

Original article

Bet v 1 homologues in strawberry identified as IgE-binding proteins and presumptive allergens

Background: No strawberry allergen has so far been identified and characterized. **Methods:** Serum samples were collected from patients with a suggestive case history of adverse reactions to strawberry and other fruits. Extracts from fresh and frozen strawberries were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and mass spectrometry. Patient blood samples were analysed for inhibition of IgE binding and basophil degranulation.

Results: Several IgE-binding proteins could be detected. In more than half of the patient sera, a 20/18-kDa doublet band was observed in Western blotting. These two bands were excised and analysed by mass spectrometry showing the presence of proteins belonging to the Bet v 1 family of allergens. Inhibition of the IgE binding to the 20/18-kDa doublet was obtained by addition of two recombinantly expressed allergens belonging to the Bet v 1 family (Bet v 1 and Mal d 1) and strawberry protein extract. In a cell-based assay of patient blood samples, basophil degranulation could be induced by strawberry protein extract and by Bet v 1 and Mal d 1.

Conclusions: We conclude that strawberry homologues to Bet v 1 may be allergens of importance for adverse reactions to strawberry.

A. L. Karlsson¹, R. Alm², B. Ekstrand¹, S. Fjellkner-Modig¹, Å. Schiött^{1,3}, U. Bengtsson⁴, L. Björk⁵, K. Hjernø⁶, P. Roepstorff⁶, C. S. Emanuelsson²

¹SIK -The Swedish Institute for Food and Biotechnology, Gothenburg; ²Department of Biochemistry, Lund University, Lund; ³Department of Immunotechnology, Lund University, Lund; ⁴Asthma & Allergy Research Group, Department of Respiratory Medicine and Allergy, Sahlgrenska University Hospital, Gothenburg; ⁵Department of Systematic Botany, Uppsala University, Uppsala, Sweden; ⁶Protein Research, University of Southern Denmark, Odense, Denmark

Key words: allergens; clinical immunology; food allergy; IgE; pollens.

Cecilia Sundby Emanuelsson
Department of Biochemistry
Center for Chemistry and Chemical Engineering
Lund University
P O Box 124
S-221 00 Lund
Sweden

Accepted for publication 31 March 2004

Strawberries are not only eaten fresh but are also used as a common ingredient in many food products such as jam, yoghurt, ice-cream, and breakfast cereals. As strawberry is an important ingredient in the food industry, it is of importance to assess how food processing can affect a supposed allergenicity. It is also of interest to know whether different strawberry varieties contain different levels of allergens. Therefore, proteins in strawberry that might be possible allergens need to be identified and characterized.

Plant allergens can be classified into two groups depending on how the primary sensitization takes place, via the gastrointestinal tract or via the airways. The first group is generally more robust and resists degradation in the gastrointestinal tract. It consists of storage proteins (prolamins, cupins) or cystein proteases, and is more commonly a problem for young children (1, 2). The other large group of plant allergens is the pollen-related allergens, for which primary sensitization takes place via inhalation.

Birch pollen-associated food allergy is a well-known clinical phenomenon especially in northern Europe (3, 4). Following a primary sensitization to birch pollen allergen, a subsequent IgE cross-reaction with homologous proteins in plant-derived food occurs. Bet v 1, the major birch pollen allergen, shares common epitopes with major food allergens in a large number of different fruits, berries and vegetables, e.g. cherry (Pru av 1), apple (Mal d 1), pear (Pyr c 1), celery (Api g 1) and carrot (Dau c 1). That the primary induction is indeed caused by the pollen allergens has been concluded from inhibition experiments (5). Many plant allergens are pathogenesis-related proteins (PR proteins), which are induced upon stress, pathogen attack and abiotic stimuli, and the Bet v 1 homologues belong to the PR-10 group (6).

So far, no single strawberry allergen has been identified and characterized. However, the presence of a strawberry homologue to the recently discovered 35-kDa Bet v 6 allergen, an isoflavone reductase, was suggested

from experiments where a strawberry protein extract yielded 50% inhibition of IgE binding in enzyme allergosorbent test (EAST) inhibition with recombinant Pyr c5, a Bet v 6 homologue, as the solid phase (7). Even more recently, a strawberry 9-kDa lipid transfer protein (LTP) with 74% homology with apple LTP (Mal d 3) has been detected, which could be a possible strawberry allergen (E. Salentijn, personal communication).

With the lack of a hitherto established, identified and characterized strawberry allergen, it has even been questioned whether adverse reactions to strawberry are IgE-mediated reactions involving a protein allergen. Although 30% of patients in northern Europe with self-reported food hypersensitivity also had adverse reactions to strawberry (8), skin-prick tests have often been negative (N. E. Eriksson, personal communication).

In this paper, an investigation including eight patients from the south-west of Sweden is reported. Data on the identification of IgE-binding proteins in strawberry by means of immunoblotting, mass spectrometry, inhibition of IgE binding and a cell-based assay involving patient basophil degranulation are presented. We conclude that strawberry homologues to Bet v 1 may be an allergen of importance for adverse reactions to strawberry.

Materials and methods

Patient sera

Seven patients at the Allergy Clinic at Sahlgrenska University Hospital (Gothenburg, Sweden) with a suggestive case history of strawberry allergy were selected on the basis of reported reactions to strawberry according to a questionnaire regarding their tolerance to various foodstuffs. Specific IgE was determined for each patient serum using the ImmunoCAP technology (Pharmacia Diagnostics, Uppsala, Sweden). Allergens coupled to the ImmunoCAP solid phase were extracts from strawberry (f44), birch pollen (t3), apple (f49) and timothy pollen (g6) (numbers within parenthesis refer to the designations used by Pharmacia Diagnostics). Serum from a strawberry allergy-diagnosed patient, which belonged to a group of sera used as reference material by Pharmacia Diagnostics was also analyzed. With this patient, prick testing with strawberry had yielded a strong reaction, with scoring for strawberry, histamine control and birch being +++(+), +++ and ++, respectively. Sera from three non-allergic individuals (negative case history, negative ImmunoCAP specific IgE test responses to strawberry, apple, birch and timothy) were also used. The study was approved by the Research Ethics Committee, Gothenburg University.

Strawberry extract

Frozen [−20°C] strawberries (*Fragaria × ananassa* Duch., variety Camarosa, from Spain, supplied by Orkla Foods AS (Eslöv, Sweden)], were thawed for 10–20 min, and homogenized in a Sorvall omni-mixer 230 (Ivan Sorvall Inc., Norfolk, CT, USA). Cellular debris was removed by centrifugation, and the supernatant was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), either directly or after storage at

−20°C. The concentration of protein was approximately 0.5 mg/ml extract.

Strawberry protein extract

Strawberries were frozen in liquid nitrogen, and ground into a fine powder using mortar and pestle. To approximately 200 mg of such frozen strawberry powder, 500 µl extraction buffer containing 0.5 M Tris-HCl pH 6.8, 0.7 M sucrose, 50 mM ethylenediamine-tetraacetic acid (EDTA), 0.1 M KCl, 0.33 g polypyrrolidone and protease inhibitor cocktail (Complete™ Mini, five tablets to 50 ml extraction buffer; Roche, Mannheim, Germany) was added, and incubated for 15 min at 4°C. Thereafter 500 µl phenol was added and the sample was thoroughly mixed by vortexing. After centrifugation at 15 000 g for 5 min the phenol phase (upper phase) was collected. The proteins therein were precipitated by adding 5 vol of 0.1 M ammonium acetate in methanol (−20°C). The precipitate was washed by repeated resuspension in 0.1-M ammonium acetate in methanol (−20°C) ×2, and acetone (−20°C) ×2. The precipitate was then allowed to air-dry at −20°C. Before use, the protein precipitate was resolved in a suitable buffer, and centrifuged at 15 000 g for 20 min to get rid of any unresolved protein. The yield of protein was approximately 200 µg per 200 mg frozen strawberry powder.

Western blotting

Strawberry extract (approximately 150 µg protein) was separated by SDS-PAGE (1 mm NuPAGE 4–12% Bis-Tris 2D well; Invitrogen, Carlsbad, CA, USA). After separation, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, and Western blotting performed according to the instructions given for the Western Breeze Chromogenic Western Blot Immunodetection kit (Invitrogen), using as primary antibody 50 µl of patient's serum diluted to 500 µl, and as secondary antibody 500 µl of a reagent containing alkaline phosphatase-labelled murine monoclonal anti-human IgE antibody (Cat. no. ABI2; DPC, Los Angeles, CA, USA). To test the specificity in IgE binding, primary antibody was mixed with a positive control, a negative control or an allergen protein as inhibitor. To 50 µl patient serum, strawberry extract corresponding to approximately 25 µg protein or 25 µg of extracted strawberry protein (positive controls), or 25 µg of bovine serum albumin (BSA) (negative control) or 2, 10 or 25 µg allergen protein was added and then diluted to 500 µl. This mixture was incubated for 1 h at room temperature before use.

Mass spectrometric analyses

Mass spectrometry was performed according to Jensen et al. (9). After excision from gels and tryptic in gel digestion, nanospray tandem mass spectrometry (MSMS) was performed on a Q-TOF-1 tandem mass spectrometer (Micromass/Waters, Manchester, UK). For liquid chromatography (LC) MSMS, the tryptic peptide mixture was separated and analysed using an nanoscale capillary high-performance liquid chromatography (HPLC) system (Dionex, Breda, the Netherlands) interfaced directly to a Q-TOF Micro tandem mass spectrometer (Micromass/Waters). Peptide mixtures were separated on a C18 reverse phase column [75 µm (inner diameter) × 90.0 mm, Zorbax SB-C18 (Agilent, Palo Alto, CA, USA) 3.5 µm] during a 1.5-h gradient of 0–90% acetonitrile (v/v) containing 1% (v/v) formic acid, 0.6% (v/v) acetic acid, and 0.005% (v/v) heptafluorobutyric acid at a flow rate of 175 nl/min. Peptide identification from raw data was performed with an in-house MASCOT server (v. 1.8) (Matrix Sciences, London, UK).

Recombinantly expressed and purified allergens

Recombinantly expressed major allergens from birch and apple, rBet v 1 and rMal d 1, respectively (10), and timothy pollen allergen (total extract and Phl p1 purified from *Phleum pratense* pollen) were obtained for non-commercial use from ALK-Abello, Hørsholm, Denmark.

Basophil degranulation assay

Blood mononuclear cells were isolated from whole blood by lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) density centrifugation. Basophil degranulation was estimated using the FASTImmune kit (Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, the isolated cells were incubated with basophil stimulation buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 0.5 mM glucose and 0.1% BSA with pH 7.4 as a negative control). Different allergens were added in a concentration of 40 µg/ml or of 10 mM N-Formyl-Met-Leu-Phe [as a positive control, a chemoattractant peptide, a component of bacterial cell walls well-known to stimulate neutrophils (11)], for 10–15 min at 37°C. The reaction was stopped by transfer of the tubes to an ice bath. The antibody cocktail [fluorescein isothiocyanate (FITC)-conjugated CD63, phycoerythrin (PE)-conjugated CD123 and PerCP-conjugated human leucocyte antigen (HLA)-DR] was added followed by incubation for 20 min and afterwards the samples were washed with phosphate-buffered saline (PBS) and resuspended in 0.5% paraformaldehyde and analysed on a FACScan (Becton Dickinson). Gates were set to exclude dead cells. The basophils were identified as low side scatter (SSC), CD123-positive and HLA-DR-negative cells. The degranulation detection was measured by the percentage CD63-positive cells within the basophil gate. The CD63 is an intracellular, lysosomal protein whose surface expression is upregulated on activated basophils (12).

Results

Characterization of patient serum samples

Patients were selected on a case history suggestive of strawberry allergy, according to a questionnaire in which the patients described their symptoms after ingestion of

strawberry (Table 1). All patients were diagnosed with allergic symptoms against other allergen sources also, which is reflected by their serum levels of various allergen-specific IgE.

IgE binding detected by SDS-PAGE and Western blotting of strawberry extract

IgE-binding was analysed using sera from eight patients and three non-allergic individuals. As shown in Fig. 1A, sera from five of eight patients yielded a doublet band at 20/18 kDa. In one patient (no. 2) another band at 35 kDa was seen. In sera from control individuals, no bands were seen. Thus the most common nominator among patients was the 20/18-kDa doublet.

The same 20/18-kDa bands could also be detected by Western blotting if, instead of the homogenized strawberry extract, extracted strawberry protein was loaded (Fig. 1B). The strawberry protein was extracted by a phenol-extraction protocol (13), designed to enrich small amounts of protein present in ripe tomatoes and remove the large amounts of pectin, polysaccharides, and secondary metabolites such as isoflavonoids. Figure 1B shows that the reactivity to IgE is not lost by the phenol-extraction procedure. Enrichment of protein by the phenol-extraction procedure even allowed an extra IgE-binding band at approximately 60 kDa to be detected.

Detection of Bet v 1 homologues in both 20- and 18-kDa bands by mass spectrometry

The 20- and 18-kDa bands to which five of eight patients' sera showed IgE-binding were cut out from the gel and subjected to mass spectrometric analysis after in-gel tryptic digestion. By tandem MS (designated MSMS), tryptic peptides were subjected to further fragmentation in the mass spectrometer to reveal a partial or complete

Table 1. Strawberry-related symptoms, specific IgE and clinical history of patients

Patient no.	Answers in questionnaire: symptoms after strawberry ingestion	Specific IgE in serum†				Clinical history		
		Strawberry	Birch	Apple	Timothy	SPT‡	Birch	Symptoms§
1	Itching: face, neck, throat, blisters in mouth after 5 min	0	2	0	0	+	A, R	45/F
2*	Itching, swelling in throat after 10–30 min	1	4	2	3	+	R	37/F
3	Eczema in arm- and kneefold, same day and day after	2	4	1	3	+	A, R, E	46/F
4	Stomach pain and irritation, flatulence, diarrhoea after 5–30 min	0	0	0	0	–	A, R	25/F
5*	Itching in throat after 0–30 min	0	4	2	0	+	A, R, E	45/F
6*	Itching in throat, eyes, rhinitis, asthma after some minutes	2	4	3	3	+	A, R, E	37/M
7*	Itching in face, throat, ears, swelling in lips, after 5–15 min	0	4	2	0	+	R, E	40/F
8*	¶	2	4	2	3	+	A, E	19/F

* Patients who were found to show the double band at 20/18-kDa by Western blotting as described in Fig. 1.

† Classes 1–6 depending on results of serum IgE using the ImmunoCAP technology (Pharmacia Diagnostics).

‡ SPT, skin-prick test.

§ A, asthma; R, rhinitis; E, eczema.

¶ Patient no. 8 was not selected on basis of the questionnaire but belongs to a group of patients used as reference material by Pharmacia Diagnostics. With this patient, prick testing with strawberry has been done, yielding a strong reaction. Comparative scoring for strawberry, histamine control and birch was +++(+), +++ and ++, respectively.

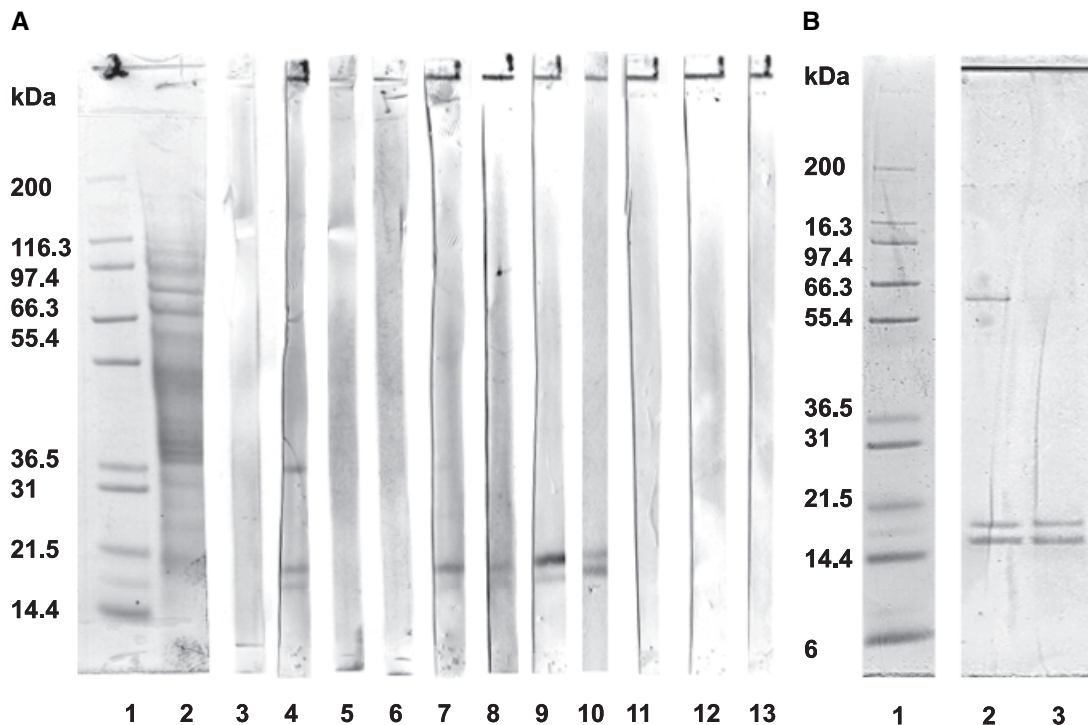


Figure 1. Patient IgE binding to strawberry proteins detected by Western blotting. Patient sera were used as first antibody and alkaline phosphatase-labelled secondary antibodies directed to IgE were used to visualize bands on PVDF membrane. (A) CB-staining of PVDF membrane: 1, molecular mass standard; 2, strawberry extract; Western blotting: 3–10, patient number 1–8; 11–13, healthy control individuals; (B) CB-staining of PVDF membrane: 1, molecular mass standard; Western blotting: 2, strawberry protein extract; 3, strawberry extract; serum from patient number 2 was used.

amino acid sequence. In the 20-kDa band, one protein was readily detected, a strawberry expansin-2, known to be highly expressed in ripe strawberries (14). In the 18-kDa band, nanospray MSMS identified a sequence AFVLDADNLIPK, which gave significant matching to the Bet v 1-homologue in apple, Mal d 1, with a score of 47, upon Mascot searching in the database NCBIInr (data not shown). The detection in the 18-kDa band of a strawberry homologue to such a well-known allergen as the birch pollen-allergen Bet v 1 was interesting. In a one-dimensional gel, each band contains several different proteins that can be detected by such a sensitive technique as mass spectrometry. A closer investigation of the 20-kDa band was therefore made by LC MSMS, whereby the tryptic digest is separated on a small reversed-phase column before being injected into the mass spectrometer, allowing detection and identification of many more peptides in the complex peptide mixture.

The result obtained after LC MSMS and subsequent Mascot searching in the database NCBIInr among plant sequences resulted in the identification of many different proteins, e.g. ribosomal proteins, isomerases and heat shock proteins (data not shown). By Mascot searching, interestingly, two peptides of the LC-MSMS were found to match Bet v 1 homologues: the A15 gene product (gi|862307) and the major allergen Mal d 1 (*Malus ×*

domestica) (gi|4590366), with scores of 63 and 42, respectively. The first peptide (Fig. 2A) was detected as a doubly charged ion, and the second peptide (Fig. 2B) as a doubly as well as a triply charged ion, and the total score was 110. The presence of the strawberry expansin-2 in the 20-kDa band was further confirmed (data not shown), by the observation of an additional peptide, GGWQSMSR, plus the one previously detected by nanospray MS.

To further confirm the identity of the strawberry Bet v 1 homologues, an error-tolerant Mascot search was made. This type of search allows for amino acid substitutions leading to identification also of proteins that are homologous to proteins listed in the database. In addition, modified peptides and peptides from non-specific enzymatic cleavage can be accounted for. The error tolerant search supported the identification of the strawberry homologue to Bet v 1 by identifying three more peptides matching the major allergen Mal d 1 (*Malus × domestica*) (gi|4590366) with a total score of 190.

To summarize, mass spectrometry pointed out the presence of strawberry homologues to the Bet v 1 allergen in both the 20- and the 18-kDa bands. In Fig. 3 a multiple alignment of Bet v 1-homologous sequences is shown, with matching peptides detected by LC MS (MS enboxed).

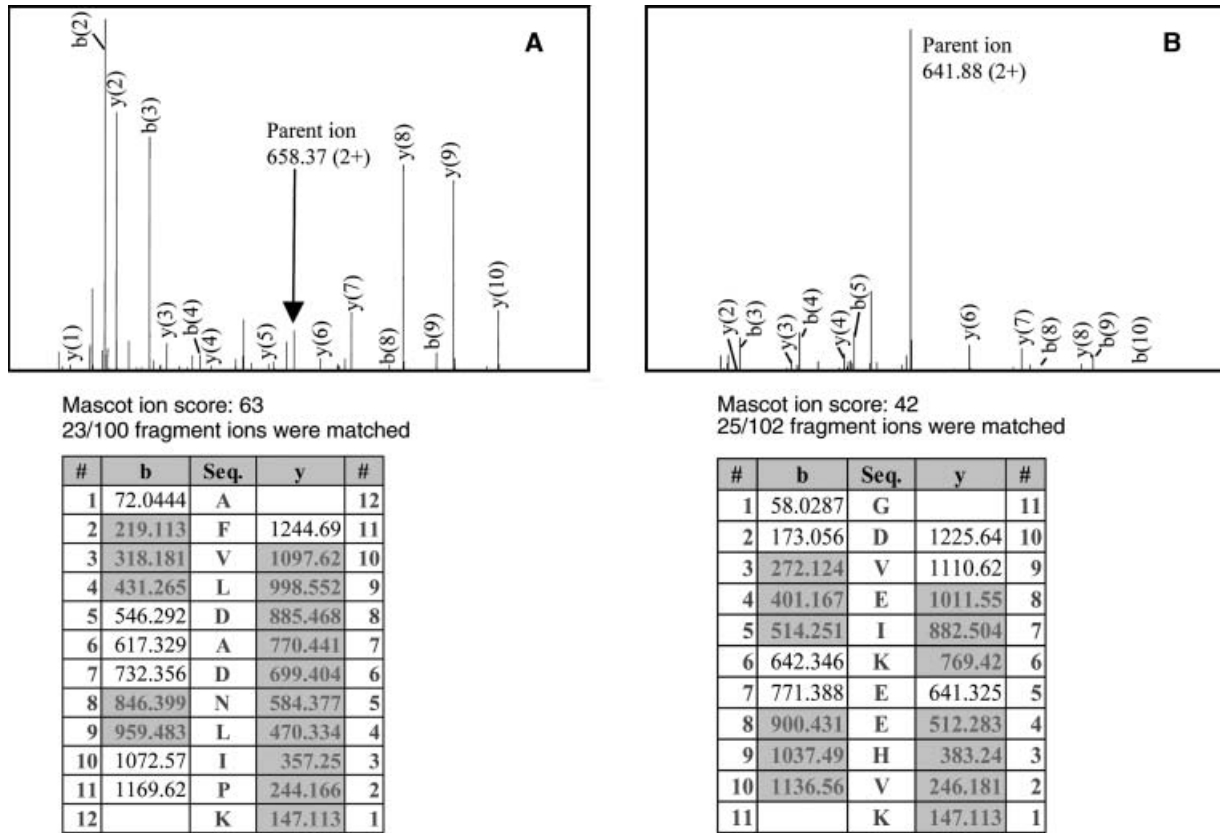


Figure 2. MSMS spectra of peptides detected in the IgE binding 20-kDa band. MSMS spectra and ion coverage for two peptides; (A) AFVLDADNLIPIK and (B) GDVEIKKEEHVK, detected by LC-MSMS of a sample from the 20 kDa band, showing IgE-binding in Fig. 1.

Inhibition of IgE binding by recombinantly expressed allergens in Bet v 1 family

The notion that several proteins are present in each band of the 20- and 18-kDa bands, and the presence of Bet v 1 homologues, reiterated the need for inhibition studies in order to identify proteins responsible for binding the patients' IgE (Fig. 1).

As seen in Fig. 4A, addition of either Bet v 1 or Mal d 1 (25 µg/50 µl patient serum) could inhibit the IgE binding to the 20/18-kDa doublet, whereas an extract from timothy pollen, containing Phl p1, did not. Titrable inhibition was made to estimate the minimal amount of allergen required for a complete inhibition of IgE binding, showing that inhibition was obtained already by 1 µg/ml Bet v 1 and 2 µg/ml Mal d 1 (Fig. 4B). The Bet v 1 and Mal d 1 allergens both belong to the Bet v 1 allergen family and show structural similarity as well as approximately 50% sequence identity. Addition of strawberry protein also inhibited the binding, whereas a negative control (BSA) did not. These inhibition data show that the IgE-binding to the 20/18-kDa doublet band is due to the Bet v 1 homologues detected in the 20- and 18-kDa bands by mass spectrometry.

Strawberry protein extract and Bet v 1 cause basophil degranulation in patient sera

To test whether strawberry allergens can cause cellular effects, cell-based assays were carried out as a complement to the IgE-binding studies. The assay used was basophil degranulation measured by flow cytometry analysis on peripheral blood mononuclear cells (PBMC) stimulated with different allergens. Gates were set to exclude all cells except the basophils and the upregulation of the intracellular marker CD63, upregulated on degranulated and activated basophils, was measured. The significance of the basophil degranulation is that it, as such, strengthens the Western blotting profoundly, which is more prone to 'false positives'.

To investigate whether strawberry protein really could evoke basophil degranulation, a screening was made to compare the response in terms of basophil degranulation to (i) negative and positive controls, (ii) purified allergen (rMal d 1, rBet v 1 protein, Phl p1), and (iii) strawberry protein. At a dose of 40 µg/ml, a basophil degranulation response was indeed obtained, by addition of either Mal d 1 and Bet v 1, or by addition of strawberry protein (Fig. 5). The grass pollen allergen



Figure 3. Multiple alignment showing homologues to Bet v 1, including partial sequences matching strawberry peptides detected in the IgE binding 20- and 18-kDa bands. Multiple alignment showing homologues to Bet v 1, including three of the 11 isoforms of Bet v 1 (Bet v 1A–C), Pru av 1, Pru ar 1, Pyr c 1 and Mal d 1 (two isoallergen sequences, P43211 and Q40280). Peptide sequences for which matches were found by Mascot searching in the strawberry 20/18-kDa bands are shown as boxed peptides. Also boxed is the conserved Glu45, which is crucial for the IgE-binding epitope [1]. Accession numbers in SWISS-PROT/TrEMBL for the sequences are given to the left. Multiple sequence alignment was performed using Clustal W.

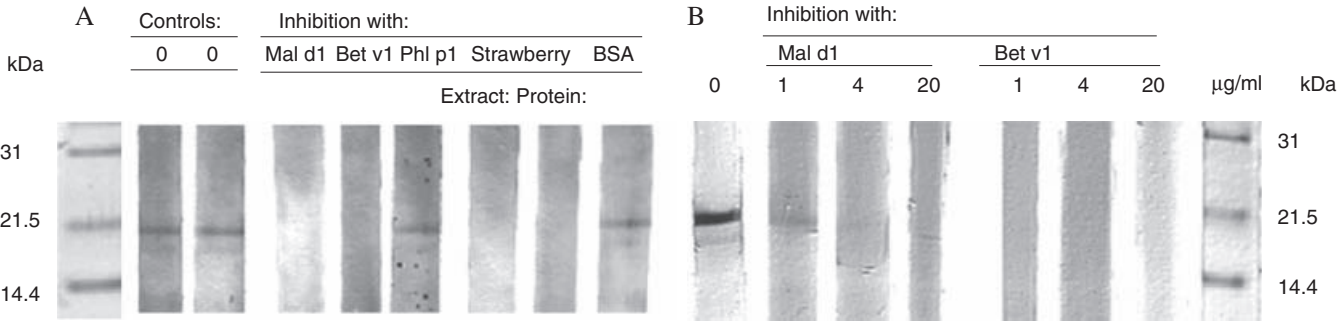


Figure 4. Inhibition of IgE binding by addition of recombinant Bet v 1-like allergens. (A) Western blotting was performed as described in Fig. 2 after prior incubation of 50 µl serum with either 25 µg allergen protein, 25 µg strawberry protein or strawberry extract corresponding to 25 µg protein, or 25 µg BSA. Lanes: 1, molecular mass standard; 2–3, no addition to serum; 4, Mal d1; 5, Bet v 1; 6, timothy pollen extract; 7, strawberry extract; 8, strawberry protein; 9, BSA. (B) Titrable inhibition was made to estimate the minimal amount of allergen required for a complete inhibition of IgE binding, using 1, 4, and 20 µg/ml Mal d 1 and Bet v 1. Serum from patient number 5 was used (patient numbers listed in Table 1).

Phl p 1 gave no response, nor did the timothy extract. This is consistent with the fact that the patient scored 0 for specific IgE to timothy in the ImmunoCAP assay (Table 1). The patient donated full blood for basophil degranulation assay twice, and the data are representative of two independent experiments. Two healthy non-

allergic individuals did not respond to any of the added proteins with basophil degranulation. To evaluate the biological activity of the allergens, a larger patient population should be analysed. Dose–response curves should then be recorded as the individuals may be both ‘low-dose’ as well as ‘high-dose’ responders.

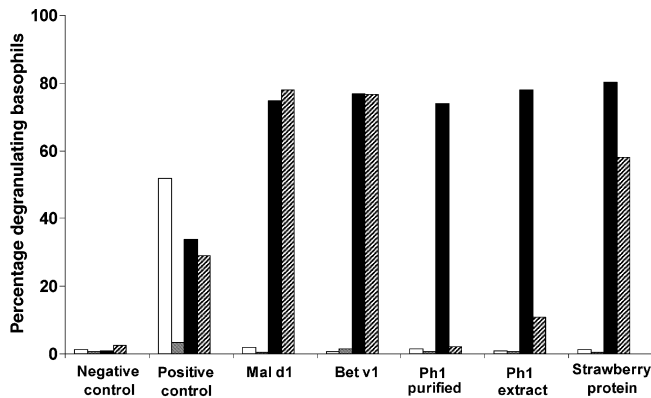


Figure 5. Effects caused by addition of allergens to patient basophils. Basophil degranulation was estimated with flow cytometry analysis. Cells were suspended in buffer only (negative control), 10 μ M of the chemoattractant peptide formyl-Met-Leu-Pro (positive control) and different allergens (40 μ g/ml) as stimuli. White bars: two different non-allergic individuals; Black bars: patient number 5 (patient numbers listed in Table 1).

At present, we can conclude from Fig. 5 that the Bet v 1-like allergens (Bet v 1, Mal d 1) and strawberry protein can cause basophil degranulation in patients with adverse reactions to strawberry.

Discussion

In this study, we have investigated Swedish patients with a case history suggestive of strawberry allergy. The actual presence of Bet v 1-homologous proteins in strawberry was detected by mass spectrometry (Fig. 2), and it could be concluded that both IgE binding (Fig. 4) and basophil degranulation (Fig. 5) is caused by Bet v 1-homologous strawberry proteins.

The Bet v 1-homologous proteins, commonly found in various fruits and vegetables, are important allergens in pollen-related food allergy (3, 4). In the patient group investigated in our study, five of eight patients showed IgE binding to the 20/18-kDa strawberry Bet v 1 homologues. This may be at least one reason for the problems caused for patients upon ingestion of strawberries. All the patients who showed IgE binding to the strawberry 20/18-kDa doublet had high scores for birch determined by ImmunoCAP (Table 1). However, the response in ImmunoCAP specific IgE to strawberry was less pronounced. This may be due to the potency of the extracts used as the solid phase in ImmunoCAP. Bet v 1 homologues are known to be easily degradable in complex fruit mixtures such as a strawberry extract. Also evident from Table 1 is that the patients which showed IgE binding to the strawberry 20/18-kDa doublet shared similar symptoms, typical for oral allergy syndrome (OAS), such as itching and swelling of lips, tongue and throat.

Thus, all patients reacting to strawberry extract or protein are sensitized to birch extract (Table 1). The reactivity to strawberry is most probably an epiphenomenon because of primary sensitization to Bet v 1 rather than a direct sensitization resulting from strawberry exposure. Such birch pollen-associated food allergy (3, 4) is common in northern Europe. That the primary induction is indeed caused by the pollen allergens is concluded from experiments showing that patient IgE has reactivity against more epitopes in pollen allergens than in food allergens, as the former are able to inhibit IgE binding to the latter but not vice versa (5).

However, other patient groups should also be investigated, as the IgE-binding pattern differs among populations. For example, cherry allergy has been shown to differ between northern and southern Europe: a cherry Bet v 1 homologue was found to be a more important allergen in a German patient group, compared with a cherry LTP homologue, which was found to be more important in an Italian patient group (15).

Why does the strawberry allergen behave as a 20/18-kDa doublet? This is not clear but the same phenomenon was reported for Pyr c 1 (16), and was suggested to be due to isoforms running differently in SDS-PAGE. By better separation of the several proteins in the 20/18-kDa bands by 2D gels, and subsequent *de novo* sequencing of the pure protein spots, a closer investigation of the strawberry Bet v 1 homologues can be carried out, with respect to quantity and quality.

Quantitative differences in content of the Bet v 1 homologue between apple varieties correlates with the IgE-binding potency (17). Qualitative differences, isoallergenic forms, also exist within a single apple (18, 19), and the different isoallergens have different epitopes, which might explain why birch pollen allergic patients react differently to apple ingestion (19). Of the part of the sequence of the strawberry Bet v 1 homologue that we have covered by MSMS peptide matching, two (AFVL-DADNLIPK and KITFGEGSQYGYVK) are present in both types of Mal d 1 sequences in the conserved, IgE-binding region (20, 21), whereas the other one (GDVEIKEEHVK) is uniquely present in only one of the two Mal d 1 sequences.

To obtain a more efficient identification of strawberry proteins, access to a strawberry EST database like the one described by Aharoni and O'Connell (22) would be very helpful, but to obtain information on the complete sequence of a strawberry protein such as the Bet v 1 homologue, a presumptive Fra a1, *de novo* sequencing must be performed, either by mass spectrometry or by cloning and sequencing of the corresponding gene.

Acknowledgments

Many thanks to Emilia Stensson for excellent technical assistance and to Arne Ståhl, Allergy Clinic, Sahlgrenska University

Hospital, for his support during development of the immunological methods. Kate Rafn is thanked for skilled expertise in MSMS, and the rest of the staff in Peter Roepstorffs laboratory for valuable discussions. Anita Kober at Pharmacia Diagnostics

is thanked for critical reading of the manuscript and useful comments. This study was supported by grants to S.F.-M. from VINNOVA (P-13642-1) and to C.E. and L.B. from FORMAS (22.6/2002-0042).

References

1. BREITENEDER H, EBNER C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000;**106**:27–36.
2. MILLS E, MADSEN C, SHEWRY P, WICHERS H. Food allergens of plant origin – their molecular and evolutionary relationships. *Trends Food Sci Technol* 2003;**14**:145–156.
3. VIETHS S, SCHEURER S, BALLMER-WEBER B. Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* 2002;**964**:47–68.
4. AALBERSE RC, AKKERDAAS J, VAN REE R. Cross-reactivity of IgE antibodies to allergens. *Allergy* 2001;**56**:478–490.
5. KAZEMI-SHIRAZI L, PAULI G, PUROHIT A, SPITZAUER S, FROSCHL R, HOFFMANN-SOMMERGRUBER K et al. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. *J Allergy Clin Immunol* 2000;**105**:116–125.
6. HOFFMANN-SOMMERGRUBER K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* 2000;**122**:155–166.
7. KARAMLOO F, WANGORSCH A, KASAHARA H, DAVIN L, HAUSTEIN D, LEWIS N et al. Phenylcoumaran benzylic ether and isoflavonoid reductases are a new class of cross-reactive allergens in birch pollen, fruits and vegetables. *Eur J Biochem* 2001;**268**:5310–5320.
8. ERIKSSON N, MÖLLER C, WERNER S, MAGNUSSON J, BENGTSSON U, ZOLUBAS M. Self-reported food hypersensitivity in Sweden, Denmark, Estonia, Lithuania, and Russia. *J Invest Allergol Clin Immunol* 2003;**13**:232–242.
9. JENSEN ON, LARSEN MR, ROEPSTORFF P. Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Prot Struct Funct Gen* 1998;**999**(Prot.Suppl.2):74–89.
10. SPANGFORT M, IPSEN H, SPARHOLT S, AASMUL-OLSEN S, LARSEN M, MORTZ E et al. Characterization of purified recombinant Bet v 1 with authentic N-terminus, cloned in fusion with maltose-binding protein. *Protein Expr Purif* 1996;**8**:365–373.
11. KELHER M, AMBRUSO D, ELZI DJ, ANDERSON S, PATERSON A, THURMAN G et al. Formyl-Met-Leu-Phe induces calcium-dependent tyrosine phosphorylation of Rel-1 in neutrophils. *Cell Calcium* 2003;**34**:445–455.
12. FUREDER W, AGIS H, SPERR WR, LECHNER K, VALENT P. The surface membrane antigen phenotype of human blood basophils. *Allergy* 1994;**49**:861–865.
13. IWAHASHI Y, HOSODA H. Effect of heat stress on tomato fruit protein expression. *Electrophoresis* 2000;**21**:1766–1771.
14. CIVELLO PM, POWELL AL, SABEHAT A, BENNETT AB. An expansin gene expressed in ripening strawberry fruit. *Plant Physiol* 1999;**121**:1273–1280.
15. SCHEURER S, PASTORELLO EA, WANGORSCH A, KÄSTNER M, HAUSTEIN D, VIETHS S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* 2001;**107**:724–731.
16. KARAMLOO F, SCHEURER S, WANGORSCH A, MAY S, HAUSTEIN D, VIETHS S. Pyr c 1, the major allergen from pear (*Pyrus communis*), is a new member of the Bet v 1 allergen family. *J Chromatogr B Biomed Sci Appl* 2001;**756**:281–293.
17. VIETHS S, JANKIEWICZ A, SCHONING B, AULEPP H. Apple allergy: the IgE-binding potency of apple strains is related to the occurrence of the 18-kDa allergen. *Allergy* 1994;**49**:262–271.
18. SON DY, SCHEURER S, HOFFMANN A, HAUSTEIN D, VIETHS S. Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* 1999;**38**:201–215.
19. HOLM J, BAERENTZEN G, GAJHEDE M, IPSEN H, LARSEN JN, LOWENSTEIN H et al. Molecular basis of allergic cross-reactivity between group I major allergens from birch and apple. *J Chromatogr B Biomed Sci Appl* 2001;**756**:307–313.
20. SPANGFORT MD, MIRZA O, IPSEN H, VAN NEERVEN RJ, GAJHEDE M, LARSEN JN. Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J Immunol* 2003;**171**:3084–3090.
21. MIRZA O, HENRIKSEN A, IPSEN H, LARSEN JN, WISSENBACH M, SPANGFORT MD et al. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J Immunol* 2000;**165**:331–338.
22. AHARONI A, O’CONNELL AP. Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. *J Exp Bot* 2002;**53**:2073–2087.