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Research Article

Characterization of Bet v 1-related allergens from kiwifruit relevant for patients with combined kiwifruit and birch pollen allergy

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Allergy to kiwifruit appears to have become more common in Europe and elsewhere during the past several years. Seven allergens have been identified from kiwifruit so far, with actinidin, kiwellin and the thaumatin-like protein as the most relevant ones. In contrast to other fruits, no Bet v 1 homologues were characterized from kiwifruit so far. We cloned, purified, and characterized recombinant Bet v 1-homologous allergens from green *(Actinidia deliciosa,* Act d 8) and gold (*Actinidia chinensis*, Act c 8) kiwifruit, and confirmed the presence of its natural counterpart by inhibition assays. Well-characterized recombinant Act d 8 and Act c 8 were recognized by birch pollen/kiwifruit (confirmed by double-blind placebo-controlled food challenge) allergic patients in IgE immunoblots and ELISA experiments. The present data point out that Bet v 1 homologues are allergens in kiwifruit and of relevance for patients sensitized to tree pollen and kiwifruit, and might have been neglected so far due to low abundance in the conventional extracts used for diagnosis.

Keywords: Bet v 1 / Food allergy / Kiwifruit / Pollen allergy / Recombinant allergen

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1 Introduction

Since 1962, when export of green kiwifruit (*Actinidia deliciosa* cv. Hayward) started from New Zealand to the US market, this cultivar became an increasinly common food in the Western world. In 2000, a new yellow fleshed species *Actinidia chinensis*, commonly called gold kiwifruit, was introduced to the European markets. Although *A. chinensis* is a species very similar to *A. deliciosa,* the gold kiwifruit is almost hairless and its flesh generally much sweeter [1].

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Abbreviations: **AP**, alkaline phosphatase; **CD**, circular dichroism; **DBPCFC**, double-blind placebo-controlled food challenge

The first adverse reactions to kiwifruit were described in 1981 [2]. Since then, many studies reported IgE-mediated allergic reactions in children and adults when eating fresh kiwifruit. Kiwifruit allergy was originally described as a "mild" allergy with symptoms confined to the oral cavity. However, a more recent study demonstrated that kiwifruit allergy can result in severe, life-threatening reactions, particularly in young children. Adults commonly reported multiple exposures before developing symptoms, while the children were more likely to react on their first known exposure to kiwifruit, indicating cross-reactivity of kiwifruit allergens with primary sensitizers [3].

Prevalence data regarding kiwifruit allergy are scarce and conflicting. In a Finnish survey, kiwifruit was identified as the most common cause of adverse reactions to foods [4] followed by cow's milk and apple, whereas in France kiwifruit is ranked third, after cow's milk and hen's egg and followed by peanuts [5].

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Up to now, seven allergens from green kiwifruit (*A. deliciosa*) have been identified and designated according to the guideline of the International Union of Immunological Societies Allergen Nomenclature Subcommittee (IUIS, http://www.allergen.org): Act d 1 (actinidin, a cysteine protease) [6], Act d 2 (a thaumatin-like protein) [7], Act d 3 (a 45-kDa protein of unknown function, Swiss-Prot acc. no. P85063), Act d 4 (cystatin) [8], Act d 5 (kiwellin) [9], Act d 6 (a pectin methylesterase inhibitor, Swiss-Prot acc. no. P83326), and Act d 7 (a pectin methylesterase, Swiss-Prot acc. no. P85076). Additionally, a class I chitinase [10] has been described, presumably responsible for the well-known latex-fruit syndrome [11]. In 2004, Bublin *et al.* [12] demonstrated IgE cross-reactivity between green and gold kiwifruit, which qualifies gold kiwifruit as a potential new allergen source for patients allergic to green kiwifruit.

The spectrum of birch pollen-related allergies to plant-derived foods is broad, including fruits such as apple, peach, and pear, nuts such as hazelnut, or vegetables such as celery and carrot. The determinants responsible for these cross-reactions are Bet v 1, the major allergen from birch pollen and its corresponding homologues in plant-derived foods, and profilins [13]. Kiwifruit has also been reported as an important allergenic food in patients with birch pollen allergy [14–16], but so far no molecular data on pollen-related allergens in this fruit have been available. This study was designed to investigate the allergenic relevance of a Bet v 1-homologous protein from green and gold kiwifruit in a well-characterized population with kiwifruit and pollen allergy. The aim of our study was to clone, express, purify, and characterize Bet v 1-homologous proteins from green and gold kiwifruit.

2 Materials and methods

2.1 Generation of recombinant Act d 8 (from green kiwifruit) and Act c 8 (from gold kiwifruit)

2.1.1 Cloning and sequencing of recombinant Act d 8 and Act c 8

Bet v 1-related sequences were identified from EST cDNA libraries from *A. deliciosa* and *A. chinensis* as described previously [17]. EST clones were derived from ripe *A. chinensis* and from dormant *A. deliciosa* buds, respectively.

The full-length ORF for Act d 8 was amplified by PCR using forward and reverse primers containing *Nco*I and *Eco*RI restriction sites (in italics), respectively (forward Act d 8: 5′-GGG *CCA TGG* GTG CCA TCA CTT ACG ATA TG-3′; reverse Act d 8: 5′-CCC *GAA TTC* CTA GCA ATC GGG ATT TGC CAA GAG-3′).

The full-length ORF for Act c 8 EST clone was amplified by PCR using primers 5′-GGG *CCA TGG* GTG TCG TTA CCT ACG-3′ (forward) and 5′-CCC *GAA TTC* TTA GCA GTA ATC AGG ATT TG-3′ (reverse), nucleotides indicating *Nco*I and *Eco*RI restriction site are shown in italics, respectively. For all PCR experiments *Pfu* DNA polymerase (Fermentas, Ontario, Canada) was used.

PCR products were purified on 1.2% agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). DNA fragments were ligated into pCR2.1-TOPO (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA, USA) and chemically competent *E. coli* cells (strain XL1-Blue; Stratagene, La Jolla, CA, USA) were transformed. Positive clones were selected by blue/white screening on plates containing X-gal and ampicillin. In addition, PCR screening was performed with insert primer pairs. DNA sequence analysis of the subsequently isolated plasmids from single clones was performed with a LI-COR fluorescent sequencer 4000L (LI-COR, Lincoln, NE, USA) or on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequence comparisons were performed using CLUSTAL W.

2.1.2 Transcript abundance in mature eating ripe fruit

Five whole mature eating-ripe Hayward and Hort16A fruits were chopped individually, flash frozen in liquid nitrogen, and ground to a fine powder using a custom turned steel bowl (pre-cooled in liquid nitrogen) with a rock crusher machine (Rocklabs, Auckland, New Zealand). Approximately 230 mg frozen powder from each fruit were combined in a 50-mL Falcon tube containing 12.5 mL lysis/binding buffer and vortexed for 2 min at max speed. Then 1 mL of this suspension was transferred to a 1.5-mL Eppendorf tube, centrifuged for 2 min at 13 000 rpm, and the supernatant transferred to a fresh tube. Poly(A+) mRNA was isolated from the supernatant using 20 μL equilibrated magnetic Dynabeads® Oligo $(dT)_{25}$ suspension (Invitrogen) per sample together with a magnetic rack apparatus (MagnaRack, Invitrogen). Buffer compositions, including lysis/binding buffer and method were as described in the Dynabeads® product manual except that LiDS was replaced by SDS (same concentration). The purified messenger RNA was eluted in 30 μL 10 mM Tris-HCl (pH 7.5) and 5 μL per sample were used in a 20-μL total volume reverse-transcription reaction using a SuperScript1 VILO™ cDNA Synthesis Kit (Invitrogen) following manufacturer instructions. For qualitative PCR, 1 μL cDNA was used per 50-μL PCR reaction. Final reaction components (all sourced from Invitrogen) included 16 × Platinum Taq buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 200 pM each primer, and 2.5 U Platinum Taq DNA polymerase. Thermocycler program: 94°C for 2 min; then 35 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 20 s. Cycling was paused at 30 cycles and 20 μL was removed for gel electrophoresis. Upon completion, 10 μL of each sample at 30 cycles was electrophoresed through a 2.5% agarose (Invitrogen) 0.56 × TBE gel stained with ethidium bromide, and visualized under UV light. Aliquots from 35 cycles were also run for Act c 8 and Act d 8 samples because they had not amplified sufficiently after

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30 cycles. Amplicon size was confirmed by running 1KB+ and Low Mass DNA ladders in the gel (both from Invitrogen). Primer sequences are as follows: actin (198-bp amplicon) (forward): 5′-CCAAGGCCAACAGAGAGAAG-3′, actin (reverse): 5′-CCAAGGCCAACAGAGAGAAG-3′; actinidin (107-bp amplicon), accession EF530131 (forward): 5′-ACAACCAAAATCACCCCAAA-3′, (reverse): 5′-AGCGCTGTACCTCTGTCCAT-3′; Act c 8 (109-bp amplicon) (forward): 5′-ACCCTTGTTCCCAAGGTTCT-3′, (reverse): 5′-GATGGCTTCCTTCACCAAAA-3′; Act d 8 (104-bp amplicon) (forward): 5′-CGCTTTGGACGT-TTTTGAAT-3′, (reverse): 5′-CGCATTTGGGGGTGTAA-ATA-3′; kiwellin (101-bp amplicon) (forward): 5′-TCGA-GACACGGGTGTGATAA-3′, (reverse): 5′-TTGTCCAAC-CCTAAGGCACT-3′.

2.1.3 Expression and purification of recombinant Act d 8

For Act d 8 expression, the full-length cDNA was ligated into the vector pETBlue-2 (Novagen, Madison, WI, USA) and the construct introduced into *E. coli* strain Tuner(DE3)-pLacI (Novagen). Cells were grown at 37°C in LB medium containing 1% glucose, 100 μg/mL ampicillin and 20 μg/mL chloramphenicol to an optical density of 0.7 measured at 600 nm. Expression of the recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 6 h, cells were harvested by centrifugation (3000 g for 20 min, 4°C) and disrupted using French pressure cell (SIM Amico Spectronic Instruments, Rochester, NY, USA). Ammonium sulfate precipitation (20% saturation) was carried out with the soluble cell fraction as a pre-purification step. The first purification step was performed using hydrophobic interaction chromatography (Phenyl Sepharose; GE Healthcare Life Sciences, Uppsala, Sweden). Bound recombinant Act d 8 (rAct d 8) was eluted with a gradient of decreasing salt concentration and increasing pH (0–100% B_1 over 10 column volumes (CVs), 1 mL/min; buffer A_1: 20 mM sodium phosphate pH 6.0, 1 M ammonium sulfate, 1 mM DTT, 1 mM EDTA; buffer B_1: 80 mM Tris-HCl, 5% isopropanol, pH 9.0, 1 mM DTT, 1 mM EDTA). Fractions containing rAct d 8 were pooled, dialyzed against 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA and further purified by anion exchange chromatography (Q Sepharose; GE Healthcare Life Sciences) using a column equilibrated with buffer A_2 and eluted with a linear salt gradient (0–100% B_2 over 10 CVs, flow rate 1 mL/min; buffer A_2: 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA; buffer B_2: buffer A_2 + 1 M NaCl). As a polishing step, fractions containing rAct d 8 were separated using gel filtration (buffer: 25 mM sodium phosphate pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mm DTT; column: HiPrep 16/60 Sephacryl S-200 High Resolution, GE Healthcare Life Sciences). Fractions containing highly pure rAct d 8 were pooled and the protein concentration determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The protein was stored at –20°C until use.

2.1.4 Expression and purification of recombinant Act c 8

Full-length Act c 8 was produced using expression vector pMW175 and *E. coli* strain BL21 (DE3) [18]. The transformed strain was grown in LB medium at 30°C for 8 h, harvested by centrifugation and disruption was performed using French pressure cell. The recombinant protein was purified from inclusion bodies.

To obtain material suitable for purification, inclusion bodies were prepared (washing twice with 50 mM NaH_2PO_4, 300 mM NaCl, 0.5% Triton X-100, 10 mM EDTA pH 8.0 and twice with 50 mM NaH_2PO_4, 300 mM NaCl, 0,1% Triton X-100 pH 8.0) and solubilized by resuspending in 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 8 M urea. Refolding was performed by stepwise dialysis in 20 mM NaH_2PO_4, pH 8.0 with decreasing concentrations of urea (6, 4, and 2 M).

Purification of rAct c 8 was performed by anion exchange chromatography (DEAE, GE Healthcare Life Sciences). The column was equilibrated with 10 mM NaH_2PO_4, pH 8.0, 1 mM DTT, 1 mM EDTA (buffer A_3). Bound protein was eluted by a linear gradient to 0.5 M NaCl in buffer A_3 over 10 CV. Fractions containing purified rAct c 8 were pooled and protein concentration was determined by BCA assay. The protein was stored at –20°C until use.

2.2 N-terminal sequencing

N-terminal sequencing of purified proteins was performed with an Applied Biosystems Procise 491 sequencer (Applied Biosystems). rAct d 8 and rAct c 8 (50 pmol) were adsorbed on a Prosorb cartridge and subjected to sequence analysis. Sequence data were compared with protein databases using the BLAST program.

2.3 Circular dichroism spectroscopy

Secondary structure of the purified recombinant kiwifruit allergens was assessed using circular dichroism (CD) spectroscopy in a Jasco J-810 spectropolarimeter (Jasco, Easten, USA) in a 0.1-cm quartz cuvette. Data from three measurements were accumulated to calculate the mean spectra.

2.4 MS

For sequence analysis, 5 μg rAct d 8 was digested with the Proteoextract trypsin digestion kit (Calbiochem, San Diego, CA, USA). The resulting peptides were separated by capillary RP-HPLC (Waters, Milford, MA, USA; pre-column Waters Nanoease Symmetry300 trap column, separating column Waters Nanoease Atlantis dC18) directly coupled

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to the mass spectrometer, connected *via* a ten-port stream select valve (Global Ultima Q-Tof instrument; Waters, Manchester, UK). The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an ACN gradient (solvent A: 0.1% formic acid, 5% ACN; solvent B: 0.1% formic acid, 95% ACN; 5–45% B in 90 min). For sequence analysis, the instrument was calibrated with the fragment ions of [Glu]-fibrinopeptide B (Sigma, St. Louis, MO, USA). Data were acquired in the Data Directed Analysis (DDA) mode. Survey and fragment spectra were analyzed using the software PLGS version 2.2.5 (Waters, UK) with automatic and manual data verification. For sequence identification, a mini database comprising the trypsin and Act d 8 sequences and a combined Swiss-Prot/Trembl database were used.

2.5 Patients' sera

Sera were collected from eight patients with a confirmed food allergy to kiwifruit according to a convincing case history and a positive double-blind placebo-controlled food challenge (DBPCFC) and with concomitant birch pollen allergy. In addition, sera from five patients with a birch pollen allergy but no kiwifruit allergy as confirmed by a negative open food provocation were tested in parallel. Each patient provided a written informed consent. DBPCFC was performed as previously described [19] up to a cumulative dose of 60 g kiwifruit. For IgE *in vitro* assays, sera from three non-allergic subjects were identified, pooled and used as controls.

2.6 IgE ELISA

Microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with 1 µg protein (diluted in 25 mM $NaHCO_3$, pH 9.6) per well. After blocking with 50 mM Tris-buffered saline containing 0.5% Tween 20 (incubation buffer) and 3% nonfat dry milk, sera (1:5 diluted in incubation buffer and 0.5% BSA) were applied to the plates and incubated overnight at 4°C. For inhibition ELISA, sera were preincubated with 100 µg/mL rBet v 1.0101, rAct d 8, or rAct c 8, respectively. After washing, incubation with an alkaline phosphatase (AP)-conjugated mouse anti-human IgE antibody (BD Pharmingen, San Diego, CA, USA; 1:1000 diluted in incubation buffer with 0.5% BSA) was carried out at room temperature in the dark. Color development was performed using *p*-nitrophenyl phosphate substrate tablets (Sigma-Aldrich, Steinheim, Germany) and the $A_{405\ nm}$ was measured. Buffer and a serum pool from three non-allergic subjects were used as negative controls. Absorbance values were considered positive if they exceeded the mean $A_{405\ nm}$ of the negative controls by >3 SD.

2.7 Kiwifruit extracts

Fresh green (*A. deliciosa* cv. Hayward) and gold kiwifruit (*A. chinensis* cv. Hort 16A) were obtained from the local market. Protein extractions were performed as previously described [12]. Purified recombinant Bet v 1.0101 (rBet v 1.0101) was obtained from Biomay (Vienna, Austria).

2.8 SDS-PAGE, IgE immunoblotting and inhibition assays

Detection of IgE binding proteins by immunoblotting was performed as described previously [12]. Briefly, the proteins were separated by 15% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Pall Corporation, East Hills, NY, USA). After blocking, the membrane was incubated with appropriately diluted sera from allergic patients. For inhibition assays, serum samples were preincubated with 100 µg/mL rBet v 1.0101, rAct d 8, or rAct c 8, respectively. Bound IgE was detected using either AP-conjugated mouse anti-human-IgE antibody (BD Pharmingen) or ^{125}I-labeled anti-human-IgE (Demeditec Diagnostics GmbH, Germany). For control experiments, buffer and the non-allergic serum pool were tested in parallel.

2.9 Immuno-tissue prints

Immuno-tissue prints were performed as described elsewhere [20]. Briefly, nitrocellulose membranes were soaked in 100 mM ascorbic acid and air dried prior to printing. Fruit slices from green and gold kiwifruit were placed on the nitrocellulose membranes, covered with filter paper and pressed onto the membrane. Membranes were air dried, unspecific binding sites were blocked using TBS containing 0.5% Tween 20 and 3% nonfat dry milk and incubated with polyclonal rabbit anti-Bet v 1 antibodies [21] at room temperature for 2 h. Detection was performed using AP-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark). As a negative control, buffer replacing rabbit anti-Bet v 1 antiserum was applied.

3 Results

3.1 Cloning of Act d 8 and Act c 8

Act d 8 and Act c 8 are encoded by ORFs of 471 and 474 nucleotides, corresponding to 157 and 158 amino acid residues, respectively (Fig. 1). To investigate polymorphisms in Act d 8, 16 clones were sequenced and conceptually translated (Table 1). Of the 157 amino acid positions, 136 were invariant and differences were found at 21 positions.

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Act d 8          MGAITYDMEIPSSISAEKMFKAFVLDGDTIIPKALPHAITGVQTLEGDGGVGTIKLTTFG 60
Act c 8          MGVVTYDMEIPSKVPPVKLYKAFILDGDTLVPKVLPHAIKCVKILEGDGCAGTIKEVTFG 60
Bet v 1          MGVFNYETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFP 60

Act d 8          EGSVHKSVKHRIDGLDKENFTYSYSIIEGGAL-DVFESISYHIKIVATPDGGCICKNRSI 119
Act c 8          EGSHHKCVKQRVDAIDKDNLTYSYTIIEGDVLAEKFESISYHIKIVACPDGGSICKNRSI 120
Bet v 1          EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNK 120

Act d 8          YTPKCDAQVSEEEIKAGKERASGIFKKVEAYLLANPDC-- 157
Act c 8          YTTKGDCKVSEEEIKLGKEKAAEIFKALEAYLLANPDY-- 158
Bet v 1          YHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN 160
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Figure 1. Amino acid sequence alignment of rAct d 8.0101 and rAct c 8.0101 with Bet v 1.0101.

Table 1. Sequence comparison of kiwifruit Bet v 1 homologues: Amino acid sequence identities (%) of Act d 8 (isoform Act d 8.0101) and its 12 isoforms (clone 1, 3, 5, 6, 7, 10–12, 16, 18–23) deduced from distinct Bet v 1-like genes, are shown. From gold kiwifruit Act c 8.0101 is presented together with deduced protein sequences from 5 distinct Bet v 1-like genes (Act c 8b, Act c 8d-g) found in HortResearch *A. chinensis* EST libraries (Crowhurst *et al.*, unpublished results).

	Clone 3	Clone 23	Clone 10	Clone 12	Clone 7,18	Act d 8.0101	Clone 21	Clone 5	Clone 19	Clone 6, 20, 22	Clone 1	Clone 11	Clone 16	Act c 8b	Act c 8f	Act c 8g	Act c 8d	Act c 8e	*Act c 8.0101*	Bet v 1
Clone 3	100	99	99	98	97	*99*	98	96	95	96	96	97	96	96	90	85	85	77	*70*	50
Clone 23		100	99	97	97	*98*	97	96	94	95	96	96	95	96	89	84	85	78	*71*	50
Clone 10			100	97	98	*99*	99	96	96	96	97	97	96	97	90	85	85	78	*71*	50
Clone 12				100	97	*98*	97	96	94	95	96	96	95	96	89	84	83	76	*68*	48
Clone 7, 18					100	*99*	98	96	94	95	96	97	96	96	90	85	83	78	*69*	50
Act d 8.0101						*100*	*99*	*97*	*96*	*97*	*97*	*98*	*97*	*97*	*91*	*86*	*85*	*78*	*70*	*53*
Clone 21							100	97	96	96	97	97	96	97	90	85	84	78	**70**	50
Clone 5								100	96	96	96	97	96	97	89	86	83	78	**70**	50
Clone 19									100	94	95	96	94	95	87	85	82	77	**69**	50
Clone 6, 20, 22										100	98	97	97	97	89	86	83	79	**69**	51
Clone 1											100	97	97	96	89	85	83	78	*69*	50
Clone 11												100	99	97	90	86	85	78	*70*	52
Clone 16													100	97	89	85	84	78	*69*	52
Act c 8b														100	92	87	84	78	*69*	50
Act c 8f															100	88	84	80	*70*	51
Act c 8g																100	81	84	*71*	53
Act c 8d																	100	82	*76*	54
Act c 8e																		100	*71*	54
Act c 8.0101																			*100*	*54*
Bet v 1																				100

At 13 of those 21 positions, variation was present in multiple, while single-clone variation occurred at 8 positions. The greatest divergence in amino acid sequence between any 2 of the 16 clones was 5.7% (9 positions). Sequence analysis of cloned 3′ and 5′ RACE products confirmed the start and stop of the Act d 8 ORF. The clone used in the work described below, designated Act d 8.0101 differed at 6 or fewer positions (≤3.8%) from the other 15 deduced sequences.

In gold kiwifruit, 6 different Bet v 1-related sequences were identified, the heterologously expressed isoform was designated Act c 8.0101 (Table 1, Fig. 1).

The amino acid sequence identity between Act d 8 and Act c 8 was 70% and to Bet v 1 (CAA54696) 53% and 54%, respectively (Table 2). Sequence identities among proteins from the Bet v 1 family ranged between 64% (Pru ar 1, AAC02632) and 51% (Aln g 1, AAB24432) (Table 2).

BLAST searching the HortResearch kiwifruit EST database [17] using apple Mal d 1d sequence (EB175267) returned six distinct clusters of sequences with greater than 95% similarity in *A. chinensis*. These are referred to as Act c 8b and d–g here with sequence identity ranging from 69 to 97% to Act c 8.0101 (Table 1), which suggests that the *A. chinensis* orthologue of Act d 8.0101 is likely to be Act c 8b (Table 1).

The predicted molecular mass of Act d 8 and Act c 8 is 16 922 Da and 17 387 Da, and their calculated p*I* 5.36 and 5.82, respectively. The sequences have been submitted to the EMBL database (acc. nos. AM489568 and AM489567) and the allergens were given the official designations Act d 8 and Act c 8, respectively, by the IUIS.

3.2 Qualitative PCR comparisons with other allergens

The relative abundance of Act c 8 and Act d 8 RNA transcripts, as well as those of other identified allergens were compared by PCR in whole mature eating-ripe green

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Table 2. Comparison of amino acid sequence identities of Act d 8 and Act c 8 to Bet v 1-related proteins (GenPep acc. no.)

Plant food allergens		Act d 8	Act c 8
Pru ar 1 (AAB97141)	Apricot	61%	64%
Mal d 1 (AAX20938)	Apple	57%	58%
Pru av 1 (AAS47037)	Cherry	56%	54%
Fra a 1 (CAJ85639)	Strawberry	55%	55%
Pru p 1 (ABB78006)	Peach	54%	57%
Pollen allergens			
Cas s 1 (CAD10374)	Chestnut	57%	60%
Fag s 1 (CAA10235)	Beech	57%	58%
Cor a 1 (AAG40329)	Hazelnut	55%	54%
Bet v 1 (CAA54696)	Birch	53%	54%
Aln g 1 (AAB24432)	Alder	50%	51%

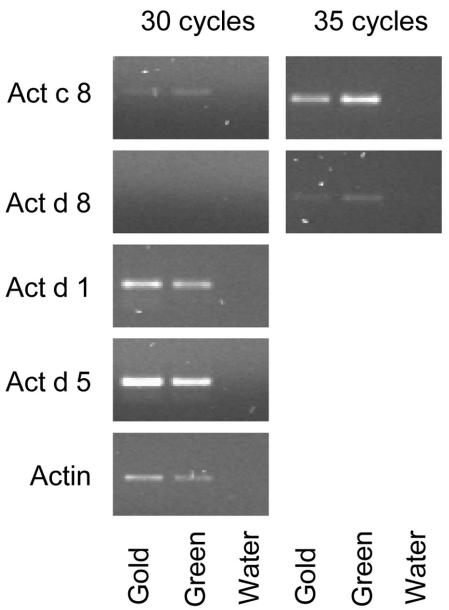

Figure 2. Qualitative PCR showing relative abundance of Act c 8 and Act d 8 transcripts in mature whole eating-ripe fruit from green and gold kiwifruit compared with those of actinidin (Act d 1) and kiwellin (Act d 5). Actin is included as a loading control. Primers specific for Act c 8, Act d 8, Act d 1, Act d 5 and actin are indicated in the left margin.

('Hayward') and gold (Hort16A) fruit after 30 and 35 cycles, respectively. Primers specifically designed to Act c 8 and Act d 8, actinidin (Act d 1), and kiwellin (Act d 5) were used (Fig. 2). Transcripts for kiwellin appeared the most abundant in both green and gold kiwifruit, followed closely by those of actinidin (acidic isoform). Transcripts for Act c 8 and Act d 8 are considerably less abundant, with Act c 8 appearing to be more highly expressed than Act d 8 in both green and gold kiwifruit. Both Act c 8 and Act d 8 primers amplified products from green (*A. deliciosa*) and gold (*A. chinensis*) cDNA at a similar level. A comparison of primer and gene sequences showed one single nucleotide polymorphism (in italics) present in the reverse primer for Act d 8 (in the middle) to its corresponding primer binding site in Act c 8b: 5′-CGCATTT*G*GGGGTGTAAATA-3′. The forward primer shared 100% sequence identity with its corresponding primer binding site in Act c 8b, so it is likely that the product amplified by Act d 8 primers from gold cDNA is Act c 8b.

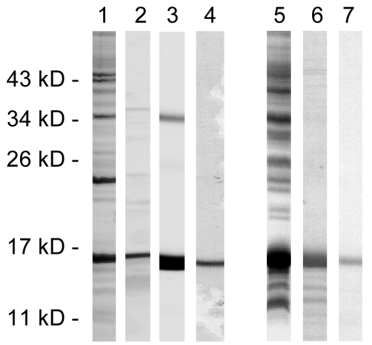

Figure 3. Coomassie-stained SDS-PAGE analysis of purification of rAct d 8 (lanes 1–4) and rAct c 8 (lanes 5–7). Crude *E. coli* lysate expressing rAct d 8 (lane 1), and rAct c 8 (lane 5), respectively. Recombinant Act d 8-enriched fraction after hydrophobic interaction chromatography (lane 2), anion exchange chromatography (lane 3) and gel filtration (lane 4). Recombinant Act c 8 after refolding (lane 6) and anion exchange chromatography (lane 7).

3.3 Purification of recombinant proteins and production of kiwifruit protein extract

3.3.1 Purification and physicochemical characterization of rAct d 8

Complementary DNA coding for Act d 8 was cloned into the expression vector pETBlue-2 and expressed as a non-fusion recombinant protein in *E. coli* Tuner (DE3)pLacI (Fig. 3, lane 1). To obtain purified rAct d 8, ammonium sulfate precipitation, hydrophobic interaction chromatography (Fig. 3, lane 2), anion exchange chromatography (Fig. 3, lane 3), and gel filtration (Fig. 3, lane 4) were performed and purity demonstrated using Coomassie-stained SDS-PAGE (15%, reducing conditions). Using this purification protocol a total yield of 3.5 mg pure protein per liter *E. coli* culture was obtained. Purified protein was stable when stored at −20°C.

Secondary structure of rAct d 8 determined by CD spectroscopy was similar to CD spectra obtained with rBet v 1.0101 (Fig. 4). N-terminal sequencing of five amino acid residues (GAITY) confirmed the identity of rAct d 8 (AM489568), showing that the initiating methionine was cleaved off. Further confirmation of the amino acid sequence was obtained after digestion with trypsin, the resulting peptides being analyzed by LC-MS-MS. Peptides with sequences matching that of rAct d 8 were identified,

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covering more than 70% of its sequence. The presence of contaminations was ruled out by using several analytical methods: mass analysis, N-terminal sequencing, and SDS-PAGE. Similarly, no chemical modifications of amino acid residues that might have occurred during the expression and purification procedures were detected as shown by tryptic digest of the protein followed by mass analysis of the obtained fragments, covering around 70% of the sequence.

3.3.2 Purification and physicochemical characterization of rAct c 8

The Act c 8 cDNA was cloned into the expression vector pMW175 and expressed as a non-fusion recombinant protein in *E. coli* BL21 [DE3] (Fig. 3, lane 5). Expression resulted in the formation of inclusion bodies and solubilization of the protein was achieved using 8 M urea. Stepwise refolding (Fig. 3, lane 6) and anion exchange chromatography (Fig. 3, lane 7) resulted in a highly pure recombinant protein, migrating at about 16 kDa in SDS-PAGE under reducing conditions. This purification protocol resulted in 2 mg purified protein per liter *E. coli* culture.

N-terminal sequencing of rAct c 8 (AM489567) verified the first five amino acid residues GVVTY, without the initiating methionine. Secondary structure of rAct c 8 was evaluated by CD spectroscopy and was similar to rBet v 1.0101 (Fig. 4).

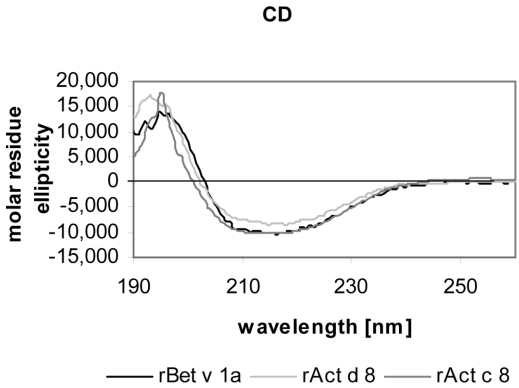

Figure 4. CD spectra of purified rAct d 8, rAct c 8 and rBet v 1.0101.

3.4 Patients' sera

The mean age of the kiwifruit/birch pollen allergic patients (n=8) included was 35 ± 18 years and that of birch pollen allergic controls (n=5) 27 ± 11 years. All patients included, were tested positive for birch pollen extract using the ImmunoCAP System (Phadia, Uppsala, Sweden) and were positive in a prick to prick skin test [19] with native kiwifruit extract (Table 3). All these patients were also positive in DBPCFC to kiwifruit. Symptoms under DBPCFC consisted of an oral allergy syndrome in four patients, dysphagia in one patient, dyspnea in two patient, and flush of the face in one patient each. All birch pollen control subjects were tested negative in the open provocation and in kiwifruit prick to prick test (data not shown).

3.5 IgE ELISA and ELISA inhibition

IgE ELISA experiments using purified rAct d 8 from green kiwifruit and rAct c 8 from gold kiwifruit were performed using sera from eight kiwifruit/birch pollen allergic patients (positive DBPCFC). Serum IgE of all eight kiwifruit/birch pollen allergic patients recognized rAct d 8, whereas five of those displayed IgE binding to rAct c 8 (Table 3).

The cross-reactivity of rBet v 1.0101, rAct d 8, and rAct c 8 was assayed in an IgE ELISA inhibition assay. Pretreatment of individual kiwifruit/birch pollen allergic patients' sera with rAct d 8 or rAct c 8 as inhibitor resulted in reduced IgE binding to rBet v 1.0101 (60%). IgE binding to rAct d 8 and rAct c 8 was completely inhibited by preincubation with rBet v 1.0101 (data not shown).

3.6 IgE immunoblotting and inhibitions

Purified rAct d 8 and rAct c 8 were tested for their IgE-binding capacity in immunoblot assays using sera from five kiwifruit allergic patients (Fig. 5). Both purified recombinant proteins were recognized by selected kiwifruit/birch

Table 3. Characteristics of patients with kiwifruit/birch pollen allergy

Pat. no.	DBPCFC	CAP classes		ELISA		Blot	
	kiwifruit	birch pollen	rBet v 1	rAct d 8	rAct c 8	rAct d 8	rAct c 8
1	+	3	4	+	-	+	-
2	+	3	4	+	+	+	+
3	+	2	0	+	+	+	+
4	+	3	4	+	-	+	-
5	+	4	4	+	-	+	-
6	+	2	3	+	+	+	+
7	+	4	5	+	+	+	+
8	+	2	0	+	+	+	+

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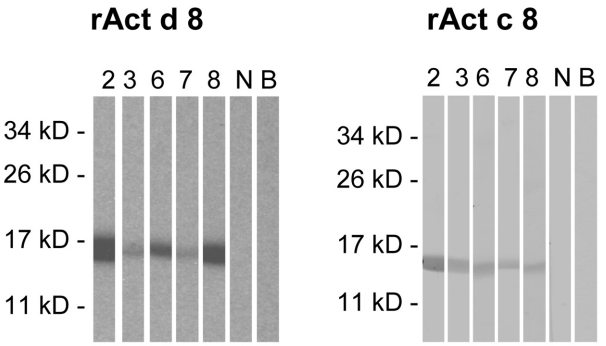

Figure 5. Recombinant Act d 8 and rAct c 8 IgE assays: IgE immunoblots were performed with five selected kiwifruit/birch pollen allergic patients' sera (corresponding to data from patients 2, 3, 6–8 in Table 3) showing IgE binding to purified rAct d 8 and rAct c 8. A pool of sera from three non-allergic subjects (lane N) and buffer (lane B) were used as negative controls.

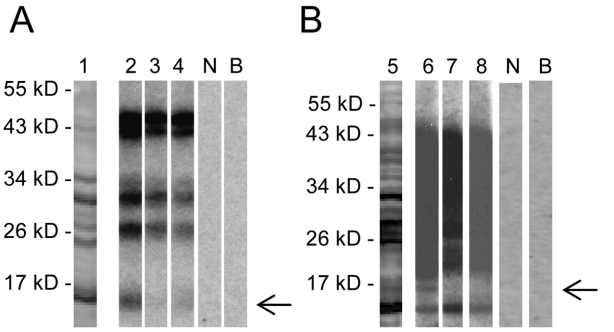

Figure 6. IgE inhibition assays with green (A) and gold (B) kiwifruit extract. Coomassie stain of green (lane 1) and gold (lane 5) kiwifruit extract. IgE immunoblot was performed using a pool of kiwifruit/birch pollen allergic patients' sera (lanes 2 and 6). Residual IgE binding of serum pool after pretreatment with either 100 μg/mL rBet v 1.0101 (A, lane 3 and B, lane 7) or 100 μg/mL rAct d 8 (A, lane 4) and 100 μg/mL rAct c 8 (B, lane 8), respectively (N, non-allergic subjects; B, buffer control. IgE binding to natural Act d 8 and Act c 8 is indicated by arrows).

pollen allergic patients' sera (patients 2, 3, 6–8; see also Table 3) and showed a distinct IgE reactive band at about 16 kDa. These sera were also tested positive with purified rAct d 8 and rAct c 8 in IgE ELISA (Table 3).

To verify the existence of a natural Bet v 1 homologous protein in green and gold kiwifruit, total protein extracts from green and gold kiwifruits were prepared. SDS-PAGE separation followed by Coomassie staining resulted in clearly distinguishable protein patterns (Fig. 6, lane 1 – green kiwifruit extract, and lane 5 – gold kiwifruit extract). Qualitative and quantitative differences of protein bands were visible, with the most prominent bands in the range of 17 and 43 kDa. Gold kiwifruit extract displayed more bands and a greater intensity of bands of >26 kDa as compared to green kiwifruit extract. In the green kiwifruit extract, bands of 11–43 kDa were clearly resolved, but poorly resolved in the higher range as compared to the gold kiwifruit extract.

In immunoblot inhibition experiments (Fig. 6), a serum pool of kiwifruit/birch pollen allergic patients' sera (n=5) was used. IgE reactivity of the serum pool to proteins ranged from 15 to 55 kDa present in the green and gold kiwifruit extract (Fig. 6A, lane 2 and Fig. 6B lane 6). Inhibition of IgE binding to a band at approximately 16 kDa in both extracts could be achieved by preincubation of the serum pool with either rBet v 1.0101 (Fig. 6A, lane 3 and Fig. 6B, lane 7) or rAct d 8 (Fig. 6A, lane 4) and rAct c 8 (Fig. 6B, lane 8).

3.7 Immuno-tissue prints

Immuno-tissue prints were performed using green and gold kiwifruit (Fig. 7). The results of the localization studies show that a Bet v 1-related protein was recognized in the peripheral pulp by a polyclonal anti-Bet v 1 antibody in green (Fig. 7A) and gold kiwifruit (Fig. 7B).

4 Discussion

In this study, we cloned, expressed, purified, and characterized Bet v 1-related proteins from green and gold kiwifruit. Expression of the recombinant proteins used in our study was performed in *E. coli*, as frequently described for Bet v 1-related proteins [22, 23]. Recombinant protein purification was performed according to standard purification protocols and the proteins were subsequently characterized (CD, N-terminal sequencing, MS).

About 95% of birch pollen allergic patients are sensitized to the major birch pollen allergen Bet v 1, and approximately 70% display allergic symptoms to plant-derived foods such as apple, peach, pear, hazelnut, celery, carrot, soybean, mung bean, and kiwifruit, because of their content of proteins homologous and cross-reactive to Bet v 1 [13].

Analysis of 16 individual clones of Act d 8 revealed differences at 21 of the 157 positions (13%) in the deduced amino acid sequence. Of these positions, 13 were variable in multiple clones, indicating that they did not represent PCR or cloning artifacts. The Act d 8 sequence chosen for further work shared 73% DNA sequence identity (70% protein sequence identity) with the chosen rAct c 8 (Act c 8.0101) from gold kiwifruit. In *A. chinensis*, six isoforms (some could be alleles) including Act c 8 (Act c 8.0101) were identified in EST libraries. They exhibited a similar range of sequence divergence to Act d 8, with one, Act c 8b, sharing 97% identity with Act d 8 (Table 1). This suggests that Act c 8b is likely to be the orthologue in *A. chinensis* of the Act d 8 in *A. deliciosa* described herein, although this would have to be confirmed by genetic mapping.

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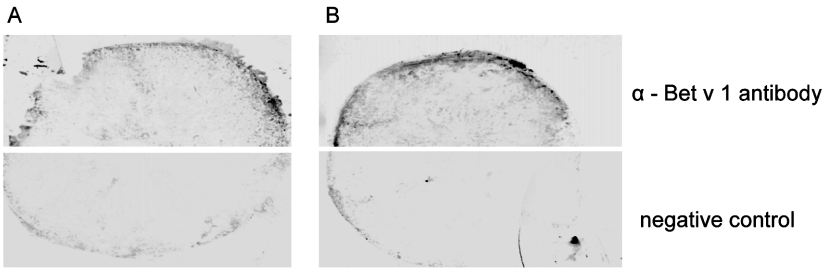

Figure 7. Immuno-tissue prints with polyclonal rabbit anti-Bet v 1 antibody cross-reactive to Act d 8 and Act c 8 in green (A) and gold (B) kiwifruit, respectively. Bound antibody was detected by AP-conjugated swine anti-rabbit antibody. Negative control was incubated with AP-conjugated swine anti-rabbit second step antibody only.

The level of sequence identity between rAct d 8 and rAct c 8 is slightly higher compared to the sequence identity of rAct d 8/rAct c 8 and other proteins from members of the Bet v 1 family (see Table 2).

Members of the Bet v 1 family usually consist of a number of isoforms. For instance, there are 20 Bet v 1 isoforms present in birch [24] and 18 (Mal d 1) in apple [25], so it is not surprising that multiple Bet v 1-like genes are present in kiwifruit. It is highly likely that the individual isoforms possess differential IgE-binding capacity as suggested by the restricted number of sera tested *in vitro*.

There are two theories on the origins of *A. deliciosa*. These are that, as a hexaploid, *A. deliciosa* either resulted from polyploidization of *A. chinensis* (diploid), or that *A. deliciosa* is an allopolyploid, with *A. chinensis* as one of the donor genomes [26]. Therefore, it can be concluded that *A. chinensis* (golden kiwifruit) and *A. deliciosa* (green kiwifruit) are closely related and share similar isoforms of Bet v 1-like proteins.

Allergic symptoms elicited by a member of the Bet v 1 family are usually confined to the oral mucosa or angioedema of the lips. Such mild symptoms have been described in Bet v 1-mediated fruit allergy such as to apple [27] and cherry [28] or Bet v 1-mediated hazelnut allergy [29]. These findings can be explained by heat lability and low resistance to digestion of the Bet v 1 homologous proteins [30].

Furthermore, they are easily degraded during extraction from their natural source or upon storage, so great care has to be taken when producing fruit extracts including Bet v 1 homologous proteins [31]. Moreover, the low abundance of some Bet v 1-related proteins in fruits like Mal d 1 from apple or Pru p 1 from peach has to be taken into account when purifying natural Bet v 1 homologous allergens [32]. Indeed, comparisons of Act c 8 and Act d 8 transcript levels with the very abundant actinidin (Act d 1) [33] and kiwellin (Act d 5) [9] allergen transcripts show that the kiwifruit Bet v 1-like genes are expressed at relatively low levels in fruit tissues. Due to these characteristics, purification of a natural Bet v 1-related protein from kiwifruit is challenging and allergens of this family might be missing in natural protein extracts prepared by conventional procedures. In particular, the endogenous protease activity of kiwifruit may cause rapid allergen degradation following tissue disruption. Even when extraction procedures preventing proteolytic degradation of allergens were applied, as in the present study, natural Act d 8 and Act c 8 were barely detectable in the total protein extracts (Fig. 3A, lane 1 and Fig. 3B, lane 5). Therefore, the expression of rAct d 8 and rAct c 8 as a recombinant non-fusion protein in *E. coli* is the method of choice. Secondary structures of rAct d 8 and rAct c 8 were highly similar to those of rBet v 1 as determined by CD spectroscopy.

Cross-reactivity between kiwifruit and birch pollen has been frequently described [14–16]. The allergenic components responsible for cross-reactions between kiwifruit and birch pollen were identified at 10–12 [16], 14, 22, 24, 38 and 41 kDa [15]. Our study demonstrated that birch pollen allergic patients with confirmed kiwifruit allergy recognized recombinant green and gold kiwifruit Bet v 1-related protein in both ELISA and immunoblot. In ELISA, rAct d 8 and rAct c 8 were recognized by 100% and 60% of the kiwifruit/birch pollen allergic patients, respectively. These findings indicate clinically relevant cross-reactivity between Bet v 1 and the homologous allergens from kiwifruit Act d 8 and Act c 8, as described for many other members of the Bet v 1 family in plant-derived foods [34, 35]. However, the primary sensitizing agent remains to be established, as well as the range of cross-reactivity since two of the patients did not display IgE binding to Bet v 1 but to both of the kiwifruit Bet v 1-related proteins.

The successful production of natural fruit extracts enabled the confirmation of Bet v 1-related proteins in green and gold kiwifruit. We identified a cross-reactive 17-kDa protein in green and gold kiwifruit extracts by immunoblot inhibition experiments using kiwifruit/birch pollen allergic patients' sera. IgE binding to natural Act d 8 and Act c 8 was inhibited by addition of recombinant Bet v 1.0101, and rAct c 8 and rAct d 8, respectively.

In ELISA inhibition assays, a similar inhibition pattern was observed. When rBet v 1.0101 was coated and kiwifruit/birch pollen allergic patients' sera were pretreated with either rAct d 8 or rAct c 8, only 40% inhibition was observed (data not shown), suggesting either that rAct d 8 and rAct c 8 lack some epitopes present on the rBet v 1.0101 molecule, or that more than one isoform of the kiwifruit allergens are responsible for cross-reactivity. The presence of more than one isoform can be assumed

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from the DNA sequence data. Tissue prints of green and gold kiwifruit indicate the existence of a Bet v 1-homologous protein in green and gold kiwifruit, with a higher abundance in the fruit skin than in the pulp.

As described previously, the diagnosis of kiwifruit allergy is challenging. Evidence of IgE-binding proteins can vary according to extraction protocols and such extracts may even lack some allergens, a fact that is relevant for reliable diagnosis. In several studies, patients reported symptoms when eating kiwifruit, although their IgE test results to kiwifruit extract were negative [36, 37]. These findings indicate the lack of allergenic activity caused by inherent enzyme or degradation during extraction process. Commercially used specific IgE tests for the diagnosis of birch pollen-related food allergies are based on protein extracts and frequently fail to detect specific IgE [31]. The identification and characterization of a Bet v 1-homologous allergen in green and gold kiwifruit could contribute to the application of component-resolved diagnosis of kiwifruit allergy or to the improvement of kiwifruit extracts for *in vitro* diagnostic purposes by spiking with the recombinant allergen. Such diagnostic applications may help to improve the medical management of allergic patients and reduce the burden of unnecessary exclusion diets for the allergic patient.

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The authors have declared no conflict of interest.

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