

Evaluation of IgE binding to proteins of hardy (*Actinidia arguta*), gold (*Actinidia chinensis*) and green (*Actinidia deliciosa*) kiwifruits and processed hardy kiwifruit concentrate, using sera of individuals with food allergies to green kiwifruit

Lingyun Chen ^a, Jane S. Lucas ^b, Jonathan O. Hourihane ^b, Julianne Lindemann ^c,
Steve L. Taylor ^a, Richard E. Goodman ^{a,*,1}

^a Food Allergy Research and Resource Program, University of Nebraska, 143 Food Industry Complex, Lincoln, NE 68583 0955, USA

^b Allergy and Inflammation Sciences, University of Southampton, Southampton, UK

^c Efficas, Inc., Boulder, CO, USA

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Abstract

Background: Allergy to green kiwifruit has become common since the fruit was introduced in North America and Europe 30 years ago. Gold kiwifruit, more recently introduced commercially, has been shown to bind IgE from some individuals allergic to green kiwifruit. Hardy kiwifruit is a third species that is now cultivated in North America with potential application as a fresh fruit and in processed foods.

Objective: To compare the IgE binding properties of proteins in hardy kiwifruit extract and processed hardy kiwifruit concentrate to each other and to extracts of green and gold kiwifruits to evaluate the potential for allergic cross-reactions.

Methods: Sera from kiwifruit-allergic subjects and individuals without allergies to kiwifruit were assayed for IgE binding to soluble proteins in green, gold and hardy kiwifruits and heat-processed concentrate from hardy kiwifruit using immunoblots and direct enzyme-linked immunosorbent assay (ELISA).

Results: Marked IgE binding to specific hardy kiwifruit proteins was identified. However, IgE binding to heat-processed hardy kiwifruit concentrate was remarkably lower than to the raw fruit extract.

Conclusions: These results suggest that some kiwifruit-allergic individuals may suffer allergic cross-reactions if they consume raw hardy kiwifruit. However, heat processing of the hardy kiwifruit alters allergenic protein structure, dramatically reducing in vitro IgE binding. Processing likely reduces the risk of eliciting an allergic response in those with allergies to raw kiwifruit.

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1. Introduction

Food allergy has become a public health issue. The prevalence of food allergy in North America, the European Union and other reporting countries has been estimated to range from 1% to 2% of adults and 6% to 8% of young children based on clinical diagnosis (Sampson, 2005). Symptoms range from mild to severe and life-threatening reactions (Sampson, 2005). Evidence exists suggesting that

Abbreviations: Act c 1, cysteine protease; Act c 2, thaumatin-like protein; DBPCFC, double-blinded, placebo-controlled food challenge; NFD, non-fat dried milk; PBST, phosphate buffered saline with Tween-20; SPT, skin prick test.

* Corresponding author. Tel.: +1 402 472 0452; fax: +1 402 472 1693.

E-mail address: rgoodman2@unl.edu (R.E. Goodman).

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allergies to kiwifruit are becoming more common as the consumption has increased across populations (Lucas et al., 2005).

Green kiwifruit (*Actinidia deliciosa*), a native of China, was introduced into New Zealand in 1904, in the US in 1962 (Lucas et al., 2003) and later in the European Union. It has become popular for consumption as a fresh fruit and as a component in some processed juice-drinks, partly because of the high vitamin C content (Nishiyama et al., 2002). The first published report of an allergic reaction to any kiwifruit was a case of contact sensitivity that resulted in systemic reactions (Fine, 1981; Falliers, 1983). As reviewed previously (Lucas et al., 2003), there is clear evidence that green kiwifruit is allergenic and that it can elicit allergic cross-reactions in some individuals with allergies to natural latex, other fruits and specific pollens due to similarities between kiwifruit proteins and hevein-like proteins or chitinases from those sources (Bublin et al., 2004; Diaz-Perales et al., 1999; Fahlbusch et al., 1998; Rudeschko et al., 1998). Based on reports that many allergic reactions to kiwi are localized in the oropharynx, that skin prick tests (SPT) often must be performed with fresh kiwifruit rather than commercial skin prick test extracts, and that in vitro kiwifruit-specific IgE assays are often quite insensitive, it appears that some of the important allergens in kiwifruit are quite labile (Aleman et al., 2004; Fiocchi et al., 2004). There is also evidence that proteins in a gold kiwifruit (*Actinidia chinensis*) can bind IgE from sera of some individuals with allergies to green kiwifruit (Bublin et al., 2004) and can induce allergic reactions in some of green kiwi-allergic patients (Lucas et al., 2004). However, the reported major allergen, actinidin (Act c 1), was not found in extracts of gold kiwifruit, while it was abundant in green kiwifruit (Bublin et al., 2004).

A third species of kiwifruit, called hardy kiwifruit (*Actinidia arguta*), is being cultivated on limited acreage in western North America (Williams et al., 2003) and is being tested as a potential processed food product. The evidence of cross-reactivity between green and gold kiwifruit leads to a prediction that some individuals with allergies to those fruits may suffer cross-reactions if they consume hardy kiwifruit. Therefore, we were interested in evaluating IgE binding to raw hardy kiwifruit. In addition, in the future this fruit may be consumed as a heat processed extract or concentrate. Since heating has little or no effect on some allergens such as the lipid transfer proteins in cherry and peaches (Scheurer et al., 2004), but dramatically reduces the allergenic activity of many proteins such as the birch pollen associated fruit allergen Mal d 1 (Vieths et al., 2002), we also tested a heat-processed concentrate of hardy kiwifruit to evaluate the affect of heating on IgE binding.

2. Materials and methods

2.1. Serum donors

Sera of 12 kiwifruit-allergic subjects were collected in a previous clinical study of responses to green and gold kiwifruit at the University of Southampton, UK (Lucas et al., 2004). The donors in that study were

clinically diagnosed as allergic to kiwi based on clinical histories and a combination of double-blinded placebo-controlled food challenge (DBPCFC), skin prick tests (SPT) and specific IgE tests (ImmunoCAP™, Pharmacia Diagnostics). Historical information and diagnostic results for all kiwifruit allergic donors (K02–K228) are presented in Table 1 along with data for donors of non-kiwifruit allergic control donors (C01–C04) collected by the University of Nebraska.

2.2. Kiwifruit sample preparation for in vitro studies

Samples for the in vitro study of IgE binding included whole freshly peeled green kiwifruit (*A. deliciosa* var. Hayward) and gold kiwifruit (*A. chinensis*) which were purchased from a local supermarket (Lincoln, NE). Fresh-frozen hardy kiwifruit (*A. arguta*) and heat-processed hardy kiwifruit concentrate were provided by Efficas, Inc. (Boulder, CO). The hardy kiwifruit concentrate was prepared from sliced, dried fruit by extraction in hot water (90 °C) in a stirred reaction vessel for several hours followed by pressure filtration (10 µm). The filtered extract was concentrated by evaporation under vacuum. Extracts of fruits were prepared by pureeing the peeled fresh fruit of green and gold kiwifruit or sliced hardy kiwifruit in extraction buffer in a Waring blender at a tissue to extraction buffer ratio of 1:2 (w:v). The hardy kiwifruit concentrate was dissolved in the same extraction buffer. The extraction buffer was phosphate buffered saline (PBS, 0.01 M, pH 7.4) containing Complete® EDTA-free protease inhibitor cocktail (Roche Applied Science) at one tablet per 50 mL of PBS. Homogenized samples were incubated at room temperature for 2 h before centrifuged at 2000g for 1 h at 4 °C to remove solids. Supernatants (extracts) were collected, assayed for protein content by the method of Lowry using a DC protein assay (Bio-Rad) and stored in aliquots at –20 °C. Preliminary tests with fresh extracts of fruit to evaluate protein content and binding characteristics in ELISA tests proved unreliable as protein content as measured for total N by the Dumas combustion method (Leco Instruments, Inc.) was significantly different from results by Lowry, and protein binding to ELISA plates even with very high concentrations of protein in coating buffer was not efficient (data not shown). Therefore samples of the frozen extracts were thawed and dialyzed using Spectra/Por® membrane MWCO 3500 (Spectrum Laboratories, Inc.) against multiple changes of PBS over 48 h at 4 °C to remove sugars, polyphenols, organic acids and other small molecular weight compounds. The retained protein solutions were reassayed using a DC protein assay (modified Lowry method from BioRad Laboratories), then diluted appropriately based on the new protein value for SDS polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and enzyme-linked immunosorbent assay (ELISA).

2.3. SDS-PAGE of kiwifruit samples

Dialyzed and non-dialyzed samples of the four kiwifruit preparations (green, gold, hardy and heat-processed hardy concentrate) were mixed at a ratio of 4:1 with 5X Laemmli buffer (containing 0.312 M Tris–HCl buffer, pH 6.8; 10% (w/v) SDS; 50% (v/v) glycerol; 25% (v/v) 2-mercaptoethanol and a trace amount of bromophenol blue), then heated in boiling water for 5 min. Ten microliters of each sample (containing 20 µg protein) was loaded onto a 15-well Novex® Tris–Glycine 10–20% gel (Invitrogen). Precision Plus Protein Dual Color Standards (Bio-Rad) were included in the gel to estimate the apparent molecular weight of kiwifruit protein bands. Non-reducing SDS-PAGE samples were prepared by mixing with 5X Laemmli sample loading buffer without 2-mercaptoethanol. The non-reducing samples were not heated prior to loading on the gel.

Electrophoresis was carried out in a SureLock™ Mini-Cell system (Invitrogen). Following electrophoresis, proteins were transferred to Invitrolon PVDF membranes using an Xcell II™ Blot Module (Invitrogen) for immuno-detection. Transfer of proteins from the gel to each of the PVDF membranes was verified by staining the membranes with Ponceau S stain (Sigma–Aldrich). Identically loaded gels were stained with Brilliant Blue G colloidal concentrate (product B2025, Sigma–Aldrich) following the manufacturer's instructions. The images were captured using a Kodak

Table 1
Characteristics of serum donors

Donor #	Age (years)	Age onset	Symptoms ^a kiwi	DBPCFC kiwi	SPT (mm)	CAPS kUA/L	Other known allergies ^b
<i>Kiwifruit allergic</i>							
K02	9	6	AE OE U	Green: FE U Gold: OP	6	3.6	Birch pollen, cat, house dust mite
K15	11	8	OE OP	Green: OP Gold: nd	3	0.6	Peanuts, house dust mite, grass pollen
K29	9	2	U (multiple occ.)	Green: AP U Gold: none	4	1.9	Peanut, tree nuts, sesame, house dust mite
K47	9	1	AP FE OE	Green: U Gold: U FE	8.5	2.35	Birch pollen, house dust mite, peanuts, tree nuts, grass pollen
K93	63	57	OE V (multiple occ.)	nd	nd	1.4	Shellfish
K96	58	57	OE OP V (multiple occ.)	nd	nd	0.6	None
K98	43	27	AE AP OE (multiple occ.)	Green: AP F Gold: nd	8	0.7	Birch pollen
K140	52	40	OS	Green: OS Gold: OS	13	6.8	None
K157	14	4	AS	nd	10	0.7	None
K165	22	19	FE OP	Green: OP LB Gold: nd	12	1.2	House dust mite, grass pollen (SPT + avocado)
K217	64	58	OE	nd	nd	2.4	Latex contact dermatitis
K228	50	48	OE OS	Green: OE Gold: nd	6	0.5	House dust mite
<i>Controls</i>							
C01	47	–		nd		<0.35	None
C02	50	–		nd		<0.35	None
C03	45	–		nd		<0.35	Pollenosis
C04	36	–		nd		<0.35	Peanut anaphylaxis

Sera from kiwifruit-allergic individuals (K01–K228), diagnosed based on clinical histories, food challenge, skin prick tests and green kiwifruit-specific IgE (ImmunoCAP from Pharmacia Diagnostics) and sera from individuals who are not allergic to kiwifruit based on questions and negative ImmunoCAP tests to green kiwifruit were used in testing for IgE binding to proteins from green, gold and hardy kiwifruit as well as heat-processed hardy kiwifruit concentrate.

^a AE: angioedema, AP: abdominal pain, AS: anaphylactic shock, F: flushed, FE: facial edema, LB: labial blister, OE: oropharyngeal edema, OP: oral puritis, OS: oropharyngeal soreness and tingling, V: vomit, U: urticaria.

^b Other causes of allergies for the individual, described by the individual in clinical history.

GL440 image station and the apparent molecular weights estimated from the protein molecular weight standards using Kodak 1D image analysis software (Eastman Kodak Company).

2.4. IgE immunoblots of reduced and non-reduced kiwifruit samples

Following protein transfer and staining with Ponceau S, PVDF membranes were rinsed briefly in distilled water and then blocked by submersion in 5% non-fat dry milk (NFDm) in PBS buffer for 2 h at room temperature. Blocked membranes were incubated overnight at room temperature in sealed plastic pouches with individual kiwifruit allergic sera or control sera, which had been diluted 1:10 (v/v) in 2% NFDm in PBST (PBS with 0.05% Tween 20) 60 min prior to adding to the membrane. Following incubation membranes were washed four times in PBST, then incubated for 1 h with biotinylated monoclonal anti-human IgE (product # 9160-08, SouthernBiotech) that was diluted 1:1000 (v/v) in 2% NFDm PBST. Membranes were then washed four times with PBST and incubated for 1 h with Neutravidin conjugated with horseradish peroxidase (HRP) (product # 31001, Pierce Biotechnology), diluted 1:5000 in 2% NFDm PBST. After four washes in PBST, the membranes were wet in Super-Signal West Dura HRP substrate (product # 34075 Pierce Biotechnology, Inc.) to visualize the antigen–antibodies–enzyme complex. The chemiluminescent image was captured using a Kodak GL440 image station (Eastman Kodak Company). The IgE bound to immunoblots of the non-reduced, non-heated samples were detected using directly conjugated anti-IgE-HRP (same monoclonal anti-IgE as anti-IgE biotin, product # 9160-05, SouthernBiotech) as we found the sensitivity and non-specific

binding of IgE or detection reagents was not satisfactory using the biotinylated antibody with the reduced sample blots. There were insufficient sera from allergic subjects to repeat the assay with reduced kiwifruit samples using the directly conjugated anti-IgE.

2.5. Direct ELISA of native kiwifruit samples

The 96-well MaxiSorp™ microtiter plates (Nalgel Nunc International) were coated with 120 µg protein/mL of dialyzed extracts of green, gold or hardy kiwifruit or heat-processed hardy kiwifruit concentrate prepared in PBS (100 µL/well) by overnight incubation at 4 °C. After coating, the free binding sites of the plate were blocked by 2% bovine serum albumin (BSA) in PBS (350 µL/well) for 2 h at room temperature. Individual kiwi specific sera or control sera or plasma, diluted 1:10 (v/v) in blocking buffer, were applied as 100 µL aliquots in three adjacent wells, to plates coated with each of the four kiwifruit samples. Sera were incubated for 1 h at room temperature and unbound antibody was removed by four washes with PBST. The biotinylated monoclonal anti-human IgE (product # 9160-08, SouthernBiotech), diluted 1:1000 (v/v) in blocking buffer, was added (100 µL) to each well. The plates were incubated at 37 °C for 1 h and then washed four times with PBST. Neutravidin (Pierce Biotechnology), conjugated with HRP diluted 1:8000 (v/v) in PBS was then added to the plate (100 µL/well) and incubated for 1 h. After four washes with PBST, TMB peroxidase substrate (KPL, Inc.) was added. Reactions were stopped after 30 min by adding 50 µL/well 6 N H₂SO₄ and optical densities (OD) were read at 450 nm on a MRX microplate reader (Dynex Technologies), with values from triplicate wells averaged. The average no-serum background

OD for each specific kiwifruit sample was subtracted from the average value for each serum sample-kiwifruit. The background corrected average OD numbers are plotted in Fig. 3. The average and standard deviation of the four control sera (C01–C04) were calculated for each kiwifruit extract. Individual kiwifruit sample readings were considered significant if the average allergic subject reading (minus the background), was greater than the average control serum reading (minus the background) plus two standard deviations for that kiwifruit extract (Fig. 3).

3. Results

3.1. Serum donor characteristics

The clinical histories, challenge test results and specific-IgE measurement for the 12 kiwifruit-allergic subjects, and specific-IgE of the four control donors are summarized in Table 1. Eight of the 12 allergic subjects had been challenged by DBPCFC (green or green and gold kiwifruit) and tested by SPT using fresh green kiwifruit. The remaining four were not challenged due to histories of severe reactions. The eight challenged individuals reacted with relatively mild symptoms typical of oral allergy syndrome to at least one active dose, and not to placebo doses. Skin prick test results were significant for all eight tested subjects. Green kiwifruit-specific IgE levels measured by ImmunoCAP were significant (by Pharmacia Diagnostic standards (0.35 kAU/L)) for all 12 kiwifruit-allergic subjects and ranged from 0.5 to 6.8 kUA/L. Control subjects were not challenged but provided historical information that they have consumed green kiwifruit without any allergic reactions. Serum samples from each of the four control subjects tested negative (<0.35 kUA/L) for specific-IgE to green kiwifruit by ImmunoCAP.

3.2. Protein staining SDS-PAGE

Colloidal blue stained protein band patterns obtained from reduced SDS-PAGE separated extracts of the three kiwifruits and the heat-processed hardy kiwifruit concentrate were distinct (Fig. 1). Proteins from extracts of the three fruits are concentrated in clearly visible bands between 21 and 28 kDa, and there were no pronounced bands greater than 37 kDa. In reduced and heated samples, the dominant band in green kiwifruit (~28 kDa) is slightly larger than the dominant band in hardy kiwifruit (~27 kDa), and clearly larger than the dominant band in gold kiwifruit (~24 kDa). The patterns of stained proteins in green and gold kiwifruit are similar to those observed in other studies (Gavrovic-Jankulovic et al., 2002; Bublin et al., 2004; Fiocchi et al., 2004; Lucas et al., 2004). There were few observable differences in protein bands between samples that were dialyzed through 3500 MWCO tubing or not for each extract (Fig. 1A and B), suggesting that the dialyzed extracts would be expected to be representative of proteins present in extracts of fresh fruit for IgE immunoblots and ELISA. Under non-reducing conditions the major protein bands of the kiwifruit extracts migrated at apparent lower molecular weights of 18–

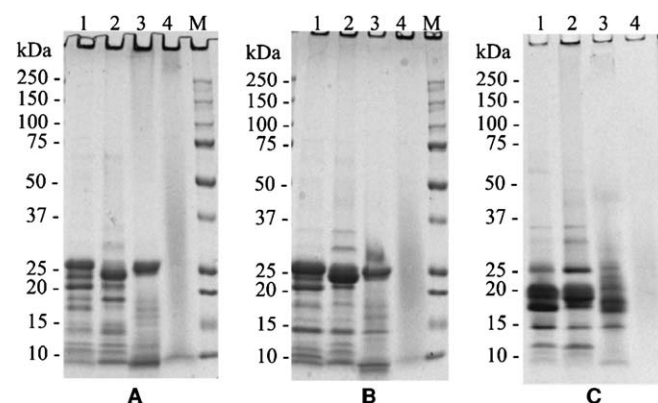


Fig. 1. SDS-PAGE of undialyzed (A) and dialyzed (B) kiwifruit extracts under reducing conditions and dialyzed samples under non-reducing conditions (C). Sample lanes: (1) green kiwifruit; (2) gold kiwifruit; (3) hardy kiwifruit; (4) processed hardy kiwifruit concentrate; M, molecular weight marker.

24 kDa (Fig. 1C). This apparent smaller size for the major stained protein bands is probably due to the compact structures of some proteins in the non-reduced condition that open up under reducing conditions, as was seen for the Thaumatin-like allergenic protein now referred to as Act c 2 (Gavrovic-Jankulovic et al., 2002).

No distinct protein bands were resolved in the heat-processed hardy kiwifruit concentrate sample under either reducing or non-reducing conditions. Instead, generally faintly stained smears were observed for the hardy kiwifruit concentrate in reduced (dialyzed or not), and in non-reduced conditions (Fig. 1A–C). These results suggest marked changes in the structure of all hardy kiwifruit proteins during processing. This result is similar to that reported for processed green kiwifruit by another group studying processing effects on the allergenic activity of green kiwifruit (Fiocchi et al., 2004), although the loss of discernable bands is greater in the sample tested here.

3.3. IgE immunoblots

The most notable observation related to one of the objectives of this study is the lack of apparent IgE binding to any protein in the hardy kiwifruit concentrate, lane 4 of Fig. 2. While serum blots are only shown for the three kiwifruit-allergic individuals with the highest levels of IgE binding in ImmunoCAPS or ELISA, similar trends were observed for all sera whether samples were reduced and heat-denatured, or not reduced or heated.

A number of other important observations can be made from the IgE immunoblots. Under reducing conditions (Fig. 2A), all sera bound to a protein of approximately 23 kDa (designated (a) in Fig. 2A) in the green kiwifruit extract (lane 1), including the non-allergic control sera (C03 and C04). Protein (a) bound to biotinylated anti-IgE and/or NeutrAvidin-HRP under reduced conditions (data not shown), which may explain why protein (a) was visible on immunoblots incubated with control sera.

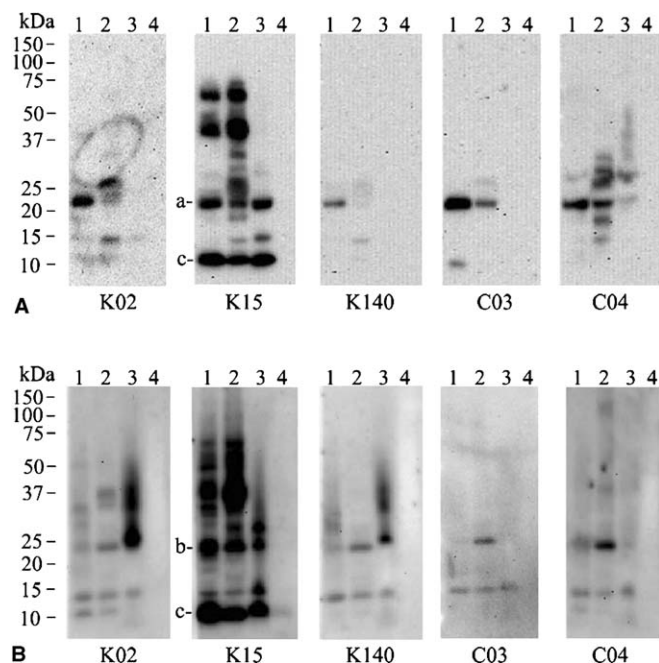


Fig. 2. Representative IgE Immunoblots are shown from samples of dialyzed kiwi protein extracts processed under reducing (A) and non-reducing conditions (B) prior to separation by SDS-PAGE. Sample lanes: (1) green kiwifruit; (2) gold kiwifruit; (3) hardy kiwifruit; (4) processed hardy kiwifruit concentrate. Serum donors K02, K15, K140 are allergic to kiwifruit and sera from control donors C03, C04 are not allergic to kiwifruit (see Table 1).

Although the apparent molecular weight of (a) is the same as the band designated (b) in Fig. 2B, the two proteins (a and b) are not the same. Protein (a) migrates at a lower molecular weight when not reduced based on observations from blots with reducing and non-reducing samples in adjacent lanes, where 2-mercaptoethanol diffuses across lanes, causing a reducing gradient that resulted in a diagonal band in the non-reduced lane (data not shown). The band marked (c) (Fig. 2A and B) is apparently the same protein under either reduced or non-reduced conditions as there is no apparent shift in molecular weight or relative binding intensity.

No serum IgE binding was detected to any sample that would correspond to a protein at the molecular weight of the major stained band in green kiwifruit, which should correspond to actinidin (approximately 27 kDa in our system). With the exception of K15, no other allergic subjects demonstrated significant apparent binding to proteins in blots of reduced, heat denatured samples except to the 23 kDa protein. The IgE from K15 bound to four proteins in reduced green kiwifruit extract and more than four proteins in gold kiwifruit. It was the only serum with obvious specific binding to reduced, heat denatured proteins from the hardy kiwifruit extract.

Serum IgE from a number of allergic sera bound to additional proteins in blots of the non-reduced kiwifruit extracts (Fig. 2B). While a direct comparison of band intensities is not reliable between reduced and non-reduced

sample blots because of the difference in the detection systems, it is clear that K02 IgE exhibits strong binding to at least two proteins in hardy kiwifruit extract, as does K140. IgE from K15 bound additional bands in green, gold and hardy kiwifruit extracts. As noted above, the band visible in green kiwifruit at about 23 kDa (lane 1, Fig. 2B) is not the same protein that is bound in the blot of a reducing gel (Fig. 2A). The non-reduced blots of all kiwifruit allergic and control sera show light but consistent and even binding to a protein of about 13 kDa in green, gold and hardy kiwifruit and 24 kDa in gold kiwifruit. That binding is probably due to a non-specific interaction of antibody and antigen through hydrophobic interactions. Interestingly, a number of bands in gold and hardy kiwifruit extracts were bound by serum C04 in the reducing blot, but were not visible in the non-reduced blot. Donor C04 is known to have severe food allergies to peanuts and soybeans, with an ImmunoCAP value of >100 kUA/L to peanut.

3.4. Direct ELISA of native kiwifruit samples

The average values (minus the no-serum background average) for each serum donor and sample extract (green, gold, hardy and processed hardy concentrate) are plotted in Fig. 3. The non-allergic control sera (C01–C04) showed little binding to any of the kiwifruit extracts, and no detectable binding to processed hardy kiwifruit concentrate. IgE binding of all kiwi-allergic sera to the hardy kiwifruit concentrate was obviously less than to hardy kiwifruit extract.

Only subjects K02 and K140 showed remarkable IgE binding to green and hardy kiwifruit extracts, at roughly 10 times the non-allergic control averages (Fig. 3). However, both sera bound much lower levels to gold kiwifruit extract. Sera from K15 had more IgE binding to gold kiwifruit than any other sera and third highest IgE binding to green and hardy kiwifruit extracts.

The OD value chosen in this study to indicate significant IgE binding in the direct ELISA tests was any value greater than two-standard deviations above the average non-allergic control OD value for the specific kiwifruit sample. Using this criteria, the number of subjects with detectable IgE binding to green kiwifruit for all kiwifruit-allergic subjects was significant (12 of 12), but fewer for gold kiwifruit (6 of 12) and hardy kiwifruit (10 of 12) extracts. The level of IgE binding to processed hardy kiwifruit concentrate was significant for 6 of 12 individuals, all with significant binding to hardy kiwifruit extract.

4. Discussion

The two aims of this study were (1) to look for evidence that individuals with known allergies to green or gold kiwifruit have IgE that binds to proteins in hardy kiwifruit and (2) to evaluate whether heat-processed hardy kiwifruit concentrate also binds IgE of the individuals and could pose the same potential risk as the raw kiwifruit.

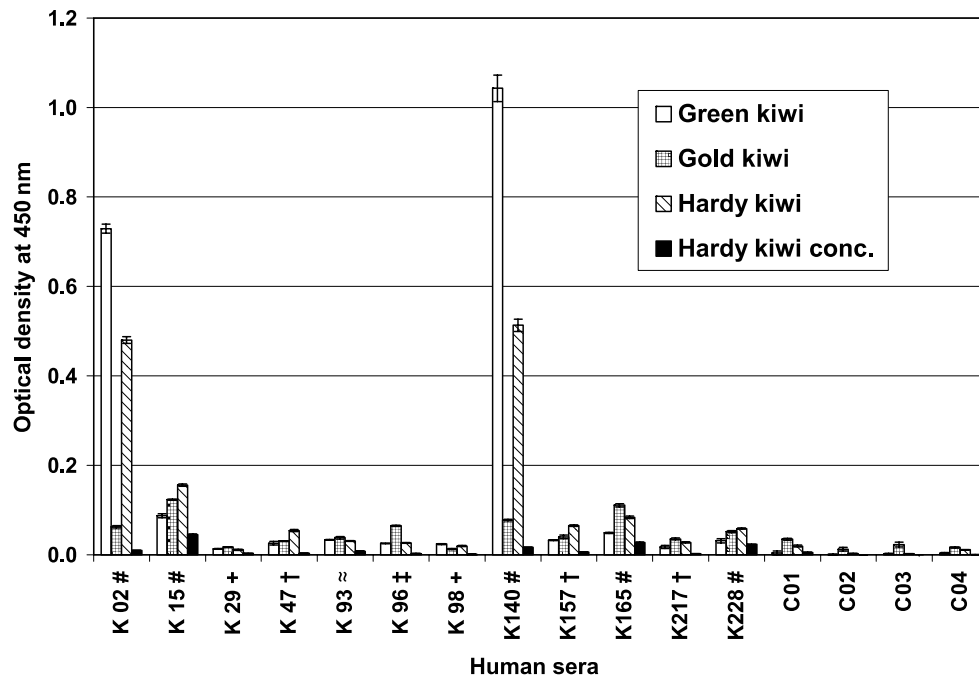


Fig. 3. Direct ELISA. Average optical density (OD) readings are represented for each kiwifruit sample, and each allergic (K02–K228) and non-kiwifruit-allergic control (C01–C04) serum, after subtracting the no-serum control values. Error bars represent standard deviation for triplicate wells. Significance of binding was defined as any average OD value for an allergic individual that was greater than two standard deviations above the average non-allergic controls (C01–C04) for that specific kiwifruit extract type (green, gold, hardy or processed hardy concentrate). Allergic individuals (K02–K228) were classified as having significant IgE binding in the direct ELISA to extracts of: green kiwifruit only (+); green, and hardy only (†); green, hardy and processed hardy concentrate (≈); green, gold and hard (§), or green, gold, hardy and processed hardy concentrate kiwifruit (#).

Results of IgE immunoblotting (Fig. 2) with reduced, heat denatured samples, and non-reduced unheated samples demonstrate that IgE from a number of these individuals bind specifically to proteins in hardy kiwifruit extract. In particular IgE binding from K02 and K140 to the non-reduced samples of hardy kiwifruit appears stronger than to non-reduced samples of green and gold kiwifruit. There were few truly distinct bands visible from immunoblots of reduced samples except to proteins at 12 and 23 kDa, except for the clear and distinct multiple proteins bound by donor K15 including proteins at ~43 and 65 kDa, bands not bound by IgE from sera of other donors. Interestingly, serum from donor K15 bound to a number of proteins with the same apparent molecular weights in both reducing and non-reducing gels. However, serum from K15 also bound to a few other proteins only under non-reducing conditions (Fig. 2B), indicating those proteins containing disulfide linkages. It is important to note that IgE from donors K02 and K140 only bind to proteins of approximately 25, 30 and 37 kDa in the hardy kiwifruit proteins of non-reducing gel membranes, while no bands were detected from hardy kiwifruit extracts of reduced gel membranes with those sera. Therefore the epitopes recognized by donors K02 and K140 are likely to be conformational in nature. Interestingly, those two individuals have the highest ELISA values to both green and hardy kiwifruits, even though no strong bands were recognized in blots of green kiwifruit extract from either reducing or non-reducing con-

ditions. Those two individuals also had the highest scoring ImmunoCAP levels, significant fresh-green kiwifruit SPT results and positive food challenges to both green and gold kiwifruit. This evidence indicates that some kiwi-allergic individuals, such as K02 and K140, are quite likely to experience allergic reactions to hardy kiwifruit if it is consumed fresh.

The apparent lack of binding to proteins in processed hardy kiwifruit concentrate, based on immunoblots, suggest that there is little likelihood that any of these kiwifruit-allergic individuals have IgE that binds specifically to proteins present in the processed material. However, the low level binding in ELISA assays to the processed hardy kiwifruit does indicate some level of specific *in vitro* IgE binding. The immunoblot results and ELISA results suggest little or no specific IgE binding on an equivalent protein loading basis between the hardy kiwifruit concentrate and that of the raw fruit. Thus a reduced risk of allergic reactions is expected from consumption of the processed hardy kiwifruit concentrate, compared to the fresh fruit.

The stained gels from SDS-PAGE run under reducing, heat denatured conditions and non-reducing unheated conditions both show the proteins from heat-processed hardy kiwi concentrate appear as diffuse smears over broad molecular weight ranges that extend from below 10 kDa to well above 37 kDa. There is a clear lack of focused bands. This result suggests there is either a generalized

partial hydrolysis of most proteins during drying or processing, with random cross-linking or aggregation of proteins and fragments, or possibly that there is extensive glycation of the proteins during drying or heating and processing (Sanz et al., 2001; Alvarez-Alvarez et al., 2005). This extensive loss of apparent protein structure is more severe than seen in samples of more solid botanical materials such as the seeds of lupine (Alvarez-Alvarez et al., 2005). In considering the potential allergenicity of processed fruits it is important to note that some fruit allergens retain their IgE binding capacity following heating, while others do not (Vieths et al., 1998; Scheurer et al., 2004). The modification of proteins during processing undoubtedly contributes to the low level of IgE binding observed in ELISA and in immunoblots to the heat-processed proteins as simple denaturation would otherwise be expected to not alter binding of IgE from allergic donor K15 to the 13, 15 and 25 kDa proteins seen in Fig. 2A and B.

Interestingly, the major stained band in green kiwifruit that migrates at approximately 27–28 kDa under reducing conditions, based on the Precision Plus Bio-Rad protein markers used in our system does not appear to bind IgE. As the dominant stained band, this should correspond to actinidin (Pastorello et al., 1996; Pastorello et al., 1998), although various investigators have reported different molecular weights ranging from 27 to 30 kDa (Bublin et al., 2004). This protein migrated at approximately 20 kDa in the non-reducing gel. It is tempting to speculate that none of these allergic patients have IgE that recognizes Act c 1 (actinidin). However, we did not purify or sequence this protein and therefore can not confirm the identity.

Based upon results using 12 well-characterized green kiwifruit-allergic subjects, heat-processed hardy kiwifruit concentrate is unlikely to cause a reaction in someone with allergy to raw green kiwifruit. The observed differences in IgE reactivity between raw kiwifruit species (e.g. ELISA, lower reactivity of gold kiwifruit) must be interpreted with some caution because differences in reactivity for various kiwi types should be expected among kiwifruit-allergic subjects (Bublin et al., 2004). These results were based only on in vitro data using sera from 12 kiwifruit-allergic individuals so caution must be exercised with respect to any broad recommendations regarding the allergenicity of heat-processed hardy kiwifruit concentrate for the entire population of kiwifruit-allergic consumers.

Finally comparing the in vitro IgE binding data of the immunoblots, ELISA and ImmunoCAP tests to the SPT with fresh fruit and food challenge demonstrates that many of the important kiwifruit allergens for most individuals are conformational or at least quite labile. That is also consistent with the observation by Lucas et al. (2004) and others that most kiwifruit-allergic individuals suffer from oral symptoms when consuming the fruit, although some individuals, including nearly half of our subjects, experience some systemic symptoms. Consequently diagnosing allergy to kiwifruit by in vitro assays alone is not highly predictive for the majority of allergic subjects.

Conflict of interest

This work was partly funded by Efficas, Inc., Boulder CO, with interest in manufacturing processed hardy kiwi-fruit concentrate. One author, Julianne Lindemann is a paid consultant of Efficas.

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References

- Aleman, A., Sastre, J., Quirece, S., de las Heras, M., Carnes, J., Fernández-Caldas, E., Pastor, C., Blázquez, A.B., Vivanco, F., Cuesta-Herranz, J., 2004. Allergy to kiwi: a double-blind, placebo-controlled food challenge study in patients from a birch-free area. *J. Allergy Clin. Immunol.* 113, 543–550.
- Alvarez-Alvarez, J., Guillaumon, E., Crespo, J.F., Cuadrado, C., Burbano, C., Rodríguez, J., Fernandez, C., Muzquiz, M., 2005. Effects of extrusion, boiling, autoclaving, and microwave heating on lupine allergenicity. *J. Agric. Food Chem.* 53, 1294–1298.
- Bublin, M., Mari, A., Ebner, C., Knulst, A., Scheiner, O., Hoffmann-Sommergruber, K., Breiteneder, H., Radauer, C., 2004. IgE sensitization profiles toward green and gold kiwifruits differ among patients allergic to kiwifruit from 3 European countries. *J. Allergy Clin. Immunol.* 114, 1169–1175.
- Diaz-Perales, A., Collada, C., Blanco, C., Sanchez-Monge, R., Carrillo, T., Aragoncillo, C., Salcedo, G., 1999. Cross-reactions in the latex-fruit syndrome: a relevant role of chitinases but not of complex asparagines-linked glycans. *J. Allergy Clin. Immunol.* 104, 681–687.
- Fahlbusch, B., Rudeschko, O., Schumann, C., Steurich, F., Henzgen, M., Schlennvoigt, G., Jager, L., 1998. Further characterization of IgE-binding antigens in kiwi, with particular emphasis on glycoprotein allergens. *Invest. Allergol. Clin. Immunol.* 8, 325–332.
- Falliers, C.J., 1983. Anaphylaxis to Kiwi fruit and related “exotic” items. *J. Asthma* 20 (3), 193–196.
- Fine, A.J., 1981. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, *Actinidia chinensis*). *J. Allergy Clin. Immunol.* 68 (2), 235–237.
- Fiocchi, A., Restani, P., Bernardo, L., Martelli, A., Ballabio, C., D’Auria, E., Riva, E., 2004. Tolerance of heat-treated kiwi by children with kiwifruit allergy. *Pediatr. Allergy Immunol.* 15, 454–458.
- Gavrovic-Jankulovic, M., Cirkovic, T., Vuckovic, O., Atanaskovic-Markovic, M., Petersen, A., Gojic, G., Burazer, L., Jankov, R.M., 2002. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J. Allergy Clin. Immunol.* 110, 805–810.
- Lucas, J.S.A., Lewis, S.A., Hourihane, J.O’B., 2003. Kiwi fruit allergy: a review. *Pediatr. Allergy Immunol.* 14, 420–428.
- Lucas, J.S.A., Grimshaw, K.E.C., Collins, K., Warner, J.O., Hourihane, J.O’B., 2004. Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin. Exp. Allergy* 34, 1115–1121.
- Lucas, J.S.A., Lewis, S.A., Grimshaw, K., Trewin, J., Warner, J.O., Hourihane, J., 2005. Comparison of the allergenicity of *Actinidia deliciosa* (green kiwi) and *Actinidia chinensis* (gold kiwi). *Pediatr. Allergy Immunol.* 16, 647–654.
- Nishiyama, I., Yamashita, Y., Yamanaka, M., Shimohashi, A., Fukuda, T., Oota, T., 2002. Varietal difference in vitamin C content in the fruit of kiwifruit and other *Actinidia* species. *J. Agric. Food Chem.* 52, 5472–5475.
- Pastorello, E.A., Pravettoni, V., Ispano, M., Farioli, L., Ansaloni, F., Rotondo, F., Invervaia, C., Asman, I., Bentsson, A., Ortolani, C.,

1996. Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *J. Allergy Clin. Immunol.* 98, 601–610.
- Pastorello, E.A., Conti, A., Pravettoni, V., Farioli, L., Rivolta, F., Ansaloni, R., Ispano, M., Incorvaia, C., Giuffrida, M.G., Ortolani, C., 1998. Identification of actinidin as the major allergen of kiwi fruit. *J. Allergy Clin. Immunol.* 101, 531–537.
- Rudeschko, O., Fahlbusch, B., Steurich, F., Schlenvoigt, G., Jager, L., 1998. Kiwi allergens and their cross-reactivity with birch, rye, timothy, and mugwort pollen. *Invest. Allergol. Clin. Immunol.* 8 (2), 78–84.
- Sampson, H.A., 2005. Food allergy—accurately identifying clinical reactivity. *Allergy* 60 (Suppl. 79), 19–24.
- Sanz, M.L., del Castillo, M.D., Corzo, N., Olano, A., 2001. Formation of Amadori compounds in dehydrated fruits. *J. Agric. Food Chem.* 49, 5228–5231.
- Scheurer, S., Lauer, I., Foetisch, K., Moncin, M.S., Retzek, M., Hartz, C., Enrique, E., Lidholm, J., Cistero-Bahima, A., Vieths, S., 2004. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J. Allergy Clin. Immunol.* 114 (4), 900–907.
- Vieths, S., Hoffmann, A., Holzhauser, T., Muller, U., Reindl, J., Haustein, D., 1998. Factors influencing the quality of food extracts for in vitro and in vivo diagnosis. *Allergy* 53 (Suppl. 46), 65–71.
- Vieths, S., Scheurer, S., Ballmer-Weber, B., 2002. Current understanding of cross-reactivity of food allergens and pollen. *Ann. N.Y. Acad. Sci.* 964, 47–68.
- Williams, M.H., Boyd, L.M., McNeilage, M.A., MacRae, E.A., Ferguson, A.R., Beatson, R.A., Martin, P.J., 2003. Development and commercialization of “Baby Kiwi” (*Actinidia arguta* Planch.). *Acta Hort. (ISHS)* 610, 81–86.