



Prevalence and peculiarities of IgE reactivity to kiwifruit pectin methylesterase and its inhibitor, Act d 7 and Act d 6, in subjects allergic to kiwifruit

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ABSTRACT

Kiwifruit is an important cause of allergy. Although several allergens have been identified in this food (www.allergome.org), data on the number and clinical relevance of kiwifruit allergenic proteins are still fragmentary. With the aim of providing a contribution to the description of the complete kiwifruit allergome, we have investigated the immunological features of pectin methylesterase (PME) and its inhibitor (PMEI) purified from the natural source. Specific IgE to PME (Act d 7) and PMEI (Act d 6) were recorded in patients with kiwifruit allergies by immunoblotting, multiplex microarray-based immunoassay and skin testing (ST). Compared to the results obtained by the microarray system, the percentage of subjects detected positive to Act d 6 was much higher in the immunoblotting. Conversely, a similar percentage of subjects positive to Act d 7 was estimated in both the in vitro tests. Few subjects compared to in vitro testing gave a positive response on ST. In the case of Act d 7 this could be ascribed to the glycan side chain recognized by cross-reactive carbohydrate determinants (CCD)-specific IgE. In fact, Act d 7 fell in the cluster of glycoallergens (including Api m 1, Cup a 1, Hor v 17, Ole e 1, Pla a 2) immobilized on the microarray and recognized by CCD-specific IgE. A comparative analysis of different kiwifruit allergens (Act d 1, 2, 5, 6, 7, 11) revealed that only Act d 7 fell in this cluster, whereas Act d 2, although reported to be glycosylated, fell outside, thus suggesting the lack of sugars bound to this molecule. The subsequent biochemical characterization confirmed that Act d 2 is not a glycoallergen. In conclusion, Act d 7 and 6 seem to be rarely involved in causing symptoms in kiwifruit allergic patients. Nevertheless they are useful to understand a single patient profile and the mechanism of protein allergenicity. In addition, in this study the use of a multiplex test system has provided more information than expected, thus contributing to both the immunological and biochemical characterization of the immobilized allergens.

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1. Introduction

Kiwifruit is reported as an important source of food allergy, sometimes associated with allergy to pollens (D'Avino et al., 2011; Pastorello et al., 1996; Rudeschko, Fahlbusch, Steurich, Schlenvoigt, & Jager, 1998; Voitenko et al., 1997), and characterized by a wide range of symptoms, including local oral mucosa reactions, laryngeal edema, urticaria, vomiting, asthma, cardiovascular collapse, and life-threatening anaphylaxis. Kiwifruit allergy has also been frequently associated with allergy to other foods including apple, avocado, banana, carrot, celery, hazelnut, and potato (Gall, Kalveram, Forck, & Sterry, 1994; Möller, Kayma, Vieluf, Paschke, & Steinhart, 1998; Möller et al., 1997; Vocks et al., 1993).

Following the analysis of green kiwifruit (*Actinidia deliciosa*) protein extracts, the presence of many IgE-binding components has been described by several authors. Data from immunoblotting experiments provided evidence of IgE-binding bands of several molecular masses, including 10–25 kDa (Voitenko et al., 1997); 12, 17, 24, and 28 kDa (Pastorello et al., 1996, 1998); 13, 22, 30, and 67 kDa (Möller et al., 1997); 15–94 kDa (Rudeschko et al., 1998); 11, 22, 26, 27, and 32 kDa (Bublin et al., 2004); 12, 24, 30, and 66 kDa (Aleman et al., 2004); and 20 and 28 kDa (Tuppo et al., 2008). Data concerning the number of allergenic proteins in kiwifruit extracts, and their clinical relevance, are still fragmentary although several allergenic proteins have been listed in the Allergome database (www.allergome.org), and most of them officially named by the WHO/IUIS subcommittee for allergen nomenclature (www.allergen.org). Kiwifruit allergens so far identified are distributed in several protein families and show different functional properties, although for some of them the function is not known yet. Act d 1 is a protease, known as

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actinidin. It is a major allergen of green kiwifruit (Bublin et al., 2011), and is almost absent in the gold species (Tuppo et al., 2008). Act d 2 is a thaumatin-like protein (Gavrovic-Jankulovic et al., 2002), belonging to the pathogenesis-related proteins group 5 (PR-5), and Act d 3 is a 40-kDa protein very poorly characterized both at the biochemical and immunological levels (Palacin et al., 2008). Act d 4 is a cystatine, that is an inhibitor of cysteine proteases (Popovic et al., 2010). Act d 5 is a protein of unknown function, named kiwellin (Tamburrini et al., 2005). It is a bi-modular protein that may undergo in vivo and in vitro proteolytic processing catalyzed by the kiwifruit allergen Act d 1, thus producing KiTH and kissper, corresponding to the C-terminal and N-terminal domains, respectively (Ciardiello, Meleleo, et al., 2008; Tuppo et al., 2008). Recently, it has been reported that the conformation and IgE reactivity of Act d 5 are affected by the physico-chemical features of the experimental conditions (Bernardi et al., 2010). Act d 8 is a Bet v 1-like protein (Oberhuber et al., 2008) and Act d 11 is a major latex protein/ripening-related protein (MLP/RRP) displaying IgE co-recognition with allergens belonging to the PR-10 family, including Bet v 1 (D'Avino et al., 2011). Act d 9 is a profilin (Bublin et al., 2011) and Act d 10 is a lipid transfer protein showing an IgE recognition pattern not totally overlapping that obtained for the peach homolog Pru p 3 (Bernardi et al., 2011). An IgE binding chitinase has also been identified in kiwifruit and included in the Allergome database (www.allergome.org), although it has not yet characterized and officially named by the WHO/IUIS.

In the framework of a study aimed at a comprehensive description of the kiwifruit allergome, here we describe an investigation of the IgE-binding properties of pectin methylesterase (Ciardiello, D'Avino, et al., 2008) and its inhibitor (Camardella et al., 2000), officially named Act d 7 and Act d 6, respectively.

Act d 7 is a glycoprotein with a molecular mass of about 50 kDa. It has an acetylated amino-terminus and five N-glycosylation consensus sequences, four of which are actually glycosylated. Act d 7 glycans belong to the complex type oligosaccharides essentially consisting of xylosylated polyfucosylated biantennary structures (Ciardiello, D'Avino, et al., 2008). Act d 6 is not glycosylated and has a molecular mass of about 17 kDa (Camardella et al., 2000). It shows structural similarities with the invertase inhibitors (INH) (Scognamiglio et al., 2003), more evident when the folds rather than the primary structures are compared (Di Matteo et al., 2005). INH and PME are grouped in the same protein family (plant invertase/pectin methylesterase inhibitors) that includes the major plane tree (*Platanus acerifolia*) pollen allergen, Pla 1.

PME and PME1 had been isolated and structurally and functionally characterized in our laboratory (Camardella et al., 2000; Ciardiello et al., 2004; Ciardiello, D'Avino, et al., 2008; Scognamiglio et al., 2003), and they were available for further studies. A preliminary characterization of the IgE binding capacity of PME (Act d 7) and PME1 (Act d 6) immobilized on the ISAC microarray was recently reported (Bublin et al., 2011). Here we describe a study of the prevalence of Act d 7 and Act d 6 positive subjects in a population of kiwifruit allergics, investigated by in vivo and in vitro experiments, immunoblotting and skin testing, respectively. The presence of oligosaccharides recognized by IgE specific for cross-reactive carbohydrate determinants (CCD) has been investigated in Act d 7, and in Act d 2 already reported to be a glycoprotein (Palacin et al., 2008).

2. Materials and methods

2.1. Allergenic molecules and inhibitors

Proteins were purified from green kiwifruit following the procedures already described for Act d 1 (Tuppo et al., 2008), Act d 2 (Tuppo et al., 2008), Act d 5 (Tamburrini et al., 2005), Act d 6 (Ciardiello et al., 2004), Act d 7 (Ciardiello, D'Avino, et al., 2008) and Act d 11 (D'Avino et al., 2011). No contaminant proteins were detected when the purified

allergens were analyzed by denaturing SDS-PAGE followed by Coomassie staining, by RP-HPLC and by N-terminal amino acid sequencing using an automated sequencer (Applied Biosystems, Foster City, CA, USA).

HSA-MUXF3 preparation (0.62 mg/mL), formed by a glycopeptide clived from pineapple bromelain (MUXF3) conjugated to human serum albumin, was kindly provided by Siemens HealthCare Diagnostics (Tarrytown, NY).

2.2. Preparation of protein solutions for skin test

Purified allergens were solubilized in 2 mM phosphate buffer, pH 7.4, and mixed with sterile glycerin in a 1:1 ratio. The final protein concentration was 0.5 mg/mL. The samples were sterilized by membrane filtration through a 0.22- μ m filter (Millex, Millipore, Bedford, MA, USA), in a sterile horizontal laminar flow hood.

2.3. Study population

This study was approved by the Institutional Review Board (CE/192/2006). All patients gave written informed consent before enrolment in the study. The study has been performed during years 2007–2008. All diagnostic and experimental data have been stored in the InterAll database (Allergy Data Laboratories s.c., Latina, Italy).

The patients were selected on the basis of (i) reported tight relationship between kiwifruit ingestion and symptoms onset, (ii) previous emergency room intervention (documented by a record report) or use of rescue medication (steroids and anti-histamine) following the symptoms onset, (iii) overall patient reliability and (iv) follow-up since years at our center. The above criteria were used when patients were evaluated for their kiwifruit allergy. Patients were enrolled in four different groups as follows: (a) sera from patients selected on the basis of a reliable history of allergic reactions to kiwifruit; (b) subjects allergic to kiwifruit tested by skin testing (ST) and microarray IgE detection; (c) subjects IgE positive to at least one kiwifruit allergen on microarray testing; and (d) a large population of random allergic subjects.

2.4. Skin testing

The patients in groups (a) and (b) were evaluated by ST with (i) a kiwi commercial diagnostic extract (Stallergenes, Antony, France), (ii) an in-house produced solution of green kiwi extract (Bernardi et al., 2010), and (iii) purified allergens obtained from the natural source as listed above. The diluent used to preserve the allergen extracts and purified allergens, and a solution of histamine 10 mg/mL, were used as negative and positive controls, respectively.

Skin test was performed as already described (Bernardi et al., 2010). Patients were asked to avoid anti-histamine intake for at least 7 days before testing. Test evaluation was done after 15 min. Ballpen-marked wheal areas, transferred on a record sheet by means of a transparent adhesive tape, were calculated by means of computer-aided design software (Autocad 22; Autodesk Inc., San Rafael, CA, USA). Any wheal area has been measured only in patients having fully negative control test with saline, and a positive result with histamine. No dropout was recorded for non specific skin test reactions. The widest surface of two test areas was considered.

Following the ST, sera were obtained from patients who consented to blood sampling for in vitro procedures.

2.5. IgE detection by immunoblotting and ImmunoCAP

To evaluate the IgE binding by immunoblotting, 1 μ g of the purified proteins reported in Table 1 was subjected to reducing 15% SDS-PAGE and transferred onto PVDF membranes. IgE immunoblotting was carried out as already described (Ciardiello et al., 2009) using 49 serum samples from patients having symptoms on kiwifruit exposure and a positive ST to kiwifruit extract.

Table 1

Forty nine kiwifruit allergic patients: IgE results from immunoblotting and ISAC microarray.

	IgE immunoblotting				IgE ISAC			
	Act d 1	Act d 2	Act d 6	Act d 7	Act d 1	Act d 2	Act d 6	Act d 7
1	+	+	+	Neg	3.67	Neg	Neg	Neg
2	+	+	+	Neg	Neg	Neg	Neg	Neg
3	++	Neg	++	++	Neg	2.82	Neg	1.20
4	+++	Neg	+	++	Neg	Neg	Neg	Neg
5	+	Neg	++	++	Neg	Neg	Neg	Neg
6	++	Neg	Neg	Neg	Neg	Neg	Neg	Neg
7	++	Neg	++	++	Neg	Neg	Neg	Neg
8	++	++	Neg	++++	Neg	13.17	Neg	8.95
9	+	Neg	+	Neg	Neg	Neg	Neg	Neg
10	++	+	+++	Neg	Neg	Neg	Neg	Neg
11	+	+	++	++	Neg	Neg	Neg	Neg
12	++	+	Neg	Neg	Neg	Neg	Neg	Neg
13	+	+	+++	Neg	Neg	Neg	Neg	0.86
14	++	Neg	++	Neg	Neg	Neg	Neg	Neg
15	+	++	++	+++	Neg	Neg	Neg	1.02
16	+++	++	++	Neg	Neg	Neg	Neg	Neg
17	+++	++	++	Neg	Neg	Neg	Neg	Neg
18	++	++	++	+++	2.13	Neg	Neg	Neg
19	++	+++	+++	Neg	Neg	Neg	Neg	Neg
20	++	+	++	Neg	2.72	Neg	Neg	Neg
21	++	++	Neg	Neg	Neg	Neg	Neg	Neg
22	+	+	Neg	Neg	Neg	Neg	Neg	Neg
23	Neg	+	+	++++	Neg	Neg	Neg	0.43
24	++	+	++	Neg	Neg	Neg	Neg	Neg
25	Neg	++	+	Neg	Neg	Neg	Neg	Neg
26	Neg	+	Neg	Neg	Neg	Neg	Neg	Neg
27	Neg	+++	Neg	Neg	Neg	Neg	Neg	Neg
28	++	+++	Neg	Neg	2.36	Neg	Neg	Neg
29	++	Neg	++	Neg	Neg	Neg	Neg	Neg
30	Neg	Neg	+++	Neg	Neg	Neg	Neg	Neg
31	+++	+	Neg	Neg	Neg	Neg	Neg	Neg
32	+	Neg	+++	++	Neg	Neg	Neg	Neg
33	+++	+	+	Neg	Neg	Neg	Neg	Neg
34	Neg	Neg	++	Neg	Neg	Neg	Neg	Neg
35	+	++	Neg	Neg	Neg	Neg	Neg	Neg
36	++	Neg	++	Neg	Neg	Neg	Neg	Neg
37	+	++	+	Neg	Neg	Neg	Neg	Neg
38	+++	Neg	++	Neg	Neg	Neg	Neg	Neg
39	++	Neg	++	++++	Neg	Neg	Neg	0.49
40	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
41	++	+++	+	Neg	Neg	Neg	Neg	0.49
42	++	++	++	++++	Neg	Neg	Neg	1.36
43	++	+++	++	+++	Neg	Neg	Neg	Neg
44	+	+	Neg	++	Neg	Neg	Neg	Neg
45	+++	Neg	+	++++	Neg	Neg	Neg	4.50
46	Neg	++	++	Neg	Neg	Neg	Neg	Neg
47	++	Neg	++	Neg	Neg	Neg	Neg	Neg
48	Neg	Neg	++	Neg	Neg	Neg	Neg	Neg
49	+++	Neg	Neg	++	Neg	Neg	Neg	Neg
Total	40	30	36	16	4	2	Neg	9
Percent	89	61	73	33	8	4	Neg	18

IgE Immunoblotting: "+" value equals band reaction strength.

Act d 1: cystein protease; Act d 2: thaumatin-like protein; Act d 6: pectin methylesterase inhibitor; Act d 7: pectin methylesterase.

Specific IgE using a singleplexed system was determined in patients' sera obtained at the time of enrolment using the ImmunoCAP system (Phadia, Uppsala, Sweden). IgE detection included the green kiwi extract as available from the manufacturer. Values were considered positive when >0.35 kU/L.

2.6. Multiplexed microarray-based IgE detection by ISAC

Quantification of specific IgE antibodies was obtained using the immuno-solid-phase-allergen chip (ISAC89 version, VBC-Genomics, Austria). ISAC is an in vitro diagnostic system developed for the semi-quantitative analysis of IgE and other types of immunoglobulins

from human serum (Harwanegg, Hutter, & Hiller, 2007). The main component of the ISAC biochip is a flat glass substrate containing four individual reaction sites designed to incubate 20 µL of serum. ISAC exclusively uses purified natural or recombinant allergen molecules, rather than crude allergen extracts, thus allowing a better standardization of allergy diagnosis, among other systems. Each allergen is immobilized in triplicate fashion in each of the four ISAC reaction sites. Therefore, data generated with ISAC are based on a multiple IgE determination in a single assay, providing more reliable diagnostic results in a single analytical step. The binding of specific IgE antibodies to immobilized allergens is detected by adding a fluorescence-labeled anti-human IgE antibody. The evaluation of the results of ISAC uses the MIA software (VBC-Genomics) and the fluorescence intensities are transformed into semi-quantitative IgE concentrations, expressed in kU/L.

2.7. Glycan detection

The purified proteins were subjected to reducing 15% SDS-PAGE and then transferred onto the nitrocellulose membrane. Glycosylation analysis was carried out by the DIG Glycan Detection Kit (Roche Diagnostics, Indianapolis, IN), following the manufacturer's instructions. Briefly, the (glyco)proteins were separated by SDS-PAGE and electro-transferred on nitrocellulose. Then, they were oxidized and labeled with digoxigenin (DIG). DIG labeled glycoconjugates were subsequently detected in an enzyme immunoassay using a DIG-specific antibody conjugated with alkaline phosphatase, followed by incubation with BCIP/NBT solution prepared according to the manufacturer.

2.8. Mass spectrometry analysis

Molecular mass of the intact protein was determined using a QSTAR Elite quadrupole time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems) equipped with a nano-electrospray ionization source. Sample was infused by the inbuilt syringe pump at 300 nL/min flow rate. ESI-MS spectra were recorded for 5 min in the m/z region from 500 to 2500 Da with the following instrument parameters: ion spray voltage = 1800 V, curtain gas = 15 L/min, declustering potential = 60 V, focusing potential = 220 V, and detector voltage = 2100 V. The obtained spectra were analyzed using the Bioanalyst 2.0 program (Applied Biosystems), and the average molecular weight protein was determined.

3. Results

3.1. Prevalence of Act d 7 and Act d 6 positive subjects in different allergic cohorts

3.1.1. Group (a): IgE reactivity evaluated by immunoblotting and ISAC testing in 49 subjects allergic to kiwifruit

A first initial group of 49 patients was selected on the basis of a reliable history of allergic reactions to kiwifruit (Table 1), though lacking further clinical investigation describing their overall clinical picture as later in the study applied to the group (b). The subjects were tested with Act d 6, Act d 7 and Act d 1 and Act d 2 for comparison. On the ISAC system, 9 (18%) subjects were detected positive for Act d 7 and no one for Act d 6, whereas 4 (8%) and 2 (4%) were positive for Act d 1 and Act d 2, respectively. IgE immunoblotting showed that 16 sera (32%) were positive for Act d 7 and 36 (73%) for Act d 6. In Fig. 1 the prevalence of subjects IgE immunoblotting positive to Act d 6 and Act d 7 is compared with that observed for other kiwifruit allergens tested in the same experiment. In IgE immunoblotting monospecificity was observed for two sera, one of them recognized only Act d 5 and the other one recognized only Act d 6.

3.1.2. Group (b): Skin test and microarray IgE reactivity in 36 subjects allergic to kiwifruit

In a second group of 36 patients, fulfilling the same clinical criteria and investigated in details about their reported symptoms on kiwi ingestion, a positive ST to Act d 7 and Act d 6 was recorded in 4 (11%) and in 1 (3%) subjects, respectively. None of the 4 patients showed specific IgE for the two molecules by ISAC microarray testing. Remaining results on other kiwifruit allergens are reported in Table 2.

3.1.3. Group (c): Microarray IgE testing of subjects positive to at least one kiwifruit allergen

A larger group of 284 patients were selected as having IgE sensitization to at least one of the green kiwifruit molecules responsible for allergy to this fruit and immobilized on the microarray system used for routine testing purposes. Their microarray IgE testing gave the following results: 38.37% Act d 7 positive and 1.76% Act d 6 positive (Fig. 2).

3.1.4. Group (d): ISAC testing of a population of random allergic subjects

A large cohort of random allergic patients (9107 subjects), representing a sample of our general allergic population, was analyzed by ISAC testing. A specific IgE positivity for PME1 (Act d 6) and PME (Act d 7) was observed in 0.05% and 1.21% of the tested subjects, respectively.

3.2. Detection of subjects having CCD-specific IgE on ISAC system

Sera of 159 subjects were detected IgE positive to Ana c 2 on the ISAC 89 microarray. Ana c 2 is the allergen name of bromelain, a protease from pineapple. It is a glycoprotein constituted of a single polypeptide chain bearing an N-linked oligosaccharide per molecule. The structure of this oligosaccharide is at least partially conserved in a large variety of plant and insect glycoproteins. This molecule detects IgE antibodies specifically recognizing CCD as true clinical allergy to bromelain is very rare (Mari, 2002).

The molecular pattern of reactivity of Ana c 2 positive subjects was analyzed and a peculiar cluster of positive molecules was observed. Overall results reported in Table 1 show that, among the tested kiwifruit allergens, only Act d 7 is recognized by the sera of kiwi-tolerant subjects containing CCD-specific IgE. Table 3 shows a group of glycosylated allergens (Ana c 2 (Bouwstra et al., 1990), Api m 1 (Blank et al., 2011), Cup a 1 (Alisi et al., 2001), Hor v 17 (Mikami, Yoon, & Yoshigi, 1999), Ole e 1 (Castro et al., 2010) and Pla a 2 (Ibarrola, Arilla, Martinez, & Asturias, 2004)) immobilized on the microarray and tested positive whenever sera containing CCD-specific IgE were used. Although Act d 2 was reported to be a glycosylated protein (Palacin et al., 2008), it does not fall in the cluster of allergens recognized by CCD-specific IgE (Table 3).

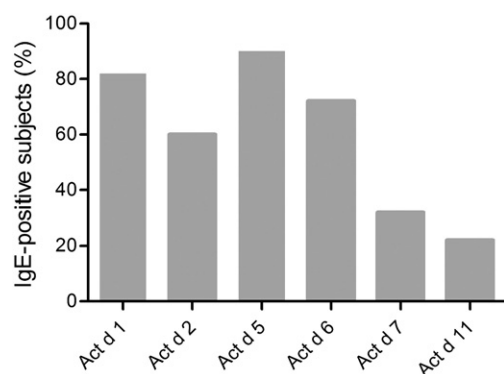


Fig. 1. Prevalence of subjects positive to kiwifruit allergens in a population of 49 subjects allergic to kiwifruit and analyzed by IgE-immunoblotting.

3.3. CCD-specific IgE binding defined by microarray IgE Single Point Highest Inhibition Achievable assay (SPHIAa)

Subjects IgE positive to Ana c 2 and to the other glycosylated allergens immobilized on the microarray were selected. Experiments were performed by running the SPHIAa assay as previously reported (Tuppo et al., 2013) and using HSA-MUXF3 and Act d 7 as inhibitors of CCD-specific IgE present in characterized individual sera.

Results obtained on the glycoproteins Act d 7, Ana c 2, Api m 1, Cup a 1, Hor v 17, Ole e 1 and Pla a 2, using one of the tested individual sera, are shown in Fig. 3. Full IgE inhibitions on Ana c 2 and Act d 7 were obtained following the pre-incubation of the serum with HSA-MUXF3 or Act d 7. In addition, full IgE inhibitions were achieved on Api m 1, Cup a 1, Hor v 17 and Ole e 1 with both the inhibitors. Only Pla a 2 was not fully inhibited by the pre-incubation with HSA-MUXF3 or Act d 7, anyhow the inhibitions were always higher than 70%. Similar results were obtained using different sera selected for the presence of CCD-specific IgE. Allergens, other than glycoproteins, used for control purposes gave no inhibition values (data not shown). No IgE binding to Act d 2 was recorded in any of the above SPHIAa experiments.

3.4. Assessment of the lack of carbohydrates linked to Act d 2

Act d 7 displayed a strong positive reaction when analyzed by glycan detection experiments (Fig. 4). In contrast, Act d 2 did not react, confirming the results obtained by the IgE microarray detection system, suggesting the absence of carbohydrates linked to this allergen.

Mass spectrometry analysis provided an experimental molecular mass of 21,598 Da. This value exactly corresponds to the theoretical one calculated on the basis of the Act d 2 amino acid sequence (Uniprot accession number P81370) assuming that all the 16 cysteine residues are involved in disulfide bridges. This result has been considered as the unequivocal evidence that natural Act d 2 purified from green kiwifruit, and used in this study, does not bear carbohydrates linked to its polypeptide chain.

4. Discussion

The allergenicity of some Act d 6 and Act d 7 homologs has already been reported in the literature. Act d 6 is the inhibitor of PME and belongs to the same protein family (plant invertase/pectin methylesterase inhibitor family) as the invertase inhibitor of the plane tree pollen, which is the major allergen Pla a 1 (Asturias, Ibarrola, Eraso, Arilla, & Martinez, 2003). The sequence identity between Act d 6 and Pla a 1 is quite low (32%). Nevertheless the two proteins are expected to share the same overall folding as suggested by literature reports describing a similar four-helix bundle 3D structure for the kiwifruit pectin methylesterase inhibitor (Act d 6) (Di Matteo et al., 2005; Scognamiglio et al., 2003) and for the tobacco invertase inhibitor (Hothorn, D'Angelo, Marquez, Greiner, & Scheffzek, 2004).

Act d 7 belongs to the PME protein family which includes some already known allergenic components. The best characterized is the PME of the Russian thistle (*Salsola kali*) pollen, Sal k 1. It is a glycoprotein and it has been proposed as a marker of sensitization to the Chenopodiaceae pollen family (Barderas et al., 2007). Ole e 11 has been described as a major allergen of the *Olea europaea* pollen (Salamanca et al., 2010) and Lyc e PME has been reported to be recognized by the IgE of subjects allergic to tomato (Foetisch et al., 2001). Act d 7 shows the highest identity with the fruit protein Lyc e PME (57%), whereas 27% of identity is observed when it is compared with the two pollen PME, Sal k 1 and Ole e 11. An additional allergenic PME, Bet v 8, has been identified in birch pollen (Mahler et al., 2001), but it has been poorly characterized.

Specific IgE to Act d 7 and Act d 6 were recorded in patients with kiwifruit allergies by immunoblotting and the ISAC microarray system.

Table 2
Demographic and diagnostic data of 36 kiwifruit allergic patients tested by IgE and skin test.

N.	Gender	Age	Symptoms	IgE(CAP) ^a	IgE (ISAC) ^a							SPT ^b							Other food allergies	
					Act d 1	Act d 2	Act d 5	Act d 6	Act d 7	Act d 11	Ana c 2	Commercial	In-house	P-P	Act d 1	Act d 2	Act d 5	Act d 6	Act d 7	ISAC IgE
1	F	30	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
2	F	56	EU	0.66	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	10.0	5.0	Neg	Neg	Neg	Neg	Neg	NEG
3	F	26	O	7.00	Neg	Neg	Neg	Neg	1.01	Neg	Neg	20.0	20.0	20.0	Neg	Neg	10.0	Neg	Neg	Profilin, LTP
4	M	57	EO	4.05	2.36	Neg	Neg	Neg	Neg	Neg	Neg	30.0	30.0	30.0	30.0	Neg	Neg	Neg	Neg	NEG
5	F	31	O-P	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	20.0	20.0	Neg	Neg	Neg	Neg	Neg	LTP
6	F	44	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
7	F	53	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	30.0	30.0	30.0	10.0	Neg	Neg	Neg	NEG
8	F	20	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
9	F	35	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	LTP
10	F	35	O	1.24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	30.0	30.0	Neg	Neg	Neg	Neg	Neg	LTP
11	M	15	AD	2.05	1.37	Neg	Neg	Neg	Neg	Neg	Neg	10.0	50.0	50.0	30.0	Neg	Neg	Neg	Neg	LTP
12	F	23	O	51.7	19.58	Neg	Neg	Neg	Neg	Neg	Neg	10.0	30.0	20.0	20.0	Neg	Neg	Neg	Neg	Bet v 1-like
13	M	28	O	0.59	Neg	0.99	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
14	F	24	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
15	F	32	AO	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	Neg	Neg	Neg	Neg	Neg	Profilin
16	M	20	Ax	6.24	Neg	2.35	Neg	Neg	Neg	Neg	Neg	10.0	20.0	20.0	Neg	20.0	Neg	Neg	Neg	NEG
17	M	14	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
18	M	25	AP	0.61	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	30.0	30.0	10.0	10.0	Neg	Neg	10.0	LTP
19	F	33	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	10.0	Neg	Neg	Neg	Neg	Neg	Neg	NEG
20	F	32	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	ND	10.0	Neg	Neg	Neg	10.0	NEG
21	F	40	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
22	F	29	Ap	1.02	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	20.0	Neg	Neg	Neg	Neg	Neg	LTP
23	F	55	A-U	0.96	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	30.0	30.0	30.0	10.0	Neg	Neg	Neg	NEG
24	F	41	AP	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	Neg	Neg	Neg	Neg	Neg	NEG
25	M	27	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	20.0	10.0	Neg	Neg	Neg	Neg	Neg	LTP
26	F	25	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NEG
27	F	40	EP	2.07	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10.0	20.0	10.0	Neg	Neg	Neg	Neg	Neg	LTP
28	F	34	O	3.34	1.13	Neg	Neg	Neg	Neg	Neg	Neg	10.0	30.0	20.0	30.0	Neg	Neg	Neg	Neg	NEG
29	F	50	O	ND	0.26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	30.0	20.0	20.0	Neg	Neg	Neg	Neg	NEG
30	M	8	V	ND	4.34	Neg	Neg	Neg	Neg	Neg	Neg	10.0	30.0	20.0	30.0	Neg	10.0	Neg	Neg	Profilin
31	F	36	O	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.0	20.0	Neg	Neg	Neg	Neg	Neg	LTP
32	M	13	V	3.41	0.73	Neg	Neg	Neg	0.40	Neg	Neg	20.0	30.0	30.0	30.0	Neg	Neg	Neg	5.0	Bet v 1-like
33	F	36	Ap	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	5.0	Neg	Neg	Neg	Neg	Neg	Neg	NEG
34	F	75	O	0.84	Neg	Neg	Neg	ND	ND	Neg	Neg	Neg	43.0	ND	31.0	Neg	Neg	Neg	Neg	Bet v 1-like
35	M	23	ND	6.24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	20.8	21.7	86.4	2.6	42.7	Neg	Neg	Neg	Bet v 1-like
36	F	21	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ND	Neg	Neg	Neg	6.0	6.0	Bet v 1-like
Tot	26 F	31.5		29	7	2	0	0	2	0	0	18	24	25	13	5	2	1	4	
%					18	5	0	0	5	0	0	50	66	69	36	14	6	3	11	

Symptoms: A = asthma; Ap = abdominal pain; Ax = anaphylaxis; C = conjunctivitis; D = atopic dermatitis; E = angioedema; H = headache; O = oral allergy syndrome; P = skin itching; R = rhinitis; U = urticaria; V = vomiting; ND = not done.

Green kiwifruit extracts or the fresh fruit were used for all testing where molecules are not specified.

Act d 1: cystein protease; Act d 2: thaumatin-like protein; Act d 5: Kiwellin; Act d 6: pectin methylesterase inhibitor; Act d 7: pectin methylesterase; Act d 11: ripening-related protein/Bet v 1-like; Ana c 2: bromelain/cystein protease/glycoprotein.

LTP: lipid transfer proteins.

^a Indicates values in kU/L.

^b Indicates values in mm².

In addition, a few positive ST to PME and PMEI have been induced. Therefore, PME and PMEI are two new allergens identified in green kiwifruit, officially named by the IUIS-WHO subcommittee for allergen nomenclature as Act d 7 and Act d 6, respectively. In comparison with

the in vitro tests, Act d 7 and Act d 6 induced a positive reaction by ST only in a few patients. In the case of PME this could be ascribed to the glycan side chain recognized by subjects with CCD-specific IgE, and known to be unable to exert any in vivo reactivity. Positive results on Act d 7 were observed when sera recognizing Ana c 2 and all the cluster

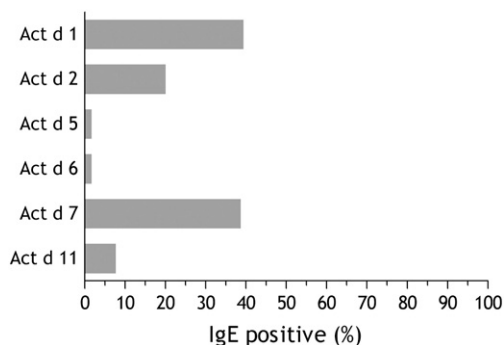


Fig. 2. ISAC protein microarray results of 284 patients positive to at least one of the kiwifruit allergen responsible for allergy to this fruit.

Table 3
Microarrayed kiwifruit allergens and glycoallergens tested with CCD IgE positive sera.

Kiwi allergens	IgE-binding on the microarray	Glycosylated allergens from other sources	IgE-binding on the microarray
Act d 1	Negative	Ana c 2	Positive
Act d 2	Negative	Api m 1	Positive
Act d 5	Negative	Cup a 1	Positive
Act d 6	Negative	Hor v 17	Positive
Act d 7	Positive	Ole e 1	Positive
Act d 11	Negative	Pla a 2	Positive

Act d 1: cystein protease; Act d 2: thaumatin-like protein; Act d 5: kiwellin; Act d 6: pectin methylesterase inhibitor; Act d 7: pectin methylesterase; Act d 11: ripening-related protein/Bet v 1-like; Ana c 2: bromelain/cystein protease; Api m 1: phospholipase A2/bee venom allergen; Cup a 1: pectate lyase; Hor v 17: β -Amylase; Ole e 1: olive pollen allergen; Pla a 2: polygalacturonase.

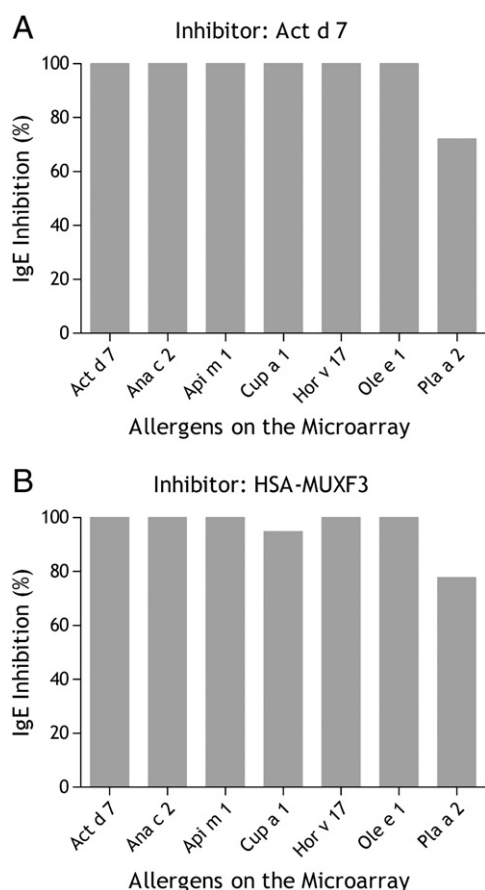


Fig. 3. SPHIA assay for IgE on ISAC89 protein microarray with Act d 7 (Panel A) and HSA-MUXF3 (Panel B) as inhibitors and individual sera of characterized CCD positive patients as controls.

of glycosylated allergens immobilized on the ISAC microarray were positive. Ana c 2 (bromelain) is a glycosylated allergen extracted from pineapple. MUXF3 is a peptide bearing the oligosaccharide bound to Ana c 2. This determinant is purified from digested Ana c 2 and widely used, sometimes linked to HSA (HSA-MUXF3), as a marker of the presence of CCD-specific IgE and as an inhibitor to assess the co-recognition between its glycan and those of other glycoproteins. Inhibition experiments carried out using sera containing CCD-specific IgE demonstrated a total inhibition of MUXF3 on Act d 7 and an inhibition of Act d 7 on the precursor of MUXF3, Ana c 2. In addition, both MUXF3 and Act d 7 inhibited the IgE binding on each of the glycosylated allergens immobilized on the ISAC microarray, such as Api m 1, Cup a 1, Hor v 17, Ole e 1, and Pla a 2.

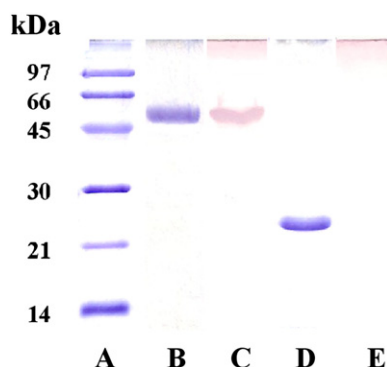


Fig. 4. 15% SDS-PAGE under reducing conditions. A, molecular markers; B, Act d 7 and D, Act d 2, detected by Coomassie blue staining; C, Act d 7 and E, Act d 2, probed by DIG Glycan Detection Kit.

The analysis of the results obtained on Act d 2 revealed that this allergen did not react with sera containing CCD-specific IgE. Since Act d 2 had been reported to be a glycoprotein (Palacin et al., 2008), we were surprised to see this result. Anyhow, the immunological observation prompted the investigation of some biochemical features of this molecule. The negative response obtained by a specific immunoassay suggested the absence of sugars bound to Act d 2, whereas a strong positive response confirmed the high level of glycosylation of Act d 7, used as a control in the same assay. The absence of oligosaccharides bound to Act d 2 was unequivocally confirmed by the mass spectrometry experiments. In fact, the molecular mass obtained by mass spectrometry (21,598 Da) corresponds to that calculated from the amino acid sequence of Act d 2 (Uniprot accession number, P81370) (Gavrovic-Jankulovic et al., 2008).

It is noteworthy that the use of a multiplex system, namely the ISAC system, provided the initial input that prompted the investigation about the glycosylation of Act d 2. In fact, Act d 2 fell outside the cluster of glycosylated allergens reacting with sera containing the CCD-specific IgE and this observation gave rise to the first doubts about its glycosylation. Conversely, we can imagine that the finding in the cluster of glycosylated allergens of a molecule described as non-glycosylated could suggest the presence on that molecule of an overlooked post-translational modification.

5. Conclusions

This paper describes an investigation of the immunological properties of two new kiwifruit allergens, Act d 6 and Act d 7. The data obtained indicate that, depending on the test system used, a different percentage of positive responses can be recorded. The knowledge of the behavior of these molecules in different experimental conditions and test systems can contribute to the description of the kiwifruit allergen profile, as well as to the achievement of a more reliable allergy diagnosis to this food.

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