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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

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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Keywords

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

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Abstract

Background: Act d 12 (11S globulin) and Act d 13 (2S albumin) are two novel relevant allergens from kiwi seeds that might be useful to 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 chromatographic procedures. Circular dichroism, mass spectrometry, concanavalin A detection, immunoblotting, enzyme-linked immunosorbent assays, basophil activation tests, and IgE-inhibition experiments were used.

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. Both purified allergens preserve the structural integrity and display typical features of their homologous counterparts from the 11S globulin and 2S albumin protein families, respectively. These allergens are released from kiwi seeds after oral and gastric digestion of whole kiwifruit, demonstrating their bioavailability after ingestion. The 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.

The consumption of kiwifruit (*Actinidia deliciosa*) has significantly risen over the last decades in Western countries (1, 2), likely due to the reported beneficial effects associated with 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 (7–9). 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 specific-

Abbreviations

BAT, basophil activation test; 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; PC, phosphatidylcholine; PG, phosphatidylglycerol; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SPT, skin prick test.

ity (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, 10).

Up to date, 13 kiwifruit allergens have been described (www.allergen.org) (6, 11–13). Almost all the kiwifruit allergens were initially described in the kiwi pulp (Act d 1–Act d 11) with the exception of Act d 10 that was also reported in seeds (14). 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 (12). 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 group of 55 Kiwifruit-allergic patients, controls from pollen-allergic patients, and nonatopic 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 three Hospitals, and written informed consent was obtained from all subjects (Detailed protocols are fully described in the supporting information of this article).

Results

Act d 12 and Act d 13 display common structural features to their homologous counterparts

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 follow-

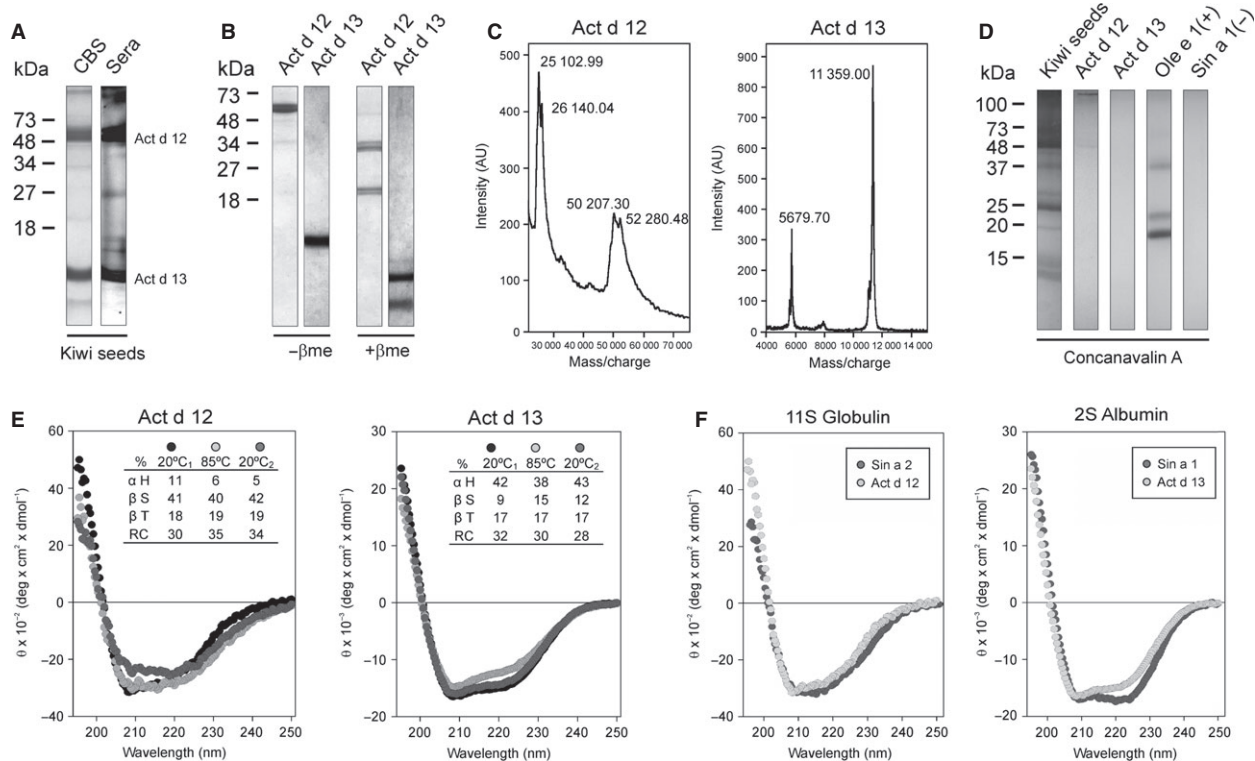


Figure 1 Structural characterization of purified Act d 12 and Act d 13. (A) coomassie blue staining (CBS) and IgE immunoblotting with a pool of sera from kiwifruit-allergic patients after SDS-PAGE of kiwi seeds extract. (B) CBS and IgE immunoblotting after SDS-PAGE of purified Act d 12 or purified Act d 13 in the absence or presence of β -mercaptoethanol (β me). (C) Molecular mass determination of the purified Act d 12 and Act d 13 by MS. (D) Sugar staining with biotinylated ConA

after SDS-PAGE of kiwi seeds extract, purified Act d 12, Act d 13, Ole e 1 (positive control), and Sin a 1 (negative control). (E) circular dichroism (CD) spectra of purified Act d 12 and Act d 13 in the far-UV at 20°C, at 85°C, and cooling down again at 20°C. Secondary structure contributions (α H, α -helix; β S, β -sheet; β T, β -turn; RC, random coil) are shown. (F) CD spectra of purified Act d 12 and Act d 13 in the far-UV at 20°C compared to those from Sin a 2 and Sin a 1, respectively.

ing the sequential chromatographic steps described in the supporting information (Fig. S1). The purity and structural integrity of Act d 12 and Act d 13 was assayed by coomassie blue staining (CBS) and IgE-binding analysis after SDS-PAGE (Fig. S1B,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 and the corresponding doubly charged ions (25102.99/26140.04 Da; Fig. 1C). For Act d 13, a single peak at 11359.0 Da with the corresponding doubly charged ion 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 circular dichroism (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°C to 80°C) 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 (Fig. 1E). Both allergens show similar secondary structure content than their homologous counterparts from yellow mustard seeds as demonstrated by CD spectra (Fig. 1F).

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, or phosphatidylcholine (PC) or phosphatidylglycerol (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

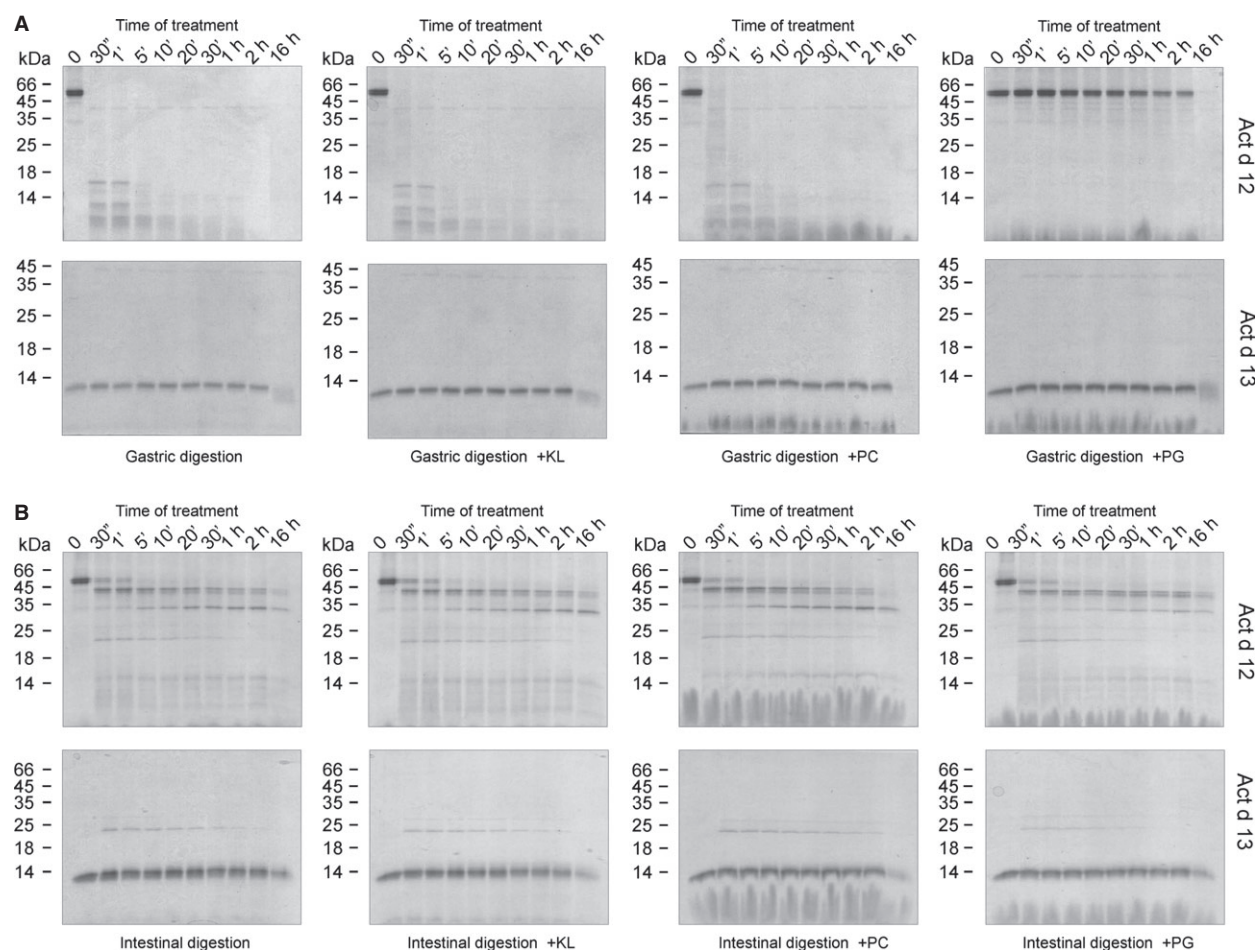


Figure 2 Simulated gastric and intestinal digestion of purified Act d 12 and Act d 13. Coomassie blue staining of the gastric digestion (A) or intestinal digestion (B) products of purified Act d 12 or Act d

13 alone or in the presence of kiwi lipids (+KL), or phosphatidylcholine (PC) (+PC) or phosphatidylglycerol (PG) (+PG) vesicles. Molecular markers are indicated in kDa in the left side.

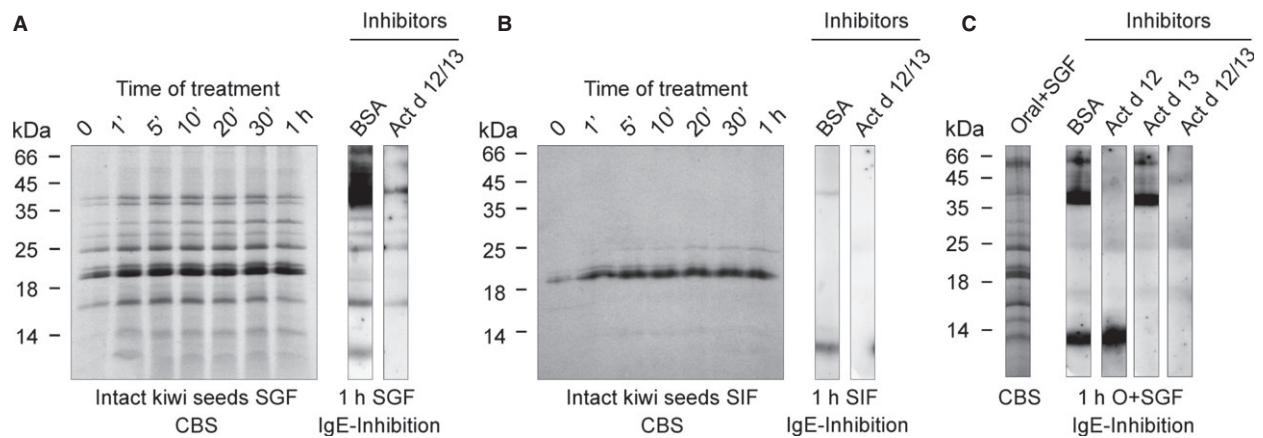


Figure 3 Bioavailability of Act d 12 and Act d 13. (A) Gastric digestion of intact kiwi seeds in SGF at the indicated time points (left panel). Inhibition of the IgE binding to Act d 12 and Act d 13 released from intact kiwi seeds after 1 h of gastric digestion by the indicated inhibitors (right panel). (B) Intestinal digestion of intact kiwi seeds in SIF at the indicated time points (left panel). Inhibition of the IgE binding to Act d 12 and Act d 13 released from intact

kiwi seeds after 1 h of intestinal digestion by the indicated inhibitors. (C) Coomassie blue staining after oral and subsequent gastric digestion (oral + SGF) of whole kiwifruit (left panel). Inhibition of the IgE binding to Act d 12 and Act d 13 released from whole kiwifruit after oral and gastric digestion by the indicated inhibitors (right panel). For all IgE-inhibition experiments, a pool of sera from kiwifruit-allergic patients was used.

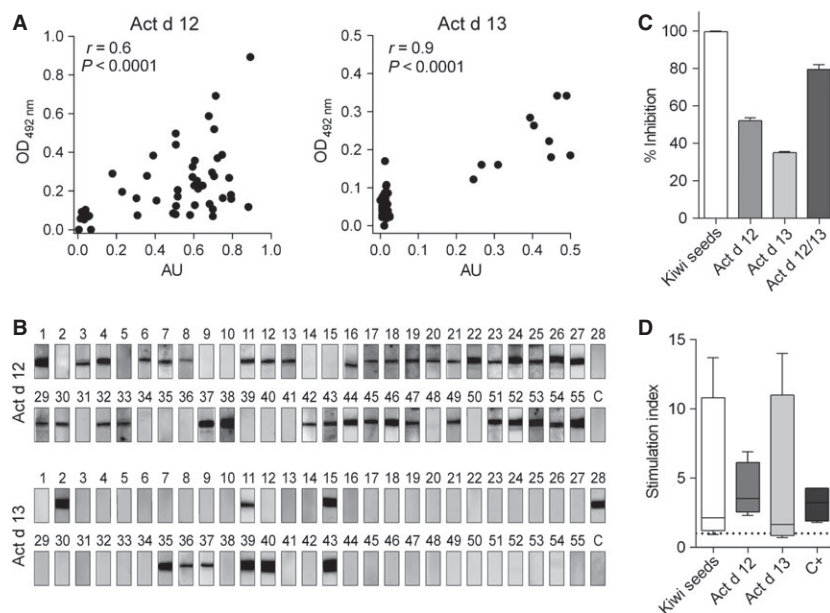


Figure 4 Allergenic characterization of purified Act d 12 and Act d 13. (A) Correlation between ELISA and immunoblotting reactivity for the purified allergens for the 55 sera from patients allergic to kiwifruit. OD, optical density at 492 nm. AU, arbitrary units showing the relative intensity for each serum after normalization of the values determined by scanning densitometry with those obtained for serum 1 (Act d 12) and 43 (Act d 13). (B) Immunoblotting for purified Act d 12 and Act d 13 with the individual sera. C, serum

from a nonatopic subject. (C) Inhibition of the IgE binding of a pool of sera from kiwifruit-allergic patients to kiwi seeds extract: with (1 mg/ml) kiwi seeds extract, (20 micrograms/ml) of purified Act d 12, Act d 13 or an equal mixture of both purified allergens. (D) Basophil activation test in four kiwifruit-allergic patients using kiwi seeds extract and purified Act d 12 and Act d 13; C+, positive control with histamine; horizontal dashed line represents the obtained values for the negative control with PBS; SI (stimulation index).

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, or 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, or 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, or PC or PG vesicles, respectively, as determined by scanning densitometry (Fig. 2B).

Bioavailability of Act d 12 and Act d 13 after oral, gastric, and intestinal digestion

To assess the bioavailability of Act d 12 and Act d 13, intact kiwi seeds were subjected to simulated gastric fluid (SGF) or to simulated intestinal fluid (SIF) without enzymes as described in the supporting information. Both allergens were released from intact kiwi seeds after 1 h of SGF treatment, which was demonstrated by the inhibition of the IgE binding to the protein bands of 51, 40, and 10 kDa with the mixture of purified Act d 12 and Act d 13 (Fig. 3A). Similar results were obtained after SIF treatment (Fig. 3B). The availability of Act d 12 and Act d 13 was also investigated after oral and subsequent gastric digestion of entire kiwifruit slices. Kiwifruit slices were chewed for 15 s without swallowing and subsequently subjected to gastric digestion for 1 h (oral + SGF; Fig. 3C). The IgE binding to the released protein bands of 51, 40, and 12 kDa was inhibited by purified Act d 12 and Act d 13, respectively, thus demonstrating that both allergens are also released from the seeds after oral and SGF treatments of the whole kiwifruit. In all the cases, Act d 12 is predominantly released as a fragment of around 40 kDa.

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 was demonstrated by ELISA and immunoblotting with the cohort of 55 kiwifruit-allergic patients (10). When we plotted the obtained ELISA values for both allergens vs the corresponding immunoblotting data (arbitrary units determined by scanning densitometry, 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. 4A). Act d 12 and Act d 13 were recognized by 70.9% and 18.2% of the tested patients, respectively, in immunoblotting (Fig. 4B).

To quantitate 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 Act d 12 (53%; Fig. 4C). 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. 4C).

The allergenic capacity of Act d 12 and Act d 13 was assessed by *in vitro* basophil activation test (BAT). Both allergens demonstrated capacity to induce IgE cross-linking in effector-circulating basophils (Fig. 4D). Kiwifruit-allergic patients with specific IgE to kiwi seeds extract and to Act d

Table 1 Clinical features of the patients selected for *in vitro* IgE inhibition experiments

Sera pool	Patient number	Sex/Age (years)	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	Yes	OAS	12	12	12	Neg
	2	M/39	U	12	0.069	0.692	0.073	Yes	A, AN	12	Neg	48	Neg
	3	F/19	OAS	9	0.066	0.52	0.051	Yes	OAS	9	20	12	9
	4	F/21	OAS	Neg	0.02	0.205	0.054	Yes	OAS	18	35	15	42
	5	F/39	OAS	Neg	0.142	0.133	0.028	Yes	OAS	ND	ND	ND	ND
Act d 13 positive	6	M/27	U	22	0.146	0	0.185	Yes	OAS	Neg	Neg	22	Neg
	7	F/20	OAS	12	0.063	0.069	0.342	Yes	OAS	12	Neg	10	12
	8	F/17	OAS	12	0.212	0.053	0.264	Yes	OAS	ND	ND	ND	ND
	9	F/36	OAS	Neg	0.112	0.068	0.181	No	OAS	12	Neg	22	22
	10	M/37	OAS	Neg	0.235	0.439	0.223	Yes	AN, OAS	22	22	22	Neg

y, year; M/F, male/female; Neg, negative; AN, anaphylaxis; 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; SPT, skin prick test.

*Skin prick test wheal area in mm².

†Specific IgE determined in ELISA as OD at 492 nm.

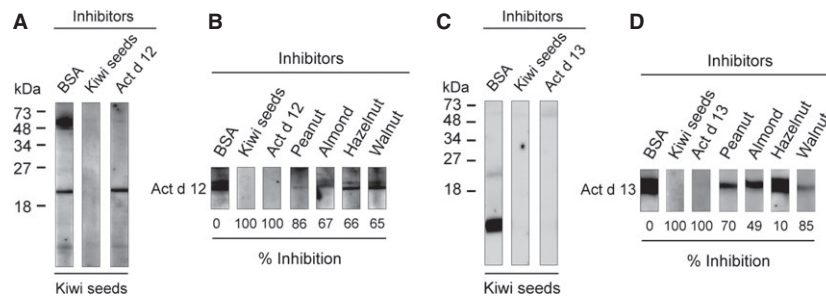


Figure 5 Immunoblotting inhibition experiments. Inhibition of the IgE binding to Act d 12 contained in kiwi seeds extract (A) or to purified Act d 12 (B) by the indicated inhibitors when using a pool of sera from kiwifruit-allergic patients sensitized to Act d 12. Inhibition of the IgE binding to Act 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).

12 or Act d 13 showed positive BAT with kiwi seeds extract, Act d 12 or Act d 13 with stimulation index values (mean $\pm$ SEM) of 4.7 ± 3 , 0, 4.1 ± 1.0 , and 4.5 ± 3.2 , respectively (Fig. 4D).

Act d 12 and Act d 13 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 1): (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 pre-adsorbed to kiwi seeds extract or to Act d 12 (Fig. 5A). The IgE binding to 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 Act d 12 (Fig. 5B). 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 pre-adsorbed to kiwi seeds extract or to Act d 13 (Fig. 5C). Inhibition of the IgE binding to Act d 13 was observed with walnut (85%), peanut (70%), and almond (49%), but not with hazelnut (10%; Fig. 5D). Complete inhibition was obtained with kiwi seeds or Act d 13.

Discussion

In this study, we performed a comprehensive structural and immunological characterization of Act d 12 and Act d 13. Our results demonstrated that both allergens preserve the structural integrity and display typical features of their homologous counterparts from the 11S globulin and 2S albumin protein families, respectively. Both 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 Act d 12 and Act d 13 as

well-defined molecules and their inclusion in CRD approaches might well contribute to improve the diagnostic sensitivity of kiwifruit allergy.

Diagnosis of kiwifruit allergy has significantly improved during the last years (2, 5, 6, 10). However, the main drawbacks are still the classical difficulties associated with 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 (12).

The use of well-defined purified allergens is very useful to improve diagnosis, management, and treatment of allergic patients (16–20). We purified and deeply characterized Act d 12 and Act d 13 from kiwi seeds. 11S globulins and 2S albumins are nonglycosylated and very stable seed-specific storage proteins composed of two polypeptide chains linked by disulfide bridges, structural features that were retained in 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 and Act d 13 to reach the intestinal gut as intact molecules able to interact with immune system cells, we subjected both allergens to different protease treatments. Act d 13, as other 2S albumins, was very resistant to simulated gastric and intestinal digestions in all the assayed conditions (28–30). In contrast, Act d 12 was rapidly digested by gastric enzymes 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. To assess bioavailability, we analyzed the release of both allergens from intact kiwi

seeds after oral, gastric, and intestinal treatments. Both allergens are released from the intact kiwi seeds after SGF/SIF treatments and from the whole kiwifruit after oral and gastric treatment. Our data suggest that Act d 12 and Act d 13 in the presence of food matrix protective molecules might well reach the oral cavity and gastrointestinal tract in an immunological active form able to sensitize or to trigger allergic reactions.

Act d 12 represents a major allergen recognized by more than 50% (39/55) of the 55 kiwifruit-allergic patients included in this study, whereas Act d 13 is a minor allergen (10/55). 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 Act d 12 and Act d 13 conserved common epitopes of homologous counterparts from peanut, almond, hazelnut, or walnut, suggesting that these allergens might be involved in cross-reactivity with these allergenic sources as have been described for 11S globulins and 2S albumins 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. Act d 12 and Act d 13 showed *in vitro* IgE cross-reactivity with homologous counterparts from tree nuts and peanut. Further detailed studies are required to confirm the clinical impact of this fact, which might also have essential implications in the management of kiwifruit-allergic patients.

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Author contributions

OP and SS conceived and designed the experiments. SS and BC performed the *in vitro* experiments. FG, NB, JCH, GC, and MB carried out clinical characterization of the patients. OP, SS, BC, FG, NB, JCH, GC, MB, RR, and MV analyzed and discussed the data. FG, NB, JCH, GC, MB, RR, MV, and OP contributed reagents/materials/analysis tools. OP wrote the paper. All the authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods and results.

Figure S1. (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).

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

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Methods and results

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

Kiwi seeds, almond, hazelnut, peanut and walnut protein extracts were obtained as previously described for yellow mustard seeds with minor modifications (1, 2). Kiwi seeds powder obtained from kiwifruit of *Actinidia deliciosa* was suspended in 0.15 M sodium borate buffer, pH 8, at a 5% (wt/vol) ratio and gently stirred for 1 h and centrifuged at 4 °C. The pellet was extracted twice with borate buffer, and the supernatants were lyophilized. The resulting material was then extracted 3 times with 5% (wt/vol) acetone. The pellets were air desiccated and dissolved in 0.15 M ammonium bicarbonate, pH 8. After centrifugation at 8,700 g, at 4 °C, supernatants were lyophilized and stored at –20 °C.

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% trifluoroacetic 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.

Formattato: Colore carattere: Testo 1

Formattato: Tipo di carattere:
Grassetto, Colore carattere: Testo 1

Formattato: Colore carattere: Testo 1

Formattato: Tipo di carattere:
Grassetto, Colore carattere: Testo 1

54

55 *Analytical procedures*

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

63

64 *Immunoblotting*

65 Immunodetection of purified Act d 12 and Act d 13 after SDS-PAGE was performed
66 with serum from patients allergic to kiwifruit (1/3 diluted) as described (5). The binding
67 of human IgE was detected by mouse anti-human IgE antibodies (diluted 1:5000; ALK-
68 Abelló), followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted
69 1:5000; Pierce). The signal was developed with the ECL-Western blotting reagent
70 (Amersham). For IgE-inhibition experiments the pool of sera (1/3 diluted) was
71 preadsorbed with 1 mg/mL of whole extracts or 20 µg/mL of purified allergens,
72 overnight at 4 °C. BSA was used as negative control of inhibition. Volummograms of
73 the reactive bands were analyzed by scanning densitometry using Multigauge software
74 (Fujifilm) and the obtained values were normalized to patient 1 (Act d 12) or to patient
75 43 (Act d 13).

76

77 *ELISA*

78 IgE quantitation was performed by ELISA in microtiter plates (Costar) coated with 100
79 µl/well of purified Act d 12 or Act d 13 (2 µg/mL) or kiwi seeds extract (20 µg/mL) (5).
80 Plates were incubated with serum from kiwifruit allergic patients (1/3 diluted) and
81 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, purified Act d 12 and Act d 13 (0.01-50 µg/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% H₂O₂ 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 °C, 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 $\times$ cm² $\times$ 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 °C. The solution was stirred every 10 min at 37 °C during 1 h, sonicated for 10 min and stored at 4 °C until use. Lipids from kiwi seeds were obtained after 3 times extraction with 5% (wt/vol) acetone of lyophilized kiwi seeds extract, dissected and dissolved in chloroform: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 μ L of SGF. The digestion was performed at 37 °C with moderate shaking. Aliquots of 15 μ L 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 μ L of Na₂CO₃ 0.2 M, adding 10 μ L 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 μ g/mL trypsin and 1.76 μ g/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 μ L. 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 °C until SDS-PAGE and CBS analysis. Control experiments without enzyme or with BSA instead of allergen were also performed.

Bioavailability studies

Gastric and intestinal digestion of intact kiwi seeds were carried out as previously reported in 50% (w/v) of SGF and SIF in the absence of proteolytic enzymes. 0.5% of each sample was used SDS-PAGE and CBS or to be transferred onto nitrocellulose membranes. For IgE-inhibition experiments we used as inhibitors purified Act d 12 and Act d 13 and a pool of sera from kiwifruit allergic patients. Kiwifruit was peeled and cut into slices of 9 g each. Four healthy individuals chewed one slice for 15 seconds and the resulting material was collected and centrifuged for 10 min 4 °C at 8500 rpm. The pellet was collected, weighted, suspended in SGF 1:1 without pepsin and gently stirred for 1 h at room temperature. After centrifugation for 10 min 4 °C at 8500 rpm, supernatant (oral+SGF) was collected and storage at -20 °C until use.

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).

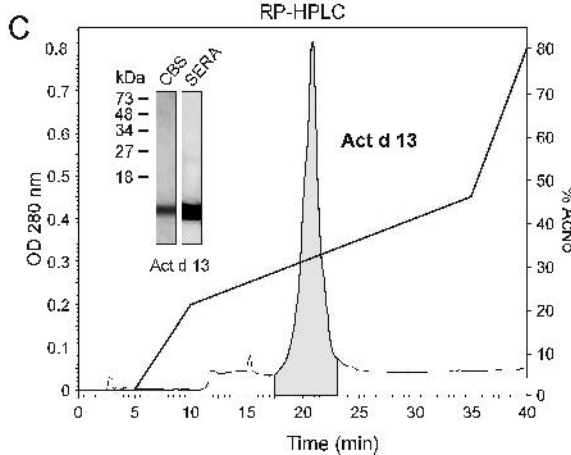
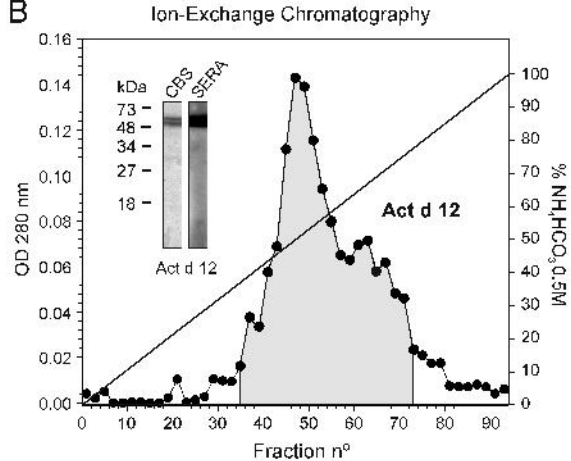
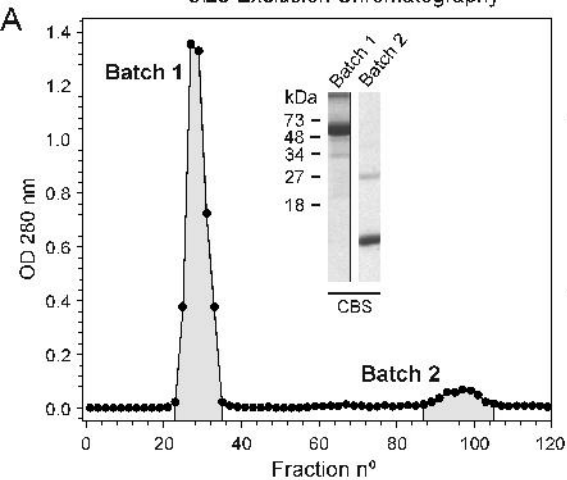


Figure Suppl1