

ORIGINAL PAPER

Immunoglobulin E recognition patterns to purified Kiwifruit (*Actinidia deliciosa*) allergens in patients sensitized to Kiwi with different clinical symptoms

A. Palacin*, J. Rodríguez†, C. Blanco‡, G. Lopez-Torres*, R. Sánchez-Monge*, J. Varela§, M. A. Jiménez†, J. Cumplido†, T. Carrillo‡, J. F. Crespo† and G. Salcedo*

*Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Madrid, Spain, †Servicio de Alergia, Hospital Universitario Doce de Octubre, Madrid, Spain, ‡Sección de Alergia, Hospital Universitario Dr. Negrín, Las Palmas de Gran Canaria, Spain and §Servicio de Química de Proteínas, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain

Clinical and Experimental Allergy

Summary

Background Green kiwifruit allergy is on the rise. However, no surveys testing purified major kiwi allergens have been carried out in a large population, including both kiwi-sensitized [skin prick test (SPT)-positive] and truly kiwi-allergic patients.

Objective To isolate major kiwifruit allergens, and to explore their relevance by *in vitro* and *in vivo* methods in a large kiwi-sensitized and -allergic population.

Methods A large group ($n = 92$) of kiwi-sensitized patients with different clinical symptoms were selected, and double-blind, placebo-controlled, food challenges to kiwi were performed in 52 of them. The three major IgE-binding proteins from kiwifruit extracts were isolated and characterized by N-terminal amino acid sequencing and molecular size and glycosylation analysis. The allergenic potency of the three kiwi allergens, and of avocado Pers a 1 as a model allergen associated with the latex-fruit syndrome, was tested by specific IgE quantitation, immunodetection assays and SPTs.

Results The isolated kiwifruit allergens were identified as actinidin Act d 1, glycosylated thaumatin-like Act d 2 and a novel 40 kDa glycoprotein designated as Act d 3.02. Specific IgE to each of the three allergens was found in over 60% of sera from kiwi-sensitized patients, and Act d 1 and Act d 2 induced positive SPT responses in over 50% of the tested patients. A significant link between IgE levels to Act d 1 and Act d 3 and anaphylaxis was uncovered. Avocado Pers a 1 showed an *in vitro* sensitization prevalence of around 45%, but a low *in vivo* reactivity.

Conclusion Act d 1, Act d 2 and Act d 3 are major allergens in the population studied. Severe symptoms after kiwi ingestion are associated with high IgE levels to Act d 1 and Act d 3.

Keywords *Actinidia deliciosa*, cross-reactive carbohydrate determinant, kiwi allergy, latex-fruit syndrome, purified allergen, specific IgE level

Submitted 22 August 2007; revised 13 November 2007; accepted 2 December 2007

Correspondence:

Gabriel Salcedo, Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain.
E-mail: gabriel.salcedo@upm.es

Introduction

IgE-mediated reactions to fruits and vegetables are among the most frequent food allergies in adults [1]. Particularly, allergy to green kiwifruit (*Actinidia deliciosa*), which was first documented in the early 1980s [2], has been reported increasingly in the recent years [3, 4]. Moreover, a closely related species, gold kiwifruit (*Actinidia chinensis*), became available in the international market in 1999 and

shares IgE cross-reactivity and common allergens with green kiwifruit [5, 6]. Although allergy to kiwifruit is commonly associated with mild and local symptoms (mainly oral allergy syndrome) and with hypersensitivity to pollens, severe anaphylactic reactions are rather common [3, 7–9]. Additionally, kiwi allergy also has to be considered in relationship with the latex-fruit syndrome, together with sensitization to avocado, chestnut and banana, which are the main plant foods linked to latex allergy

[10, 11]. In spite of this extensive background on kiwi allergy, its objective immunological evaluation is limited by the low predictive value of the commercial tools used currently, both *in vitro* (i.e. UniCAP System, Phadia, Uppsala, Sweden) and *in vivo* [skin prick test (SPT)] [3, 4, 9].

Several putative kiwifruit allergens have been detected in recent years. However, only two of them have been studied sufficiently in different groups of kiwi-allergic patients until now. Act d 1 (originally Act c 1) corresponds to the 30 kDa thiol-protease actinidin, which is well established as a major kiwi allergen [5, 8, 9, 12], although recently reported data point to its lack of *in vitro* reactivity in the case of the kiwi-sensitized population from the United Kingdom [13]. Act d 2 is a 24 kDa thaumatin-like protein, whose sensitization prevalence is still controversial [8, 9, 14]. Besides, N-terminal amino acid sequences of putative relevant allergens, namely an 11 kDa cystatin (Act d 4) [5], a 28 kDa kiwellin (Act d 5) [12, 15], a 43 kDa [16] and a 45 kDa (Act d 3; accession number P85063) protein, have been reported, but their actual importance in kiwi sensitization is not fully clarified. On the other hand, none of these allergens appears to be primarily responsible for the latex-kiwi cross-sensitization described in the context of the latex-fruit syndrome [10, 11]. Class I chitinases with an N-terminal hevein-like domain and latex hevein have been identified as the major cross-reactive components involved in this syndrome, regarding avocado, chestnut and banana [11, 17]. Despite the detection of a putative homologous chitinase from gold kiwifruit with IgE-binding capacity [5], as well as the presence in green kiwifruit of a 41 kDa band whose recognition by IgE of sera from patients with the latex-fruit syndrome is inhibited by the avocado chitinase allergen Pers a 1 [18], the role of class I chitinases as kiwifruit allergens is currently unknown.

The aim of this study was to search for the major IgE-binding proteins in a large group of Spanish patients from two different geographical areas, including kiwi-sensitized subjects, as well as clinical reactors with a systemic anaphylaxis or a positive double-blind, placebo-controlled, food challenge (DBPCFC) on kiwifruit ingestion. The major kiwi allergens, Act d 1 and Act d 2, as well as a new 40 kDa allergen, designated as Act d 3 according to the IUIS Allergen Nomenclature Sub-Committee, together with avocado class I chitinase Pers a 1, were isolated and tested by *in vitro* and *in vivo* assays.

Materials and methods

Patients and sera

Ninety-two kiwi-sensitized patients were selected at the Servicio de Alergia, Hospital Universitario Doce de Octubre (Madrid; $n=62$), and at the Servicio de Alergia, Hospital Universitario de Gran Canaria Dr. Negrin (Las Palmas de Gran Canaria, Spain; $n=30$). Selection criteria were a

convincing clinical history of allergic reactions after green kiwifruit ingestion and a positive prick to prick test to the fresh fruit. All patients from the Hospital Universitario Doce de Octubre (Madrid), except 10 of them showing systemic anaphylaxis on kiwi ingestion (i.e., an adverse reaction involving at least three target organs or with demonstrated vascular collapse), were further analysed by DBPCFC with kiwifruit. Demographic and clinical data of the studied patients were as follows: gender (75 females/17 males); age (mean 33 years; range 6–55); number of patients mono-sensitized to kiwi, 30; and number of patients sensitized to latex, 19. The diagnosis of natural rubber latex sensitization was made by a convincing clinical history of immediate reactivity on latex exposure, as well as a positive SPT response to commercial latex extract (ALK-Abelló, Madrid, Spain) and specific IgE determination to latex (>0.35 kU/L; CAP system FEIA, Phadia, Uppsala, Sweden).

Individual sera from all 92 patients were assayed for specific IgE determination. Additionally, a serum pool ($n=92$) and 26 individual sera – randomly selected from the whole group – were used in IgE immunodetection assays.

A subgroup of 25 patients – those who agreed to undergo additional *in vivo* tests in a second study phase – were subjected to an SPT with the purified allergens.

Written informed consent was obtained from all patients or from their parents. The study was approved by the Ethical Committees of both hospitals.

Double-blind, placebo-controlled, food challenge

Actual clinical reactivity to kiwi was first evaluated by an open food challenge.

Subjects showing a positive reaction on open provocation were subsequently challenged in a double-blind fashion [19].

A piece of fresh green kiwifruit (100 g) was masked in a vehicle containing a mix of orange and pineapple juices, sugar, wheat meal and liquid colouring (McCormick, Hunt Valley, MD, USA). This amount was divided into five doses administered in increasing quantities (4.5, 8.0, 12.5, 25.0 and 50 g), masked as described above. The total volume was 400 mL, for both the placebo and the verum day. The interval between doses was the latency period reported by the patient (time between ingestion and onset of first symptoms plus 15 min). A panel of six healthy volunteers could not detect the taste, flavour or texture when the kiwifruit recipe was tested for proper blinding.

Randomization and preparation of the challenges were performed in the Allergy Laboratory (Hospital Universitario Doce de Octubre). Subjects were first challenged randomly with either food or placebo (vehicle), and the interval before the second part of the DBPCFC was at least 24 h. Confirmation by DBPCFC was accepted if the subjects had symptoms after provocation with the active substance and no symptoms after the placebo challenge.

An open feeding with a whole piece fresh green kiwi (100 g) followed all negative blinded challenges. An oral challenge was not performed when a patient had a convincing history of a recent severe anaphylaxis to kiwi, defined as objective findings (marked laryngeal oedema, significant wheezing or hypotension), which developed immediately after the isolated ingestion of this fruit and required emergency management within the last year.

Preparation of kiwi extract

Fruits of green kiwi (*A. deliciosa* cv. Hayward) were peeled, cut into small pieces and freeze dried. The dry pulps were defatted with cold acetone [$2 \times 1:5$ (w/v), 1 h, 4°C] and then with ethanol:ether [$1:3$ (v/v); $3 \times 1:5$ (w/v), 1 h, 4°C]. After drying, the defatted pulps were extracted with phosphate-buffered saline (PBS) buffer [0.1 M sodium phosphate, pH 7.0 and 0.15 M NaCl; $1 \times 1:5$ (w/v), 1 h, 4°C]. After centrifugation ($12\,100 \times g$, 30 min, 4°C), the supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation), and then centrifuged under the same conditions. The precipitate was dissolved in PBS buffer, dialysed (cut-off point 3.5 kDa) against H_2O and freeze dried.

The protein concentration was quantified according to the method of Bradford [20].

Isolation and characterization of allergens

The kiwi extract was fractionated by cation-exchange chromatography on a Mono S HR 5/5 column (Amersham Bioscience, Uppsala, Sweden). The column was equilibrated with 20 mM formic acid (pH 4.0) buffer, eluted with a linear salt gradient to 0.5 M NaCl in the same buffer ($0\text{--}0.5\text{ M}$ in 140 min; 0.5 mL/min) and fractions were dialysed (cut-off point 3.5 kDa) against H_2O and freeze dried. Fractions containing the different kiwi allergens were identified by SDS-PAGE fractionation and immunodetection with the serum pool from kiwi-sensitized patients or anti-thaumatococcus antibodies. Fractions eluting at 0.00 (non-retained material), 0.11 and 0.41 M contained single SDS-PAGE bands that corresponded to Act d 1, Act d 2 and Act d 3.02, respectively.

Pers a 1, the major allergen and class I chitinase from avocado fruit, was purified by a method described previously [21]. Isolated allergens were quantified using a commercial bicinchoninic acid test (Pierce, Cheshire, UK).

SDS-PAGE was performed on Bio-Rad Miniprotein III System (Bio-Rad Laboratories, Hercules, CA, USA) gels (15% polyacrylamide) under reducing conditions, according to Laemmli [22]. N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis were carried out using standard methods with an Applied Biosystems Procise 494A gas-phase sequencer (Foster City, CA, USA) and a Biflex-III spectrometer with delayed extraction (Bruker-Franzen Analytik, Bremen, Germany).

Immunodetection and immunoblot inhibition assays

Samples (15 μg of protein of crude extract from kiwifruit and 3 μg of purified allergens) were fractionated by SDS-PAGE and then electrotransferred (transfer buffer: 50 mM Tris, 50 mM boric acid, pH 8.3) onto polyvinylidene difluoride membranes. After blocking with 5% bovine serum albumin (BSA) (w/v) in PBS buffer, membranes were incubated overnight with a pool of sera from all 92 kiwi-sensitive patients ($1:5$ dilution), washed and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid ($1:3000$ dilution, 1 h) [23]. After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody ($1:5000$ dilution, 1 h; Dako A/S, Glostrup, Denmark) was added and detection of IgE-binding components was carried out by enhanced chemiluminescence following the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK). A serum pool from patients ($n=10$) with dustmite, but not plant food or pollen allergy, was tested as a negative control.

Immunoblot inhibition assays were performed by the same method, except that the appropriate serum was pre-incubated with 9 $\mu\text{g/mL}$ of each inhibitor (purified Act d 1, Act d 2 or BSA as a negative control).

Additionally, blocked membranes were immunodetected with rabbit antibodies to chestnut thaumatococcus (1:1000 dilution; kindly provided by Dr. L. Gomez, ETSIM, UPM, Madrid, Spain) [24] or with an anti-complex asparagine-linked glycans serum (1:40 000 dilution; kindly provided by Dr M. J. Chrispeels, University of California, USA) [18]. Membranes were then treated with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution; Sigma, St Louis, MO, USA) and revealed with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution.

Specific immunoglobulin E determination

Specific IgE levels to purified Act d 1, Act d 2, Act d 3 and Pers a 1 were determined in the 92 individual sera from kiwi-sensitized patients by an ELISA method, as described previously in Díaz-Perales et al. [21]. Purified allergens were tested at 3 $\mu\text{g/mL}$ as a solid phase, and each individual serum at a $1:6$ dilution. BSA [3 $\mu\text{g/mL}$; $n=24$; mean optical density (OD) $\pm$ SD = 0.064 ± 0.044] as a solid phase and a serum pool from 10 patients with dust mite but not plant food or pollen allergy [$n=12$; mean (OD) $\pm$ SD to Pers a 1 = 0.061 ± 0.043 , to Act d 1 = 0.056 ± 0.051 , to Act d 2 = 0.071 ± 0.060 and to Act d 3 = 0.070 ± 0.066] were tested as negative controls. Specific IgE levels were considered to be positive when >0.27 OD units (the highest value of mean $\pm 3 \times$ SD of the above-described determinations; $0.070 \pm 3 \times 0.066 = 0.268$, corresponding to Act d 3 using negative control serum pool).

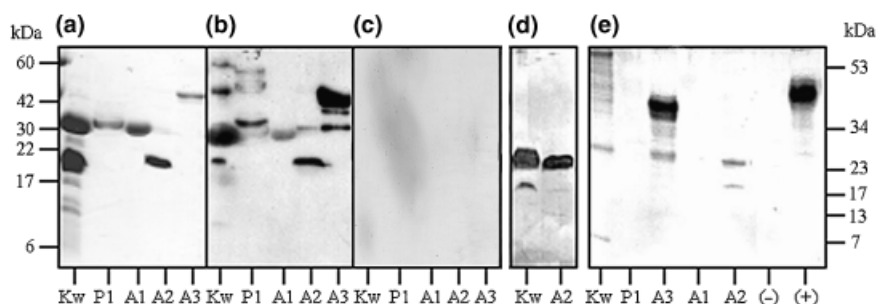


Fig. 1. Protein staining (a) and immunodetection with IgE of a serum pool from kiwi-sensitized (b), or from dustmite but not plant food- or pollen-allergic patients (c), or rabbit polyclonal antibodies to chestnut thaumatin (d) or plant complex glycans (e) of the following samples separated by SDS-PAGE: kiwifruit extract (Kw) and purified allergens Pers a 1 (P1), Act d 1 (A1), Act d 2 (A2) and Act d 3 (A3). Bovine serum albumin (–) and horseradish peroxidase (+) were used in (e) as negative and positive control, respectively.

Skin prick tests

SPTs with purified allergens [Act d 1, Act d 2 and Pers a 1; 50 µg/mL in a 1:1 (v/v) PBS buffer/glycerol solution] were carried out by standard procedures. Histamine dihydrochloride (10 mg/mL) and a PBS buffer/glycerol solution served as positive and negative controls, respectively. A mean weal diameter of 3 mm or larger (15 min after puncture) compared with that produced by the negative control was considered to be a positive response. Five healthy subjects were tested with the purified allergens as a control group, obtaining negative responses in all cases. Duplicates of each sample were tested.

Statistics

For statistical purposes, patients were stratified according to (i) the severity of symptoms reported on kiwi ingestion (systemic anaphylaxis vs. other reactions), (ii) the result of DBPCFC to kiwi (positive vs. negative challenge tests); and (iii) associated sensitizations (monosensitized to kiwi vs. kiwi-latex vs. other sensitization patterns). All statistical tests were two sided, and a level of significance <5% ($P=0.05$) was considered to be significant. For each individual kiwi allergen, the median of specific IgE (kU_A/L) with a 95% confidence interval was calculated. The Wilcoxon rank-sum test was used to compare the median levels of specific IgE with different kiwi allergens regarding the severity of symptoms, latex sensitization and concomitant sensitizations to other plant-food allergens.

Results

Detection of immunoglobulin E-binding proteins in the kiwifruit extract

Protein staining of the kiwifruit extract separated by SDS-PAGE showed two major bands of approximately 20 and 30 kDa (Fig. 1a). Both bands, together with two additional ones of around 40 and 60 kDa, comprised the main IgE-binding kiwifruit proteins, as found by immunodetection

using the serum pool from 92 kiwi-sensitized patients (Fig. 1b). The relevance of these proteins as potential major kiwi allergens was further confirmed by immunodetection with 26 individual sera (Fig. 2).

Isolation and characterization of Act d 1, Act d 2 and Act d 3

The 20, 30 and 40 kDa putative allergens were isolated by cation-exchange chromatography (not shown). The purity of the isolated allergens was checked by protein staining after SDS-PAGE (Fig. 1a) and N-terminal amino acid sequencing, and its IgE-binding capacity was verified by immunodetection with the serum pool (Fig. 1b) and individual sera (Fig. 2) from kiwi-sensitized patients.

The 30 kDa component exhibited a single band of the expected molecular size (Fig. 1a), was not glycosylated (Fig. 1d) and yielded the same N-terminal amino acid sequence (LPSYV) as kiwi actinidin [12]. Therefore, it could be identified as Act d 1 allergen. Act d 1 was clearly recognized by the serum pool (Fig. 1b) and 23 out of 26 (88%) individual sera (Fig. 2) from kiwi-sensitized patients.

The molecular size, N-terminal amino acid sequence (ATFNI) [14] and strong reaction with anti-chestnut thaumatin antibodies (Fig. 1c) led to define the isolated 20 kDa protein as Act d 2 (kiwi thaumatin). The purified allergen presented a major band of around 20 kDa and a minor form of 30 kDa, the latter being clearly detected only when higher amounts of sample were analysed (Fig. 3a). The N-terminal amino acid sequence of the 30 kDa form (ATFNI) was identical to that of Act d 2. Furthermore, Act d 2, but not Act d 1, inhibited its IgE binding in immunoblot inhibition assays using an individual serum (no. 8 in Fig. 2), which mainly recognized the 30 kDa form (Fig. 3b). Interestingly, both Act d 2 forms, corresponding to the 20 and 30 kDa bands, were glycosylated, as indicated by their reaction with anti-complex asparagine-linked glycans antibodies (Fig. 3a). Moreover, considering the relative protein stain band intensities and the response to such

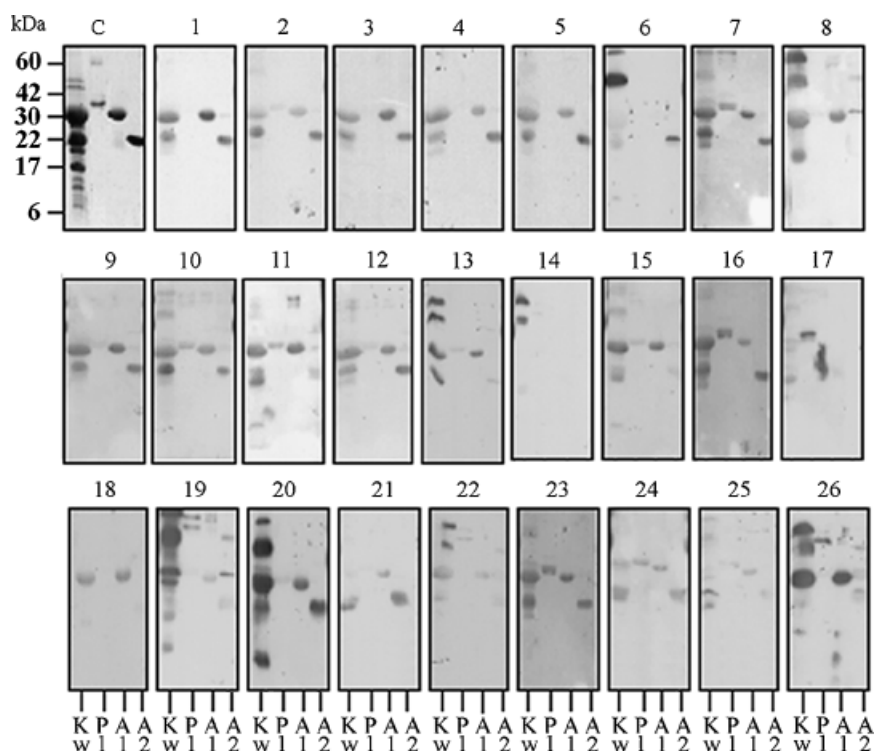


Fig. 2. IgE immunodetection with 26 individual sera (1–26) from kiwi-sensitized patients of the following samples separated by SDS-PAGE: kiwifruit extract (Kw) and purified allergens Pers a 1 (P1), Act d 1 (A1) and Act d 2 (A2). Protein staining of the same samples is shown in (C). Individual sera are from patients with anaphylaxis (no. 1–6, 21 and 24–26), positive (no. 7–13) or negative (no. 14–20) double-blind, placebo-controlled, food challenge (DBPCFC) to kiwifruit, or a positive skin prick test response to kiwifruit extract, but without DBPCFC still performed (no. 23).

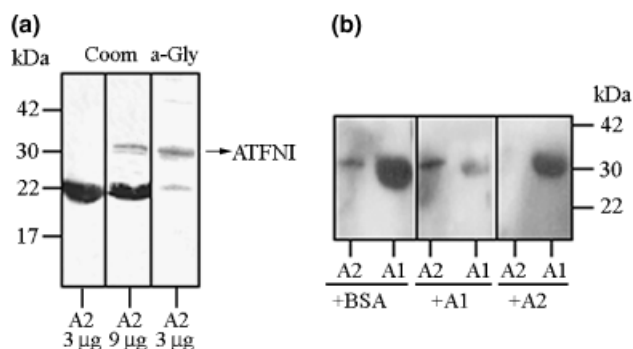


Fig. 3. (a) Protein staining (Coom; 3 and 9 µg of sample) and immuno-detection with polyclonal antibodies to plant complex glycans (a-Gly) of purified allergen Act d 2 (A2). The N-terminal sequence of the 30 kDa form is indicated. (b) IgE immunodetection with individual serum 8 (see Fig. 2) of purified allergens Act d 1 (A1) and Act d 2 (A2). The serum was pre-incubated with each allergen (+A1 or +A2) or bovine serum albumin (BSA) (+BSA) as negative control.

antibodies, it seemed that the 30 kDa band was more heavily glycosylated than the 20 kDa band. Act d 2 was recognized by 23 out of 26 (88%) individual sera from kiwi-sensitized patients (Fig. 2).

The third isolated kiwifruit allergen corresponded to the approximately 40 kDa IgE-binding band (Fig. 1). The purified protein displayed a single band of the expected

apparent molecular size in SDS-PAGE, but a major peak at 40 007 Da in MALDI analysis (not shown). Its first 15 N-terminal amino acid residues fully matched those of Act d 3.01 (accession no. P85063), but differences were found between both sequences in positions 16–25 (Fig. 4). Thus, the purified allergen should be designated as Act d 3.02 (accession no. P84992). Significant sequence identity between Act d 3.02 and a hypothetical hydrolase (accession no. O24329-EMBL; predicted molecular weight 40 006 kDa; positions 21–45 of its deduced amino acid sequence; see Fig. 4) from castor bean (*Ricinus communis*) was also found on searching the sequence database. Act d 3.02 seemed to be highly glycosylated (Fig. 1d), and both human IgE from kiwi-sensitized patients (Fig. 1b) and anti-complex glycan antibodies (Fig. 1d) detected, besides the 40 kDa around band, additional components of lower apparent molecular sizes, probably corresponding to degradation products. Unfortunately, the low yield of purified Act d 3.02 did not allow to include it either in immunodetection assays using individual sera from kiwi-sensitized patients or in SPTs.

Pers a 1, the class I chitinase and major avocado fruit allergen involved in the latex-fruit syndrome, was also tested for reactivity. It was recognized by the serum pool (Fig. 1b), as well as by 10 out of 26 (38%) individual sera from kiwi-sensitized patients.

Allergen/protein	N-terminal amino acid sequence																							% id		
	1	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200					
45 kDa allergen (Actd 3.02)	F	T	D	G	L	M	K	N	G	N	F	E	L	A	P	K	P	E	D	M	X	G	T	V	R	-
Act d3.01	F	T	D	G	L	M	K	N	G	N	F	E	L	A	P	V	-	E	S	L	K	A	-	V	K	68
<i>R. communis</i> hypothetical hydrolase	I	R	D	G	L	V	K	N	G	N	F	E	L	A	P	K	P	S	D	M	K	G	T	Q	V	72

Fig. 4. Alignment of the N-terminal amino acid sequences of the purified 40 kDa allergen (Act d 3.02), Act d 3.01 (accession no. P85063) and a hypothetical hydrolase from *Ricinus communis* (accession no. O24329; positions 21–45). Sequence identities to Act d 3.02 (% id) are indicated on the right side.

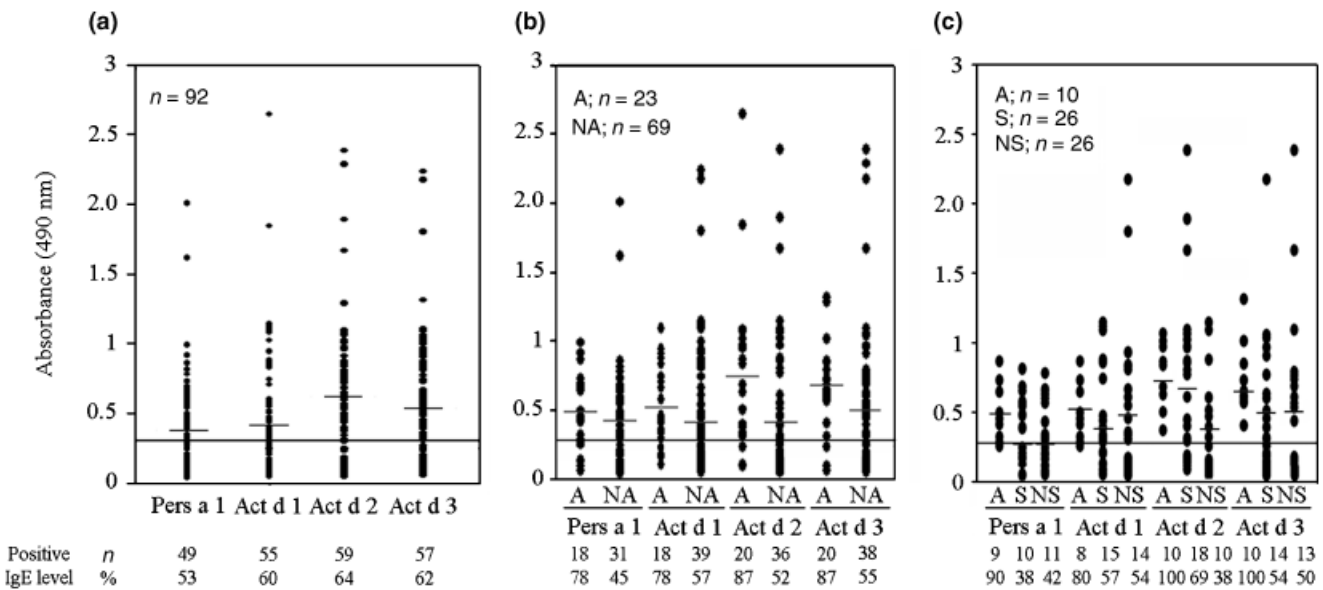


Fig. 5. Specific IgE levels in optical density units to purified kiwifruit allergens Act d 1, Act d 2 and Act d 3.01, and avocado Pers a 1. Values ≥ 0.27 U were considered to be positive (see 'Materials and methods'). (a) Individual sera from 92 kiwi-sensitized patients. (b) Individual sera from 92 kiwi-sensitized patients grouped according to symptoms: anaphylaxis (A; $n = 23$) and non-anaphylaxis (NA; $n = 69$). (c) Individual sera from 62 kiwi-sensitized patients grouped according to a positive (S; $n = 26$) or a negative (NS; $n = 26$) double-blind, placebo-controlled food challenge to kiwi, and anaphylaxis (A; $n = 10$). Short horizontal lines (–) indicate the mean values (absorbance units) for each solid phase and serum pool.

Specific immunoglobulin E levels to purified kiwifruit allergens and Pers a 1 in individual sera from kiwi-sensitized patients showing different clinical symptoms

ELISA assays using the different purified allergens as a solid phase led to determine the specific IgE levels to each allergen in the individual sera from the 92 kiwi-sensitized selected patients. The results are summarized in Fig. 5a. Positive IgE levels to Act d 1, Act d 2 or Act d 3 were found in around 60% of the sera assayed, and to Pers a 1 in 53% of the samples.

The group of patients ($n = 23$) with systemic anaphylaxis exhibited specific IgE to the different isolated allergens tested more frequently (78–87%) than those patients ($n = 69$) with other symptoms (45–57%) ($P = 0.015$, 0.022, 0.015 and 0.007 for Act d 1, Act d 2, Act d 3 and Pers a 1, respectively; Fig. 5b). Moreover, significant statistical differences regarding specific IgE levels to Act d 1 ($P = 0.023$) and Act d 3 ($P = 0.036$), but not to Act d 2 and Pers a 1, were found between the group of patients with or without systemic anaphylaxis. A further approach to cut up groups of patients with different clinical

symptoms was carried out with kiwi-sensitized subjects from the Madrid area ($n = 62$). Besides 10 patients with systemic anaphylaxis, two subgroups ($n = 26$ each) were defined based on positive or negative DBPCFC to kiwi (Fig. 5c). Once again, patients with anaphylaxis showed higher sensitization rates (100–80%) to the three kiwifruit allergens or to Pers a 1 than the kiwi-sensitive subjects included in the other two subgroups (69–38%). However, no significant differences in the IgE level or the prevalence of sensitization to any of the four allergens tested were observed between the subgroups of patients with positive or negative DBPCFC to kiwi.

Additionally, no statistical differences in the IgE level to Pers a 1 or any of the kiwifruit allergens were detected either between patients with or without latex sensitization or between subjects monosensitized to kiwi and those also sensitized to other plant foods. In contrast, sensitization rates (in a subgroup of patients tested to latex, $n = 60$) to each of the four purified allergens were higher in patients with latex-kiwi ($n = 19$; 63–77%) than in the group lacking latex sensitization ($n = 41$; 49–63%).

Table 1. Skin prick test responses to purified allergens Act d 1, Act d 2 and Pers a 1 in 25 kiwi-sensitized patients

	Positive SPT responses		
	Act d 1	Act d 2	Pers a 1
Total <i>n</i> = 25	18	13	3
%	72	52	12
Anaphylaxis, <i>n</i> = 9	7	5	1
Subgroup (%)	78	55	11
Other symptoms, <i>n</i> = 16	11	8	2
Subgroup (%)	69	50	12

SPT, skin prick test.

In vivo reactivity of Act d 1, Act d 2 and Pers a 1 in kiwi-sensitized patients

SPTs with the isolated allergens Act d 1, Act d 2 and Pers a 1 were performed in 25 kiwi-sensitized patients (Table 1). Act d 1 and Act d 2 elicited positive responses in over 70% and 50% of the tested patients, respectively, whereas Pers a 1 induced skin reactions in only three (12%) of them. The percentage of positive results was similar in patients with anaphylaxis or with other symptoms for the three allergens assayed.

Discussion

The aim of this work was to evaluate the relevance of three kiwifruit protein allergens, which are among the strongest IgE-binding components included in salt extracts from this plant food. To achieve this objective, the three main kiwifruit allergens were isolated, and a large population of 92 kiwi-sensitized patients was recruited in two hospitals located in different Spanish regions. A substantial proportion (25%) of the selected population developed severe symptoms (systemic anaphylaxis) after kiwifruit ingestion. Additionally, 26 out of 52 (50%) patients from Madrid showed a positive DBPCFC to kiwi, a proportion fully in agreement with that reported previously for kiwi-sensitized patients [4, 9].

The most abundant and strongest IgE-binding component of the kiwifruit extract is a 30 kDa protein, which was identified as the thiol protease actinidin and Act d 1 allergen [12]. The high proportion of individual sera with specific IgE to Act d 1 (60% in ELISA and 88% in immunodetection assays), and patients with positive SPT responses (72%) to the isolated allergen, support its designation as a major allergen in the population studied here. Furthermore, the group of patients with anaphylaxis showed a significantly higher level of IgE, as well as sensitization rates, to Act d 1 than the patients with other symptoms, thus suggesting its potential clinical relevance. Other authors have also emphasized the high recognition prevalence of this allergen in kiwi-sensitized patients

from different European areas [5, 8, 9, 12], but this scenario seems to be different in the United Kingdom, where Act d 1 is not recognized by any of 76 sera from kiwi-sensitized patients, thus not being a major allergen in this population [13].

A second isolated IgE-binding protein of 20 kDa, also abundant in the kiwifruit extract, corresponds to the thaumatin-like allergen Act d 2 [14]. *In vitro* (specific IgE detected in 64% and 88% of individual sera by ELISA and immunodetection assays, respectively) and *in vivo* (52% of positive SPT responses) reactivity point to Act d 2 as the other major allergen in the kiwi-sensitized population analysed here. These prevalence rates are in agreement with data reported previously by Gavrovic-Jankulovic et al. [14], who studied a small number (*n* = 7) of kiwi-allergic subjects, but are clearly in contrast with the lower proportion of Act d 2-sensitized patients reported by other authors (30–15%) [9, 25]. Interestingly, two Act d 2 forms, a major one of around 20 kDa and a minor one of approximately 30 kDa, have been detected. Both forms probably varied in their glycosylation patterns, but both harbouring complex asparagine-linked glycans. Glycosylation of Act d 2 has been suggested previously based on its ability to bind to concanavalin A, but only one 24 kDa form was described [14]. The frequency and type of glycosylation and the role of the attached glycans on IgE binding regarding members of the thaumatin-like allergen family are still controversial [26]. Nevertheless, the presence of cross-reactive carbohydrate determinants in Act d 2, together with the presence of homologous allergens in other foods and pollens, should be considered in the context of kiwifruit/plant food/pollen cross-reactivity.

A novel kiwifruit allergen, designated as Act d 3.02, has also been isolated. This strongly glycosylated 40 kDa protein is a genetic variant of Act d 3.01 [25], and is also homologous to hypothetical hydrolases from castor bean and other plant species. The high sequence identity with the putative *R. communis* protease suggests a functional role of Act d 3 in kiwifruits that could explain the presence in preparations of the purified allergen of possible self-degradation products retaining the IgE-binding potency. Act d 3.02 seems to be a minor component of kiwifruit extracts, but, in contrast, represents a major kiwi allergen based on its high specific IgE prevalence (62%) in sera from kiwi-sensitized patients. The potential clinical relevance of Act d 3.02 is further supported by the statistical correlation between IgE levels to this allergen and anaphylactic symptoms. All these data are in agreement with those observed previously for Act d 3.01, both *in vitro* (66% of sera with specific IgE) and *in vivo* (13 out of 15 patients with positive SPT responses) [25]. Moreover, Act d 3 could probably correspond to the 38 kDa protein recently described as the major kiwifruit allergen in the United Kingdom by Lucas et al. [13]. The complex glycans (cross-reactive carbohydrate determinants) attached to

Act d 3 can be a source of cross-reactivity between kiwifruit and other plant foods and pollens, whose actual clinical relevance remains to be explored.

Taking together the data presented here, besides the relationship found between anaphylaxis and specific IgE levels to Act d 1 and Act d 3, no clear-cut junction between sensitization to any of the three purified kiwifruit allergens and clinical symptoms has been found. Comparison of the groups of patients with positive and negative DBPCFC to kiwi (Fig. 5) clearly highlighted this fact. Previous works have also failed to establish a link between clinical symptoms and *in vitro* reactivity to any particular kiwi allergen [9].

A high rate of kiwi-sensitized subjects (25% of the patients studied here) is cosensitized to latex, suffering from the denominated latex-fruit syndrome [10]. This syndrome is also frequent in subjects with hypersensitivity to other fruits or nuts, mainly avocado, chestnut and banana [10]. The kiwifruit allergens involved in cross-reactivity with latex have not been identified convincingly, whereas fruit class I chitinase harbouring an N-terminal hevein-like domain, such as avocado Pers a 1, seems to be mainly responsible in the case of avocado, banana and chestnut [11]. A putative allergen homologous to Pers a 1 has been detected in gold kiwi (*A. chinensis*), but neither its sensitization prevalence in kiwi-sensitized patients nor its role in latex cosensitization has been clarified [5]. We have failed to isolate, or even to detect, such Pers a 1 homologous in green kiwi (R. Sanchez-Monge et al., unpublished data). Therefore, the avocado chitinase was selected as a model allergen implicated in the latex-fruit syndrome, and was included in the screening carried out in this work. Specific IgE to Pers a 1 has been found in 53% (49 out of 92) and 38% (10 out of 26) of the sera analysed by ELISA and IgE immunodetection assays, respectively. However, only 12% (three out of 25) of the tested patients showed a positive SPT response to Pers a 1, indicating a low *in vivo* reactivity. Additionally, a drastic difference in the sensitization rate to the avocado allergen has been found between those with or without latex cosensitization (91% vs. 30%), but no significant differences in the corresponding IgE levels have been detected. Moreover, both characteristics are also true regarding Act d 1, Act d 2 and Act d 3. Together, all these data suggest a potential role of class I chitinases as minor kiwi allergens, as well as the need for further research to define their relevance in kiwi-latex cross-reactivity fully.

Acknowledgements

This article was supported by Fondo de Investigacion Sanitaria, Ministerio de Salud y Consumo (grants PI050375 and RD07/0064/0003).

References

- 1 Woods RK, Abramson M, Bailey M, Walters EH. International prevalences of reported food allergies and intolerances. Comparisons arising from the European Community Respiratory Health Survey (ECRHS) 1991–1994. *Eur J Clin Nutr* 2001; 55:298–304.
- 2 Fine AJ. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, *Actinidia chinensis*). *J Allergy Clin Immunol* 1981; 68:235–7.
- 3 Lucas JSA, Lewis SA, Hourihane JO'B. Kiwi fruit allergy: a review. *Pediatr Allergy Immunol* 2003; 14:420–8.
- 4 Lucas JSA, Grimshaw KEC, Collins K, Warner JO, Hourihane JO'B. Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin Exp Allergy* 2004; 34:1115–21.
- 5 Bublin M, Mari A, Ebner C et al. IgE sensitization profiles toward green and gold kiwifruits differ among patients allergic to kiwifruit from 3 European countries. *J Allergy Clin Immunol* 2004; 114:1169–75.
- 6 Lucas JSA, Lewis SA, Trewin JB, Grimshaw KEC, Warner JO, Hourihane JO'B. Comparison of the allergenicity of *Actinidia deliciosa* (kiwi fruit) and *Actinidia chinensis* (gold kiwi). *Pediatr Allergy Immunol* 2005; 16:647–54.
- 7 Gall H, Kalveram KJ, Forck G, Sterry W. Kiwi fruit allergy: a new birch pollen-associated food allergy. *J Allergy Clin Immunol* 1994; 94:70–6.
- 8 Pastorello A, Pravettoni V, Ispano M et al. Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *J Allergy Clin Immunol* 1996; 98:601–10.
- 9 Aleman A, Sastre J, Quirce S et al. Allergy to kiwi: a double-blind, placebo-controlled food challenge study in patients from a birch-free area. *J Allergy Clin Immunol* 2004; 113:543–50.
- 10 Blanco C. Latex-fruit syndrome. *Curr Allergy Asthma Rep* 2003; 3:47–53.
- 11 Salcedo G, Diaz-Perales A, Sanchez-Monge R. The role of plant panallergens in sensitization to natural rubber latex. *Curr Opin Allergy Clin Immunol* 2001; 1:177–83.
- 12 Pastorello EA, Conti A, Pravettoni V et al. Identification of actinidin as the major allergen of kiwi fruit. *J Allergy Clin Immunol* 1998; 101:531–7.
- 13 Lucas JSA, Nieuwenhuizen NJ, Atkinson RG et al. Kiwifruit allergy: actinidin is not a major allergen in the United Kingdom. *Clin Exp Allergy* 2007; 37:1340–8.
- 14 Gavrovic-Jankulovic M, Cirkovic T, Vuckovic O et al. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J Allergy Clin Immunol* 2002; 110:805–10.
- 15 Tamburrini M, Cerasuolo I, Carratore V et al. Kiwellin, a novel protein from kiwi fruit. Purification, biochemical characterization and identification as an allergen. *Protein J* 2005; 24:423–9.
- 16 Moller M, Kayma M, Steinhart H, Paschke A. Isolation and characterization of a major allergen in kiwi fruit. *Z Lebensm Unters Forsch* 1997; 205:364–9.
- 17 Diaz-Perales A, Collada C, Blanco C et al. Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. *J Allergy Clin Immunol* 1998; 102:127–33.
- 18 Diaz-Perales A, Collada C, Blanco C et al. Cross-reactions in the latex-fruit syndrome: a relevant role of chitinases but not of

- complex asparagine-linked glycans. *J Allergy Clin Immunol* 1999; **104**:681–7.
- 19 Bock SA, Sampson HA, Atkins FM *et al.* Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* 1988; **82**:986–97.
- 20 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–54.
- 21 Díaz-Perales A, Blanco C, Sánchez-Monge R, Varela J, Carrillo T, Salcedo G. Analysis of avocado allergen (Pers a 1) IgE-binding peptides generated by simulated gastric fluid digestion. *J Allergy Clin Immunol* 2003; **112**:1002–7.
- 22 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**:680–5.
- 23 Sánchez-Madrid F, Morago G, Corbi AL, Carreira J. Monoclonal antibodies to three distinct epitopes on human IgE: their use for determination of allergen-specific IgE. *J Immunol Methods* 1984; **73**:367–78.
- 24 García-Casado C, Collada C, Allona I *et al.* Characterization of an apoplastic basic thaumatin-like protein from recalcitrant chestnut seeds. *Physiol Plant* 2000; **110**:172–80.
- 25 Merima B, Radauer C, Lebens A, Knulst A, Scheiner O, Breiteneder H. Purification and partial characterization of a 40 kDa allergen from kiwifruit (*Actinidia deliciosa*). XXV Congress Eur Acad Allergol Clin Immunol (Abstract Book) 2006; p 148, poster 505.
- 26 Breiteneder H. Thaumatin-like proteins – a new family of pollen and fruit allergens. *Allergy* 2004; **59**:479–81.