p 1 and rMal d 1) and Betulaceae (rCor a 1). These data also suggest that members of the PR10 Bet v 1 family are likely to be highly susceptible to gastric proteolysis although further studies using other Bet v 1 homologues are needed to confirm this hypothesis. rApi g 1 the most distant sequentially and structurally from other homologues (Supporting Information Table 3) was clearly slightly different in terms of its properties, being more stable to the low pH conditions of the stomach than the other homologues, and lacking any evidence of interaction with PC. Currently, studies comparing different structural families (e.g. lipid-transfer proteins, etc.) are being carried out to test the theory that 3D structure is predictive of biological properties, such as denaturation by the low pH conditions in the stomach, interactions with gastrointestinal biosurfactants and susceptibility to proteolysis. The general applicability of this hypothesis would need to be tested by subjecting other homologues of the Bet v 1 superfamily to simulated gastric digestion. Should this hypothesis hold, these biological properties may be amenable to in silico prediction and hence further contribute to the allergenic risk assessment process [29].

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Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: Impact on structure and allergenic activity

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Supporting information

Table 1 Specific IgE to rApi g 1, rCor a 1, rMal d 1 and rPru p 1 as determined by CAP-FEIA. Patients' sera were used in experiments as follows: I; Immunoblot (Figure 3), E; ELISA inhibition (Figure 6), B; basophil histamine release (Figure 7)

	rApi g 1			rCor a 1			rMal d 1			rPru p 1		
Patient no.	kU/L	CAP class	Assay	kU/L	CAP class	Assay	kU/L	CAP class	Assay	kU/L	CAP class	Assay
1				53.2	5	E/I/B	20.1	4	E/I	47.2	4	E/I
2				30.9	4	E/I	20.1	4	E/I	27.5	4	
3				98.5	5	E/I	82.0	5	E/I/B	89.4	5	E/I/B
4				28,6	4		19.1	4		38.2	4	E/I
5	11.5	3	E/I									
6	11.5	3	E/I/B									
7	18.5	4	E/I									

Figure 1 Far UV CD spectra of rCor a 1 (a, c) and rPru p 1 (b, d) obtained at pH 7 (black), 5 (orange), 4 (green), 3 (red) and 2.5 (grey) using either 10 mM sodium phosphate buffer (pH 7, I=0.02) or 10 mM citrate buffer (pH 2.5-5, I=0.001-0.025) (a, b) or at pH 7. I= 0.02 (black line), pH 2.5, I=0.001 (grey line) or at pH 2.5, I= 0.15 (SGF) alone (red line) or following the addition of DMPC (green line) (c, d).

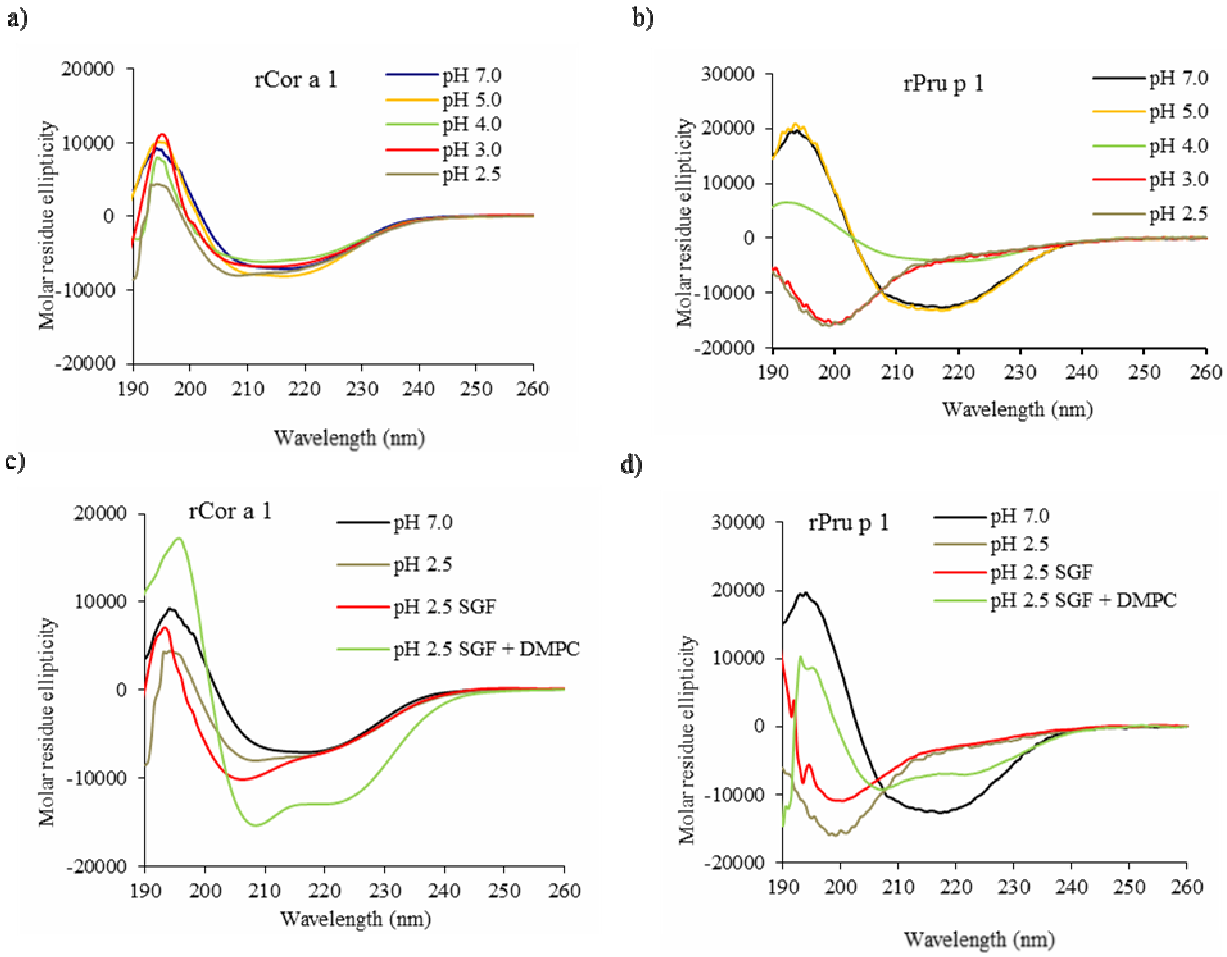
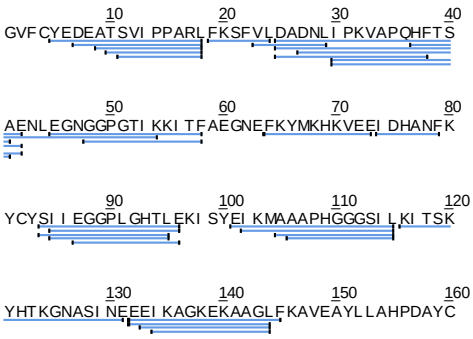


Figure 2 Location of peptides in the Bet v 1 homologues primary structure identified after 60 min incubation with pepsin in simulated gastric fluid in the absence of PC

Cor a 1



Pru p 1

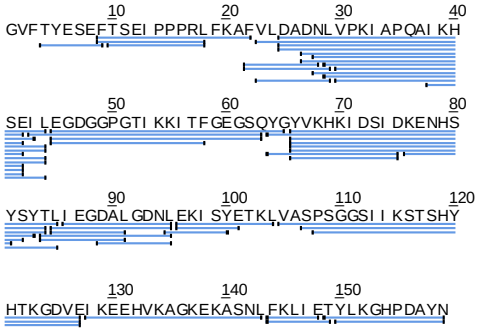


Figure 3 ELISA inhibition of IgE binding to undigested rPru p 1 (filled circle), rCor a 1 (filled square), rApi g 1 (filled triangle), and digested rPru p 1 for 1 min (open circle), and rCor a 1 (open square) and rApi g 1 for 10 min (open triangle) in the absence of PC. Sera from patient number 1, 4 and 6 were used. All determinations were run in triplicate.

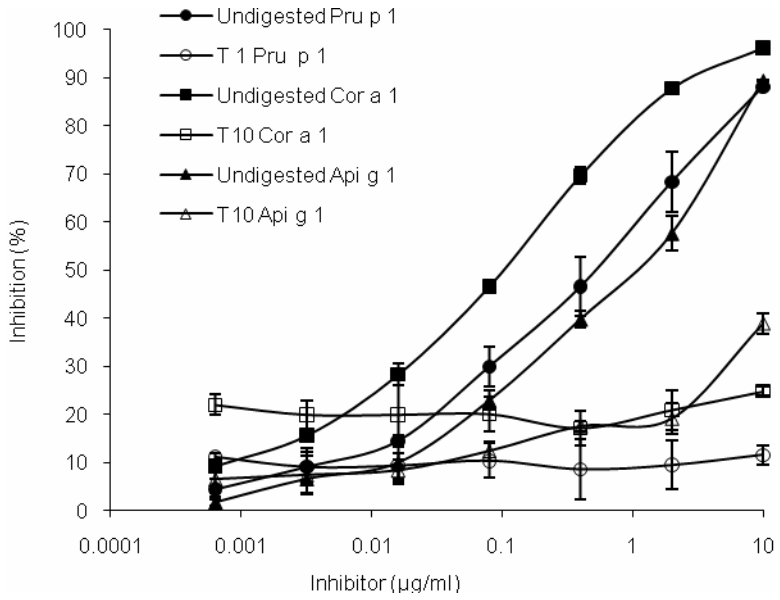


Table 2 Histamine release stimulated by intact and digested Bet v 1 homologues (µg/ml) with passively sensitized basophils. Results are given as concentration inducing 50% of maximal release. Different buffy coats were used for experiments (a) and (b). Patient's sera no. 6 for rApi g 1, no. 1 for rCor a 1 and no. 3 for rMal d 1 and rPru p1.

	rApi g 1		rCor a 1		rMal d 1		rPru p 1	
Digestion time point (min)	No PC	+ PC	No PC	+ PC	No PC	+ PC	No PC	+ PC
0[(a)]	0.009	0.008	0.156	0.046	0.208	0.0204	0.001	0.001
0[(b)]	<0.31	<0.31	<0.03	<0.03	<0.31	<0.31	<0.31	<0.31
0.2	<0.31	<0.31	<0.03	<0.03	<0.31	<0.31	0.9	<0.31
1	<0.31	<0.31	1.0	0.4	<0.31	<0.31	5.7	1.8
2	<0.31	<0.31	1.5	0.7	2.0	<0.31	6.3	2.1
5	<0.31	<0.31	5.4	1.9	>10	1.1	>10	2.1
10	1.5	1.2	>10	2.2	>10	3.7	>10	2.3
20	5.5	4.6	>10	5.0	>10	5.2	>10	1.4
40	>10	>10	>10	5.4	>10	7.3	>10	1.9
60	>10	>10	>10	5.3	>10	>10	>10	1.9

Table 3 Sequence identity bottom half (%) and RMSD upper half (Å) between structure pairs. Sequences between pairs were aligned before carrying out RMSD calculations on backbone atoms (N, Ca, C, O) using Pymol.

	rApi g 1	rCor a 1	rMal d 1	rPru p 1
rApi g 1		1.1 Å	1.4Å	1.4 Å
rCor a 1	42%		1.2 Å	1.3 Å
rMal d 1	43%	64%		0.3 Å
rPru p 1	44%	68%	88%	

Supporting information Materials and Methods

2.1 **Allergens and other reagents**

Vesicles were prepared as described by Moreno et al. [18]. Briefly, solvent was removed from PC stock solution under rotary evaporation in order to form a dry thin film of phospholipids, then dissolved in 16.5 mL of warmed simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). The suspension was then sonicated at 5 °C (30% full power, 9/10 power cycle) for 10 min and the resulting phospholipid vesicles were collected and filtered through Millex-HA 0.45 µm mixed cellulose (Millipore, Billerica, MA) to remove possible titanium particles.

2.6 Mass Spectrometry (MS) analysis

This mass limit of $m > 700$ Da was selected to minimise false mass-only identifications due to chemical noise and, in MS/MS, to ensure reliable sequencing by avoiding fragmentation spectra of small precursor ions.

2.7 Structure modelling

Verify scores for both Mal d 1 and Cor a 1 structures are higher than expected based on statistical analysis of high-resolution structures in PDB. Thus we concluded that in spite of low sequence identity between template proteins and homology models it is likely that both structures are correct.

<u>Evaluation of modeled structures</u>

Verify Protein (Profiles-3D) results for Mal d 1 structure:

Verify Score = 78.72

Verify Expected High Score = 71.5285

Verify Expected Low Score = 32.1878

Verify Protein (Profiles-3D) results for Cor a 1 structure:

Verify Score = 77.71

Verify Expected High Score = 72.4412

Verify Expected Low Score = 32.5985

2.10 Basophil histamine release assay (BHR)

In brief, peripheral blood mononuclear cells (PBMCs) from selected buffy coat blood were isolated by Lymphoprep gradient centrifugation. IgE was stripped off the basophils by a rebound in pH from 7.4 to 3.55 and back to 7.4. The PBMCs were then incubated with patient or control (non-allergic) serum to re-sensitize the basophils, which subsequently were challenged in glass fibre coated microtiter plates with untreated, intact or digested allergen (0.3–10 µg/mL for rApi g 1, rMal d 1 and rPru p 1 or 0.03–10 µg/mL for rCor a 1) or a control digestion mix without allergen. Released histamine bound to the glass fibres was coupled to 0.05% (v/v) o-phtahaldialdehyde, stabilized by 0.6% (v/v) $HCLO_4$, and measured fluorometrically as described in Stahl Skov et al. [25].