Detailed characterization of Act d 12 and Act d 13 from kiwi seeds: implication in IgE cross-reactivity with peanut and tree nuts

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Short title: Structural and immunological characterization of Act d 12 and Act d 13

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Abstract

Background: Act d 12 (11S globulin) and Act d 13 (2S albumin) are two relevant allergens from kiwi seeds recently discovered. Their inclusion in component-resolved diagnosis of kiwifruit allergy could improve the diagnostic sensitivity and the management of kiwifruit allergic patients.

Objective: To perform a comprehensive structural and immunological characterization of purified Act d 12 and Act d 13 from kiwi seeds.

Methods: Sera from 55 well-defined kiwifruit allergic patients were used. Act d 12 and Act d 13 were purified by conventional chromatographic procedures. Circular dichroism, mass spectrometry, concanavalin A detection, immunoblotting, enzyme-linked immunosorbent assays, basophil activation tests and IgE-inhibition experiments were used for structural and immunological characterization and IgE cross-reactivity studies.

Results: Act d 12 and Act d 13 were purified from kiwi seeds to homogeneity by combining size-exclusion, ion-exchange and RP-HPLC chromatographies. Purified Act d 12 and Act d 13 preserve the structural integrity and display typical features of their homologous counterparts from the 11S globulin and 2S albumin protein families, respectively. Both purified allergens retain the capacity to bind serum IgE from kiwifruit allergic patients, induce IgE cross-linking in effector circulating basophils and display *in vitro* IgE cross- reactivity with homologous counterparts from peanut and tree nuts.

Conclusion: Purified Act d 12 and Act d 13 from kiwi seeds are well-defined molecules involved in *in vitro* IgE cross-reactivity with peanut and tree nuts. Their inclusion in component-resolved diagnosis of kiwifruit allergy might well contribute to improve the diagnostic sensitivity and the management of kiwifruit allergic patients.

Key words: kiwifruit allergy, 11S globulin Act d 12, 2S albumin Act d 13, component-resolved diagnosis, peanut and tree nuts cross-reactivity.

Abbreviations

CBS: Coomassie blue staining
CD: Circular dichroism
CRD: Component-resolved diagnosis
DBPCFC: double-blind placebo-controlled food challenges
ELISA: Enzyme-linked immunosorbent assay
MS: Mass spectrometry
OAS: Oral allergy syndrome
OD: Optical density
SPT: Skin prick test

Introduction

IgE-mediated food allergy is a common health problem affecting around 5% of adults and 8% of children in industrialized countries (1). The consumption of kiwifruit (*Actinidia deliciosa*) has significantly risen over the last decades in western countries (2), likely due to the reported beneficial effects associated to the inclusion of this fruit into the diet (3). Since the first case of kiwifruit allergy was reported in 1981 (4), the prevalence has enormously increased, being currently considered among the top 10 food allergies (2, 5, 6).

The gold standard approach to accurately diagnose kiwifruit allergy remains double-blind placebo-controlled food challenges (DBPCFC), but the main inconveniences include the difficulty to mask kiwifruit and the risk of anaphylactic reactions (7). Different attempts at improving the diagnosis of kiwifruit allergy have been performed (2, 5, 6). Prick to prick test with fresh kiwifruit is a highly sensitive method for diagnosis (83-100%) with low specificity (around 31%). In contrast, skin prick test (SPT) and *in vitro* serum specific-IgE determinations with commercially available kiwifruit extracts improved specificity (40-45%) but showed low sensitivity (17-60%). Recent studies showed component-resolved diagnosis (CRD) with purified kiwifruit allergens as an alternative not only to increase the diagnostic sensitivity (up to 65%) but also to link specific IgE-sensitization patterns with clinical features (2, 5, 8).

Up to date, 13 kiwifruit allergens have been described according to the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee (www.allergen.org) (6, 9, 10). Almost all the kiwifruit allergens were initially described in the kiwi pulp (Act d 1 to Act d 11) with the exception of Act d 10 (LTP) that was also reported in kiwi seeds (11). We have recently demonstrated that kiwi seeds, which are usually ingested together with the kiwi pulp, constitute a source of additional potent allergens such as the 11S globulin Act d 12 and the 2S albumin Act d 13(10). Around 85%

of the kiwifruit allergic patients were sensitized to at least one of these allergens from kiwi seeds, suggesting that their inclusion in CRD of kiwifruit allergy might well contribute to improve the diagnostic sensitivity. Prior to the inclusion of these molecules in CRD approaches, a comprehensive characterization of both purified allergens is mandatory.

The aim of this study was to characterize from structural and immunological point of view the purified Act d 12 and Act d 13 from kiwi seeds and to investigate the potential implication of these allergens in IgE cross-reactivity involving kiwi, peanut and tree nuts.

Material and methods

Patients' sera

Serum samples were obtained from a well-defined cohort of 55 Kiwifruit allergic patients, controls from pollen-allergic patients and non-atopic donors from the Allergy Service of Hospital Carlos Haya, Málaga, Spain, and Hospitals Fundación Jiménez Díaz and Infanta Leonor, Madrid, Spain (10). The study was approved by the Ethic Committee of the 3 Hospitals, and written informed consent was obtained from all subjects.

Detailed protocols for the purification of Act d 12 and Act d 13, protein extracts, analytical procedures, immunoblotting, ELISA, basophil activation test, carbohydrate detection, circular dichroism (CD), preparation of phospholipid vesicles, isolation of lipids from kiwi seeds, simulated gastric and intestinal digestion methods are fully described in the online repository material of this article.

Results

Act d 12 and Act d 13 display common structural features to their homologous counterparts from the 11S globulin and 2S albumin protein families

Act d 12 (11S globulin) and Act d 13 (2S albumin) of around 51 and 12 kDa, respectively, are two novel allergens contained kiwi seeds extract that display IgE-reactivity in immunoblotting with a pool of sera from kiwifruit allergic patients (Fig. 1A). We purified both allergens to homogeneity following the sequential chromatographic steps described in the online repository (Supplementary Fig. 1). The purity and structural integrity of the purified Act d 12 and Act d 13 was assayed by CBS and IgE-binding analysis after SDS-PAGE (Supplementary Fig. 1B,C), Edman degradation and MS-fingerprint analysis (data not shown). Both purified allergens consisted of two polypeptide subunits of around 32 and 20 kDa (Act d 12) and of around 8 and 4 kDa (Act d 13) that can be separated under reducing conditions (Fig. 1B). MS of purified Act d 12 yielded a heterogeneous profile with two main peaks at 50207.3 and 52280.5 Da (Fig. 1C). For Act d 13, a single peak at 11359.0 Da was obtained (Fig. 1C). None of the purified allergens carried glycan moieties as demonstrated by negative staining with the lectin Concanavalin A (Fig. 1D). The CD spectra in the far-UV of purified Act d 12 and Act d 13 showed that both allergens display a structured folding (Fig. 1E). Temperature-dependent unfolding experiments (from 20 ºC to 80 ºC) of Act d 12 and Act d 13 revealed slight and reversible changes of secondary structure, indicating that both allergens are highly stable to thermal denaturation (Figure 1E).

Act d 12 and Act d 13 show different resistance to gastric and intestinal digestion

Purified Act d 12 and Act d 13 were subjected to *in vitro* simulated gastric and intestinal digestions alone or in the presence of lipids from kiwi seeds, PC or PG vesicles. Gastric digestion of Act d 12 rendered fragments of around 17, 13 and 10 kDa within the first

seconds that were completely digested after 2 h of treatment (Fig. 2A). No differences were observed in the presence of kiwi lipids or PC vesicles. In contrast, the addition of PG vesicles significantly increased the resistance of Act d 12 to gastric digestion remaining the allergen intact even after 2 h of treatment (Fig. 2A). Act d 13 was very resistant to gastric treatment as it was only digested after 16 h without significant differences in the presence of kiwi lipids, PC or PG vesicles (Fig 2A). Intestinal digestion of Act d 12 resulted in the rapid appearance of proteolytic fragments of around 40, 34 and 16 kDa that were detectable even after 16 h of treatment. The presence of kiwi lipids, PC or PG vesicles did not significantly modify the resistance of Act d 12 to intestinal digestion (Fig. 2B). Act d 13 was very resistance also to intestinal degradation and after 16 h of treatment 53%, 54%, 42% and 51% of Act d 13 remained undigested when assayed alone, in the presence of kiwi lipids, PC or PG vesicles, respectively, as determined by scanning densitometry (Fig. 2B).

Act d 12 and Act d 13 retain IgE-binding capacity and allergenicity

The IgE-binding capacity of purified Act d 12 and Act d 13 from kiwi seeds was demonstrated by ELISA and immunoblotting with the cohort of 55 kiwifruit allergic patients (10). When we plotted the obtained ELISA values for Act d 12 and Act d 13 versus the corresponding immunoblotting data (arbitrary units determined by scanning densitometry and relative to patient 1 for Act d 12 and to patient 43 for Act d 13, respectively), we found significant correlations in both cases (Fig. 3A). Purified Act d 12 and Act d 13 were recognized by 70.9% and 18.2% of the tested patients, respectively, in immunoblotting (Fig. 3B).

To quantify the contribution of Act d 12 and Act d 13 to the total allergenicity of the kiwi seed extract, we performed ELISA inhibition experiments. The IgE binding to kiwi seeds extract was notably abolished after pre-incubation of the pool of sera with purified

Act d 12 (53%) (Fig. 3C). When Act d 13 was used as inhibitor, a 34% of inhibition was reached, and when the pool of sera was pre-incubated with the mixture of both allergens, the inhibition was around 80% (Fig. 3C).

The allergenic capacity of Act d 12 and Act d 13 was assessed by *in vitro* BAT. Both purified allergens demonstrated capacity to induce IgE cross-linking in effector circulating basophils (Fig. 3D). Kiwifruit allergic patients with specific IgE to kiwi seeds extract and to Act d 12 or Act d 13 showed positive BAT with kiwi seeds extract, purified Act d 12 or Act d 13 with median SI values of x ± y, z ± w and m ± n, respectively (Fig 3D).

Act d 12 and Act d 13 from kiwi seeds show in vitro IgE cross-reactivity with homologous counterparts from peanut and tree nuts

We performed *in vitro* IgE-inhibition experiments in immunoblotting. We pooled the sera from kiwifruit allergic patients sensitized to peanut and tree nuts into two groups (Table I): i) patients with specific IgE to Act d 12, and ii) patients with specific IgE to Act d 13. The IgE reactivity to the protein of 51 kDa in kiwi seeds extract was totally abolished when the pool of sera from patients sensitized to Act d 12 was preadsorbed to kiwi seeds extract or to the purified Act d 12 (Fig. 4A). The IgE-binding to purified Act d 12 was significantly inhibited by peanut, almond, hazelnut and walnut (86%, 67%, 66% and 65%, respectively) and completely inhibited by kiwi seeds extract or purified Act d 12 (Fig. 4B). The IgE-reactivity to the protein of 12 kDa in kiwi seeds extract was also completely inhibited when the pool of sera from patients sensitized to Act d 13 was preadsorbed to kiwi seeds extract or to the purified Act d 13 (Fig. 4C). Inhibition of the IgE-binding to the purified Act d 13 was observed with walnut (85%), peanut (70%) and almond (49%) but not with hazelnut (10%) (Fig. 4D). Complete inhibition was obtained with kiwi seeds or the purified Act d 13.

Discussion

In this study, we performed a comprehensive structural and immunological characterization of purified Act d 12 and Act d 13 from kiwi seeds. Our results demonstrated that purified Act d 12 and Act d 13 preserve the structural integrity and display typical features of their homologous counterparts from the 11S globulin and 2S albumin protein families, respectively. Both purified allergens retained the capacity to bind serum IgE from kiwifruit allergic patients in ELISA and immunoblotting, induced IgE cross-linking in effector circulating basophils and displayed *in vitro* IgE-cross reactivity with homologous counterparts from peanut and tree nuts. The availability of purified Act d 12 and Act d 13 from kiwi seeds as well-defined molecules and their inclusion in CRD approaches might well contribute to improve the diagnostic sensitivity of kiwifruit allergy. Further detailed studies are required to confirm the clinical relevance of the *in vitro* IgE cross-reactivity among kiwi, peanut and tree nuts involving Act d 12 and Act d 13, which might also have essential implications in the management of kiwifruit allergic patients.

The prevalence of kiwifruit allergy has significantly increased over the last decades in western countries (2, 5). Kiwifruit allergy is frequently associated to pollen or latex allergy and clinical symptoms vary from mild local oral allergy syndrome (OAS) (12) to severe systemic reactions including anaphylaxis (13, 14), which compounds accurate diagnosis. Diagnosis of kiwifruit allergy has significantly improved during the last years (2, 5, 6, 8). However, the main drawbacks are still the classical difficulties associated to DBPCFC, the low specificity for prick to prick tests and the low sensitivity for *in vivo* SPT and *in vitro* serum specific-IgE determinations. Low sensitivity of diagnostic tests has been attributed to the different allergenic protein content of the kiwi varieties (15) and to the lack of relevant allergens in the used commercial kiwifruit extracts (6). At this regard, we recently showed that kiwi seeds represent an important allergenic source to be considered in the context of kiwifruit allergy as they contain relevant allergens such as Act d 12 or Act

d 13 (10). Seeds from other species are also well-recognized potent inducers of food allergy due to their high content of very stable seed-specific storage proteins (16-18).

The use of well-defined purified allergens is very useful to improve diagnosis, management and treatment of allergic patients (19, 20). We purified and deeply characterized Act d 12 and Act d 13 from kiwi seeds. 11S globulins and 2S albumins are non-glycosylated and very stable seed-specific storage proteins composed of two polypeptide chains linked by disulphide bridges, structural features that were retained in purified Act d 12 and Act d 13. Other members from these protein families have been previously described as potent food allergens able to induce primary sensitization at the gastrointestinal level (21-27). To assess the capacity of Act d 12 an Act d 13 to reach the intestinal gut as intact molecules able to interact with immune system cells, we subjected purified Act d 12 and Act d 13 to different protease treatments. Act d 13, as other 2S albumins such as Sin a 1 (28) BnIb (29), or Ber e 1 (30), was very resistance to simulated gastric and intestinal digestions in all the assayed conditions. In contrast, Act d 12 was rapidly digested by simulated gastric fluid showing higher resistance to intestinal degradation as previously shown for homologous counterparts (31). Interestingly, the presence of PG vesicles significantly increased the resistance of Act d 12 to gastric digestion without affecting intestinal treatments, suggesting that acidic phospholipids could specifically hide the pepsine-sensitive proteolytic sites of Act d 12 and increase its resistance to gastric digestions.

Act d 12 represents a major allergen recognized by more than 50% of the 55 kiwifruit allergic patients included in this study, whereas Act d 13 is a minor allergen. There was a significant correlation between the reactivity observed in ELISA and immunoblotting for both allergens. A mixture of Act d 12 and Act d 13 inhibited around 80% of the IgE-reactivity to kiwi seeds extract, indicating that most of the IgE epitopes of kiwi seeds are represented within these two allergens in the tested populations. Importantly, we also

demonstrated that Act d 12 and Act d 13 were able to induce *in vitro* IgE cross-linking in circulating basophils from kiwifruit allergic patients, thus demonstrating that both purified allergens retain allergenic capacity.

Around 44 % of the patients included in this study were also allergic to tree nuts or peanut. Among these patients, 91.6 % (22/24) were sensitized to Act d 12 or Act d 13, suggesting a potential implication of these allergens in cross-reactivity with peanut and tree nuts. *In vitro* IgE-inhibition experiments demonstrated that purified Act d 12 and Act d 13 conserved common epitopes of homologous counterparts from peanut, almond, hazelnut or walnut, suggesting that Act d 12 and Act d 13 might be involved in cross-reactivity with these allergenic sources. Further detailed studies are required to elucidate the potential clinical relevance of the observed *in vitro* IgE-cross reactivity. Different studies have previously shown that 11S globulins and 2S albumins constitute two families of allergenic proteins that might be involved in IgE cross-reactivity among mustard, peanut and tree nuts (32-35).

Collectively, our data indicated that Act d 12 and Act d 13 purified from kiwi seeds constitute well-defined molecules that might be included in future CRD of kiwifruit allergy, which could contribute to improve the diagnostic sensitivity. Purified Act d 12 and Act d 13 showed *in vitro* IgE cross-reactivity with homologous counterparts from tree nuts and peanut. Further studies are required to confirm the clinical impact of this fact, which might be also relevant for the management of kiwifruit allergic patients.

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Author contributions

Conceived and designed the experiments: OP and SS. Performed the *in vitro* experiments: SS and BC. Clinical characterization of the patients: FG, NB, JCH, GC and MB. Analyzed and discussed the data: OP, SS, BC, FG, NB, JCH, GC, MB, RR, and MV. Contributed reagents/materials/analysis tools: FG, NB, JCH, GC, MB, RR, MV and OP. Wrote the paper: OP. All the authors read and approved the final manuscript.

Conflict-of-interest statement

The authors declare to have no conflict of interest in relation to this manuscript

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Table I. Clinical features of the patients selected for in vitro IgE inhibition experiments

Sera pool	Patient number	Sex/Age (y)	Kiwifruit Symptoms	Kiwifruit SPT*	ELISA†			Other food allergies	Pollen allergy	Tree nuts and peanut symptoms	SPT (mm^2)			
					Kiwi	Act d 12	Act d 13				Peanut	Almond	Hazelnut	Walnut
Act d 12 positive	1	F/26	OAS	22	0.068	0.212	0.029	r,n,t,p	Yes	OAS	12	12	12	Neg
	2	M/39	U	12	0.069	0.692	0.073	r,n,p	Yes	A, AN	12	Neg	48	Neg
	3	F/19	OAS	9	0.066	0.52	0.051	r,n,p	Yes	OAS	9	20	12	9
	4	F/21	OAS	Neg	0.02	0.205	0.054	r,n,p,b,t,pn,gr	Yes	OAS	18	35	15	42
	5	F/39	OAS	Neg	0.142	0.133	0.028	n,p	Yes	OAS	ND	ND	ND	ND
Act d 13 positive	6	M/27	U	22	0.146	0	0.185	r,n,p	Yes	OAS	Neg	Neg	22	Neg
	7	F/20	OAS	12	0.063	0.069	0.342	r,n,p	Yes	OAS	12	Neg	10	12
	8	F/17	OAS	12	0.212	0.053	0.264	r,n	Yes	OAS	ND	ND	ND	ND
	9	F/36	OAS	Neg	0.112	0.068	0.181	r,n,p,tr	No	OAS	12	Neg	22	22
	10	M/37	OAS	Neg	0.235	0.439	0.223	r,n,p,m,f	Yes	AN, OAS	22	22	22	Neg

y: year; M/F: male/female; Neg: negative; AN: anaphylaxia; OAS: oral allergy syndrome; U: urticaria; av. avocado; b: banana; f: fig; gr: grape; n: tree nuts including almond; p: peanut; pn: pineapple; r: rosaceae excluding almond; t: tomato; tr: tropical; ND: not done

* Skin prick test wheal area in mm2

†Specific IgE determined in ELISA as OD at 492 nm

384 **Figure legends**

385

386 **Figure 1**. Structural characterization of purified Act d 12 and Act d 13. (A) CBS and IgE
387 immunoblotting with a pool of sera from kiwifruit allergic patients after SDS-PAGE of kiwi
388 seeds extract. (B) CBS and IgE immunoblotting after SDS-PAGE of purified Act d 12 or
389 purified Act d 13 in the absence or presence of β-mercaptoethanol (βme). (C) Molecular
390 mass determination of the purified Act d 12 and Act d 13 by MS. (D) Sugar staining with
391 biotinylated ConA after SDS-PAGE of kiwi seeds extract, purified Act d 12, Act d 13, Ole e
392 1 (positive control) and Sin a 1 (negative control). (E) CD spectra of purified Act d 12 and
393 Act d 13 in the far-UV at 20 ºC, at 85 ºC and cooling down again at 20 °C. Secondary
394 structure contributions (α H, α-helix; β S, β-sheet; β T, β-turn; RC, random coil) are shown.

395

396 **Figure 2.** Simulated gastric and intestinal digestion of purified Act d 12 and Act d 13.
397 Coomassie blue staining of the gastric digestion (A) or intestinal digestion (B) products of
398 purified Act d 12 or Act d 13 alone or in the presence of kiwi lipids (+KL), PC (+PC) or PG
399 (+PG) vesicles. Molecular markers are indicated in kDa in the left side.

400

401 **Figure 3.** Allergenic characterization of purified Act d 12 and Act d 13. (A) Correlation
402 between ELISA and immunoblotting reactivity for the purified allergens for the 55 sera from
403 patients allergic to kiwifruit. OD, optical density at 492 nm. AU, arbitrary units showing the
404 relative intensity for each serum after normalization of the values determined by scanning
405 densitometry with those obtained for serum 1 (Act d 12) and 43 (Act d 13). (B)
406 Immublotting for purified Act d 12 and Act d 13 with the individual sera. C, serum from a
407 non-atopic subject. (C) Inhibition of the IgE-binding of a pool of sera from kiwifruit allergic
408 patients to kiwi seeds extracts with (1 mg/mL) kiwi seeds extract, (20 micrograms/mL) of
409 purified Act d 12, Act d 13 or an equal mixture of both purified allergens. (D) Basophils

activation test using kiwi seeds extract and purified Act d 12 and Act d 13; C+, positive control with histamine; horizontal dashed line represent the obtained values for the negative control with PBS.

Figure 4. Immunoblotting inhibition experiments. Inhibition of the IgE-binding to Act d 12 contained in kiwi seeds extract (A) or to purified Act d 12 (B) by the indicated inhibitors when using a pool of sera from kiwifruit allergic patients sensitized to Act d 12. Inhibition of the IgE-binding to Act d 13 contained in kiwi seeds extract (C) or to purified Act d 13 (D) by the indicated inhibitors when using a pool of sera from kiwifruit allergic patients sensitized to Act d 13. BSA was used as negative control of inhibition. Percentages of inhibition are shown at the bottom of the figures (B) and (D).

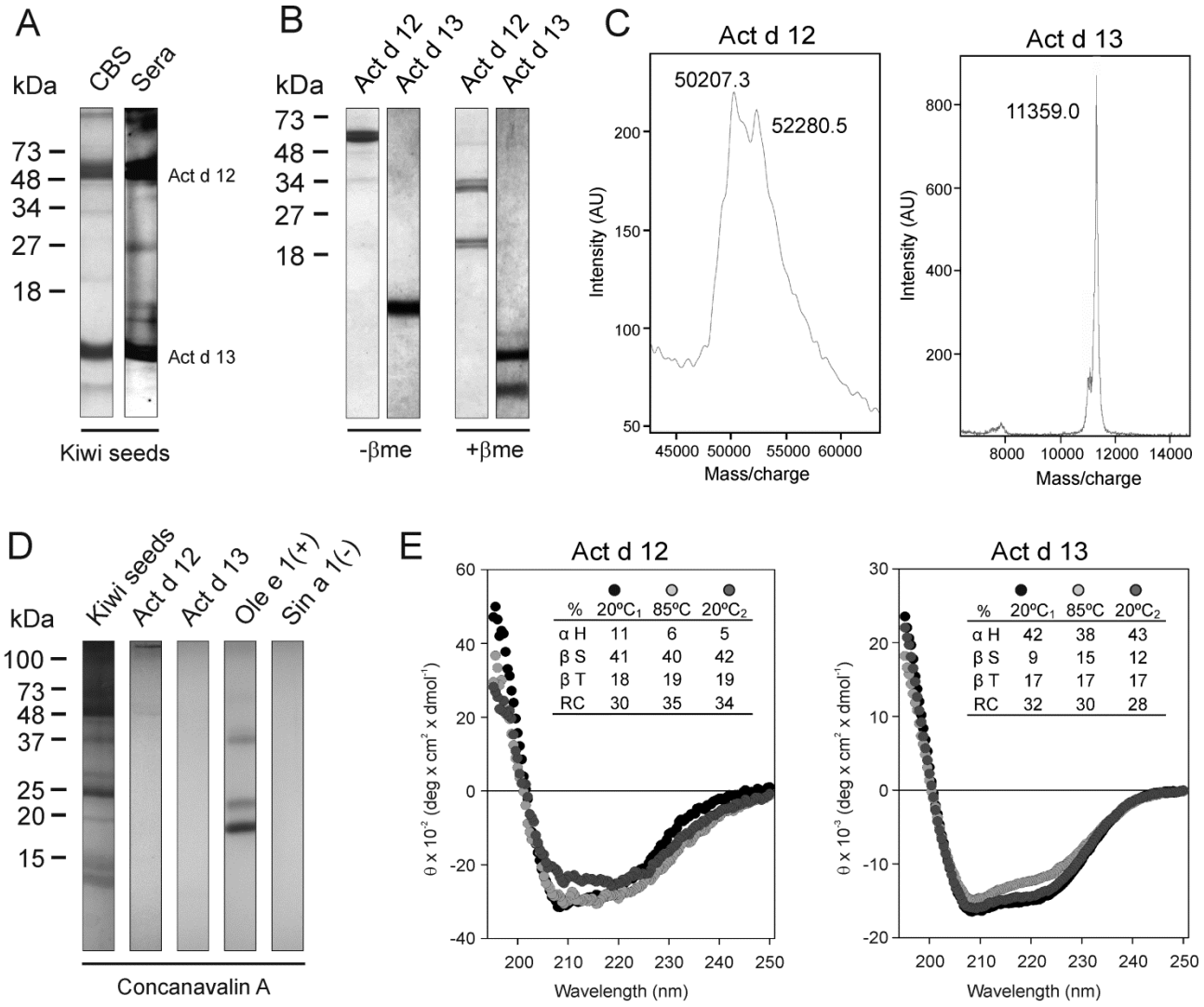
A
kDa
CBS
Sera
73
48
34
27
18
Act d 12
Act d 13
Kiwi seeds
B
kDa
Act d 12
Act d 13
Act d 12
Act d 13
73
48
34
27
18
-βme
+βme
C
Act d 12
50207.3
52280.5
Intensity (AU)
Mass/charge
Act d 13
11359.0
Intensity (AU)
Mass/charge
D
kDa
Kiwi seeds
Act d 12
Act d 13
Ole e 1(+)
Sin a 1(-)
100
73
48
37
25
20
15
Concanavalin A
E
Act d 12
θ x 10-2 (deg x cm2 x dmol-1)
Wavelength (nm)
% 20ºC1 85ºC 20ºC2
α H 11 6 5
β S 41 40 42
β T 18 19 19
RC 30 35 34
Act d 13
θ x 10-3 (deg x cm2 x dmol-1)
Wavelength (nm)
% 20ºC1 85ºC 20ºC2
α H 42 38 43
β S 9 15 12
β T 17 17 17
RC 32 30 28

Figure 1

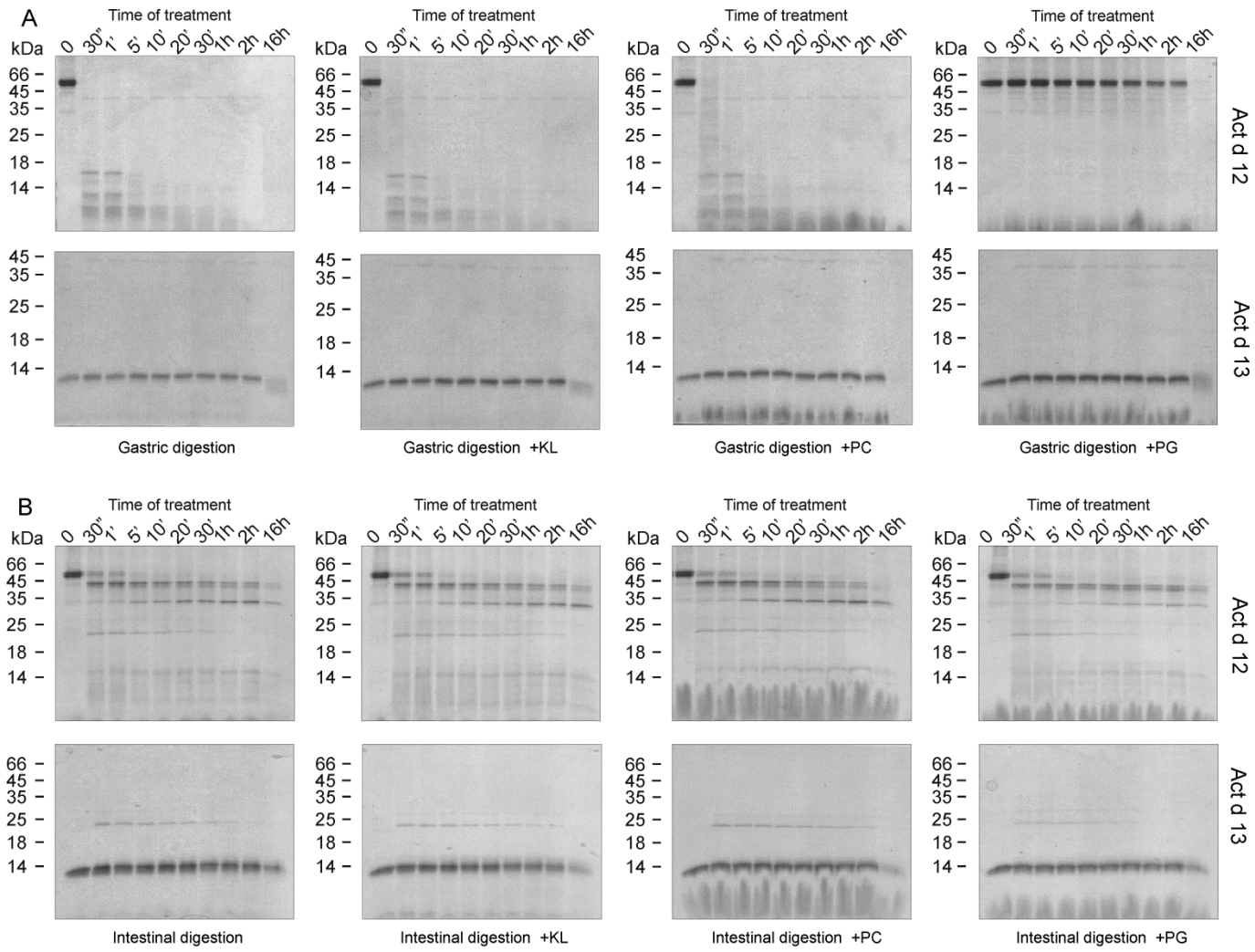
A
Time of treatment
kDa
0 30" 1' 5' 10' 20' 30' 1h 2h 16h
66
45
35
25
18
14
Act d 12
Act d 13
Gastric digestion
Gastric digestion +KL
Gastric digestion +PC
Gastric digestion +PG
B
Intestinal digestion
Intestinal digestion +KL
Intestinal digestion +PC
Intestinal digestion +PG

Figure 2

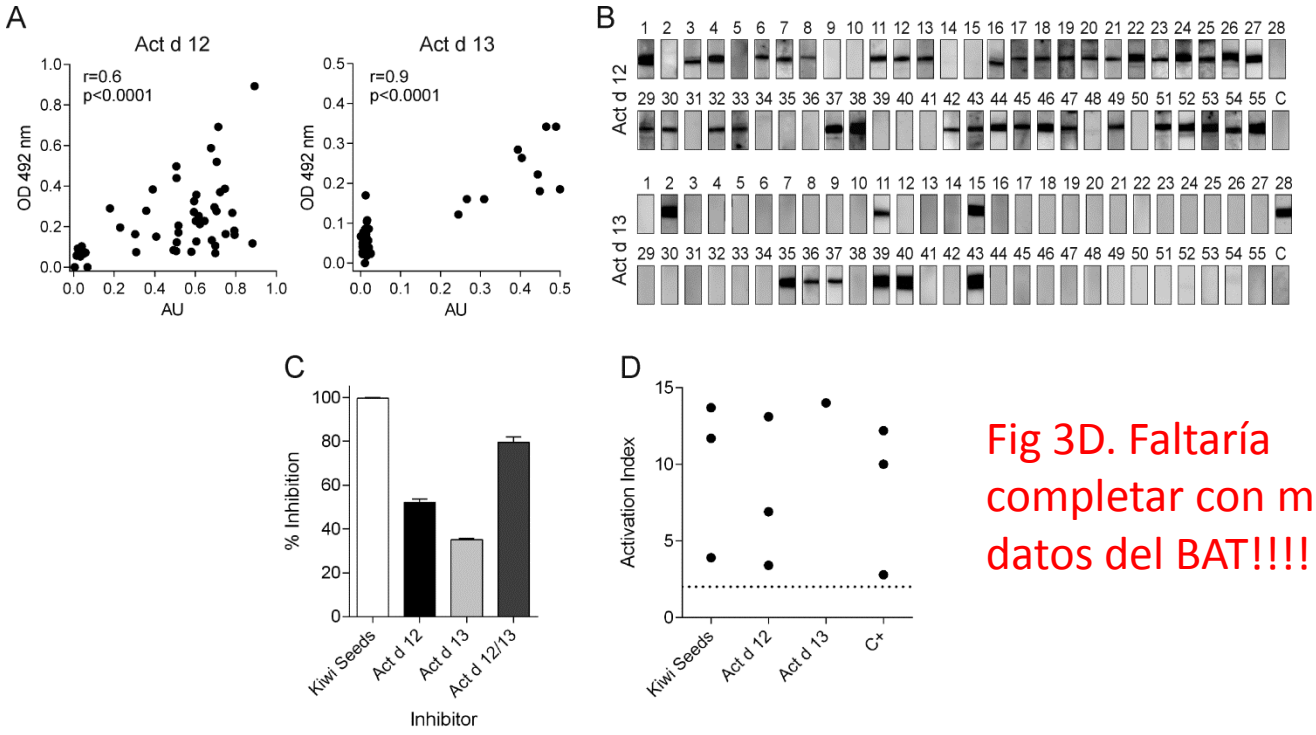

Fig 3D. Faltaría completar con más datos del BAT!!!!

Figure 3

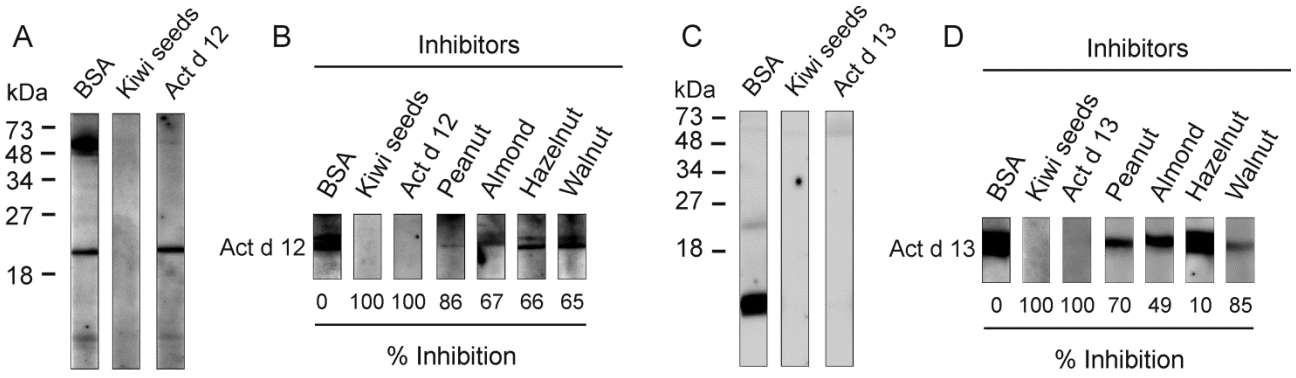
A
kDa
BSA
Kiwi seeds
Act d 12
73
48
34
27
18
B
Inhibitors
BSA
Kiwi seeds
Act d 12
Peanut
Almond
Hazelnut
Walnut
Act d 12
0 100 100 86 67 66 65
% Inhibition
C
kDa
BSA
Kiwi seeds
Act d 13
73
48
34
27
18
D
Inhibitors
BSA
Kiwi seeds
Act d 13
Peanut
Almond
Hazelnut
Walnut
Act d 13
0 100 100 70 49 10 85
% Inhibition

Figure 4

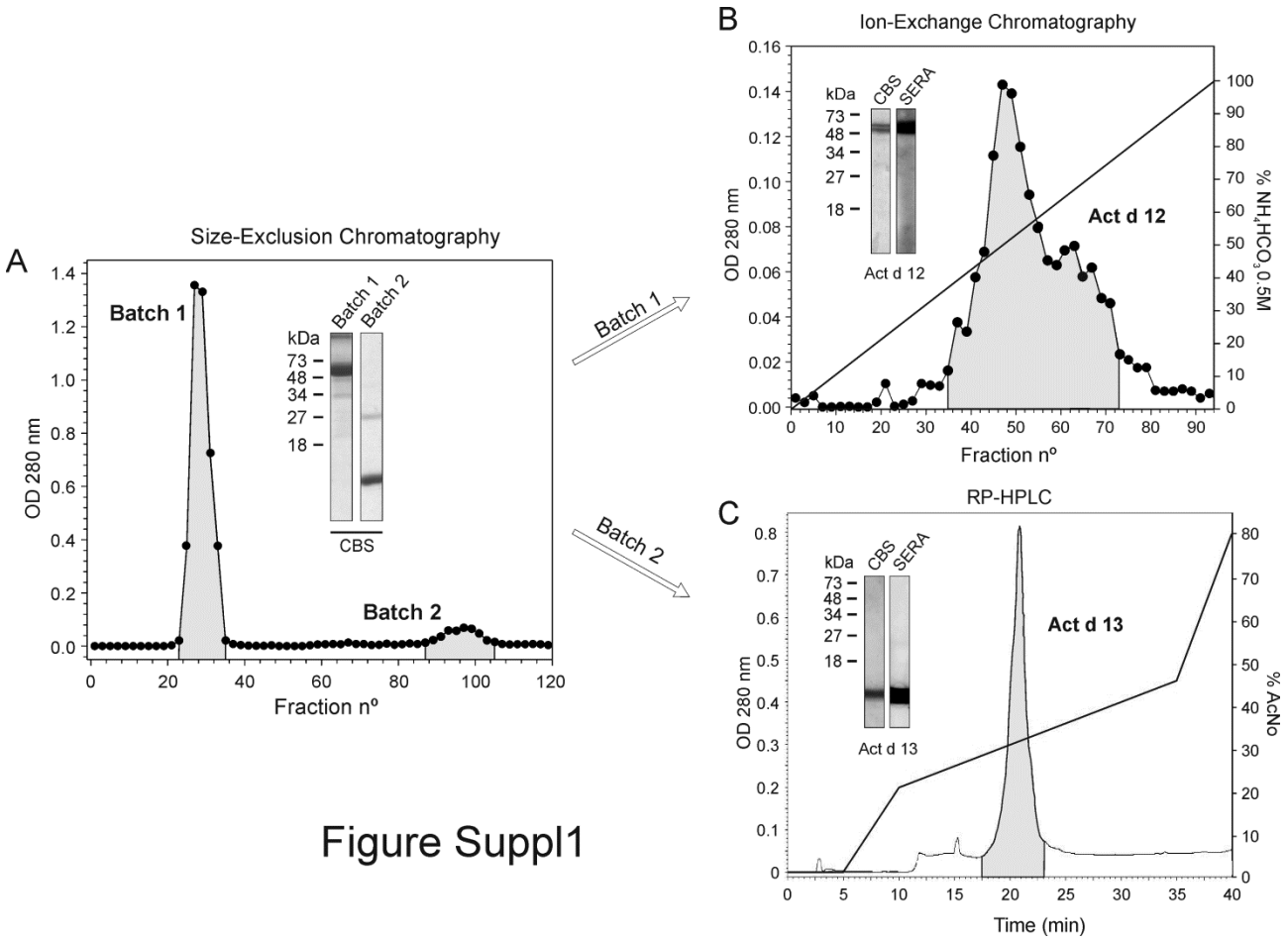

Figure Suppl1

Detailed characterization of Act d 12 and Act d 13 from kiwi seeds: implication in cross-reactivity with peanut and tree nuts

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Short title: Structural and immunological characterization of Act d 12 and Act d 13

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Word count: 2537

Methods

Purification of 11S globulin Act d 12 and 2S albumin Act d 13 and protein extracts

Kiwi seed extract was applied onto a Sephadex G-50 column equilibrated in 0.15 M ammonium bicarbonate, pH 8.0. Fractions containing protein bands of around 51 and 12 kDa were visualized by Coomassie blue staining (CBS) after SDS-PAGE and pooled in two independent batches, respectively (Supplementary Figure 1). The batch 1 containing the 11S globulin Act d 12 was further subjected to ion-exchange chromatography on a DEAE-Cellulose column equilibrated in 20 mM ammonium bicarbonate buffer, pH 8.0. Proteins were eluted with a gradient from 20 to 500 mM ammonium bicarbonate buffer, pH 8.0. The fractions containing the 11S globulin Act d 12 were pooled together. The batch 2 containing the 2S albumin Act d 13 was resolved on a reverse-phase high-performance liquid chromatography (RP-HPLC) nucleosil C-18 column with a gradient (25-45 %) of acetonitrile in 0.1% trifluoracetic acid. SDS-PAGE and CBS were carried out for all purification steps. The IgE reactivity of Act d 12 and Act d 13 was analyzed for each purification step by using a pool of sera from kiwifruit allergic patients diluted 1/3. Protein extracts for kiwi seeds, almond, hazelnut, peanut and walnut were obtained as previously described (1-3)

Analytical procedures

SDS-PAGE was performed in 17 % polyacrylamide gels. Proteins were visualized by Coomassie blue staining (CBS) or alternatively transferred to nitrocellulose membranes (Amersham). The protein concentration was determined using the method of bicinchoninic acid (Pierce Chemical Co) or by Lowry method. Molecular mass determinations were performed by mass spectrometry (MS) using a Bruker Reflex IV matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik), as described (4).

Immunoblotting

Immunodetection of purified Act d 12 and Act d 13 after SDS-PAGE was performed with serum from patients allergic to kiwifruit (1/3 diluted) as described (5). The binding of human IgE was detected by mouse anti-human IgE antibodies (diluted 1:5000; ALK-Abelló), followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1:5000; Pierce). The signal was developed with the ECL-Western blotting reagent (Amersham). For IgE-inhibition experiments the pool of sera (1/3 diluted) was preadsorbed with 1 mg/mL of whole extracts or 20 µg/mL of purified allergens, overnight at 4ºC. BSA was used as negative control of inhibition. Volummograms of the reactive bands were analyzed by scanning densitometry using Multigauge software (Fujifilm) and the obtained values were normalized to patient 1 (Act d 12) or to patient 43 (Act d 13).

ELISA

IgE quantitation was performed by ELISA in microtiter plates (Costar) coated with 100 µl/well of purified Act d 12 or Act d 13 (2 µg/mL) or kiwi seeds extract (20 µg/mL) (5). Plates were incubated with serum from kiwifruit allergic patients (1/3 diluted) and binding of IgE was detected as described above. Peroxidase reaction was developed using fresh enzyme substrate and measuring optical density (OD) at 492 nm. Each value was calculated as the mean of 2 determinations after blank subtraction. OD values <0.1 were considered negative responses. For IgE-inhibition experiments, the corresponding pool of sera (1/3 diluted) was preadsorbed to 20 µg/mL of purified Act d 12 or Act d 13, or a mixture of both or to 1mg/ml of kiwi seeds extract prior to incubation with plates coated with kiwi seeds extract.

Basophil activation test

Basophil activation tests (BAT) were performed with kiwi seeds extract (x mg/mL), purified Act d 12 (y mg/ml) and Act d 13 (z mg/ml) using heparinized whole blood from

kiwifruit allergic patients and healthy controls as described.(6) Anti-human IgE antibodies (BD PharMingen) and physiologic saline solutions were used as positive and negative controls, respectively. Stimulation indexes (SI) were calculated as the ratio between the percentage of degranulated basophils with the stimulus and the negative control. SI values higher than 2 were considered positive according to previously established receiver operating characteristic curves comparing BAT results between patients and control subjects (6).

Carbohydrate detection and circular dichroism

Carbohydrate detection of protein transferred to nitrocellulose membranes was performed by using a biotinylated concanavalin A solution. The staining was developed by horseradish peroxidase reaction with 0.05% diaminobenzidine/0.03% of 30% H_2O_2 in Tris/NaCl reaction buffer. The circular dichroism (CD) spectra were obtained using a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co.) fitted with a 150 W xenon lamp and connected to a Nestlab RTE-111 thermostabilizer bath, at 20 °C and 85 ºC, as described (7). Far-UV spectra (190−250 nm) were registered using optical-path cell of 0.1 cm. The protein concentration was 0.2 mg/mL in 20 mM ammonium bicarbonate, pH 8. Mean residue mass ellipticities were calculated based on 110 as the average molecular mass/residue and expressed in terms of θ (degree × cm^2 × $dmol^{-1}$). Final spectra were corrected by subtracting the corresponding baseline spectrum obtained for the buffer alone under identical conditions.

Preparation of phospholipid vesicles and isolation of lipids from kiwi seeds

Ten milligrams of dried phosphatidylcholine (PC), phosphatidylglycerol (PG) (Avanti Polar Lipids) or kiwi lipids were rehydrated with 1 mL of simulated gastric fluid (SGF), (30 mM NaCl, 48 mM HCl, pH 1.2), or simulated intestinal fluid (SIF), (4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM bis-Tris buffer, 30 mM NaCl, pH 6.5) without enzymes at 37 ºC. The solution was stirred every 10 min at 37 ºC

during 1 h, sonicated for 10 min and stored at 4 ºC until use. Lipids from kiwi seeds were obtained after 3 times extraction with 10% (wt/vol) acetone of lyophilized kiwi seeds extract, dissecated and dissolved in cholophorm:methanol 1:2, aliquoted and dried under nitrogen stream.

Simulated gastric and intestinal digestion

For gastric digestions, purified Act d 12 or Act d 13 were dissolved in SGF without enzyme at 0.2 mg/mL as previously described (8). For some experiments the allergens were mixed with kiwi lipids, PC or PG vesicles to a final lipid concentration of 6.7 mM. Porcine pepsin (Sigma, activity: 4720 U/mg) was added at a ratio of enzyme:substrate 1:20 w/w to a final volume of 200 µl of SGF. The digestion was performed at 37 ºC with moderate shaking. Aliquots of 15 µL were taken at 0, 30 sec, and 1, 5, 10, 20, 30, 60, 120 min and 16 h for SDS-PAGE analysis. The digestion was stopped by increasing the pH with 5 µL of Na_2CO_3 0.2 M, adding 10 µL of 3x loading buffer and keeping samples into liquid nitrogen until use. For intestinal digestions, purified allergens with or without lipids were dissolved in SIF in the presence of 35 µg/mL trypsin and 1.76 µg/mL α-chymotrypsin (Worthington Biochemical Co. 200 U/mg and 50 U/mg, respectively) at a trypsin:chymotrypsin:substrate ratio of 34.5 U:0.44 U:1 mg at final volume of 200 µL. Digestions were stopped by adding phenylmethylsulfonyl fluoride (2 mM). In all the cases, the aliquots withdrawn at different times were frozen and stored at -20 ºC until SDS-PAGE and CBS analysis. Control experiments without enzyme or with BSA instead of allergen were also performed.

Results

First, proteins contained in lyophilized defatted kiwi seeds extract were separated by size-exclusion chromatography (Supplementary Fig. 1A). Fractions containing proteins of around 51 and 12 kDa were pooled in two different batches according to CBS and IgE-reactivity after SDS-PAGE (Supplementary Fig. 1A, inset). Act d 12 was further purified to homogeneity from batch 1 by ion-exchange chromatography (Supplementary Fig. 1B). The purified Act d 12 migrated as a single protein in SDS-PAGE under non-reducing conditions and retained the IgE-binding capacity (Supplementary Fig. 1B, inset). Act d 13 was purified from batch 2 by RP-HPLC and also migrated as a single monomer of around 12 kDa under non-reducing conditions and retained the IgE-binding capacity (Supplementary Fig. 1C, inset).

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Legend to figure

Supplementary Figure 1. (A) Elution profile of kiwi seeds extract after a size-exclusion chromatography. Two main proteins of around 51 and 12 kDa were separated into Batch 1 and 2 (shadow in grey). The kiwi seeds extract and the proteins of each batch were visualized by CBS after SDS-PAGE (inset). **(B)** Elution profile of Batch 1 after ion-exchange chromatography. Fractions containing the 11S globulin Act d 12 is shadowed in grey. Purified Act d 12 was visualized by CBS and its IgE-binding capacity was analyzed with sera from kiwifruit allergic patients (inset). **(C)** Elution profile of Batch 2 in RP-HPLC. Fractions containing the 2S albumin Act d 13 is shadowed in grey. Purified Act d 13 was visualized by CBS and its IgE-binding capacity was analyzed with sera from kiwifruit allergic patients (inset).