

Research Article

Effects of gastrointestinal digestion and heating on the allergenicity of the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin-like protein

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Kiwifruit is a significant elicitor of allergy both in children and adults. Digestibility of two kiwifruit allergens, actinidin (Act d 1) and thaumatin-like protein (Act d 2), was assessed using an *in vitro* digestion system that approximates physiological conditions with respect to the passage of food through the stomach into the duodenum. Act d 1 precipitated in simulated gastric fluid at pH 2 and digestion of the aggregated protein proceeded slowly. The residual precipitate redissolved completely in simulated duodenal fluid at pH 6.5 and was partially digested. Forty percent of Act d 2 remained intact during gastric digestion and were cleaved by duodenal proteases into large fragments covalently linked by disulfide bonds. Both digested allergen samples displayed nearly unchanged IgE binding abilities. Circular dichroism spectra were used to analyze heat and acid-induced unfolding. Thermal stability of both allergens was strongly pH dependent. While Act d 1 was irreversibly destabilized in acidic solutions, heat-induced denaturation of Act d 2 at pH 2 was fully reversible. IgE binding to Act d 2 but not Act d 1 was detected in processed food products. The stability of Act d 1 and Act d 2 provides one explanation for the allergenic potency of kiwifruit.

Keywords: Actinidin / Allergen / Food allergy / Kiwifruit / Thaumatin-like protein

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1 Introduction

In recent years, kiwifruits (*Actinidia deliciosa*) have become major elicitors of plant food allergy both in children and adults [1–3]. So far, four kiwifruit allergens have been identified: actinidin (Act d 1) [4], a thaumatin-like protein (TLP) (Act d 2) [5], kiwellin (Act d 5) [6], and cystatin (Act d 4) [7]. Act d 1 and Act d 2 are considered to be important kiwifruit allergens. However, very little work describing the biochemical properties of kiwifruit allergens has been published.

Act d 1 is a 27.4 kDa protein described as major allergen in previous studies and identified as actinidin by N-terminal sequencing [4]. The allergen, a papain-like cysteine pro-

tease, represents about 50% of the total soluble kiwifruit protein. Act d 1 seems to be a kiwi-specific allergen as no crossreactivity with birch or grass pollen has been observed [8]. Act d 2, a 21.5 kDa protein, has been identified as an *in vivo* reactive allergen among a group of five kiwifruit allergic patients among whom four had positive skin-prick tests (SPT) to purified Act d 2 [5]. Act d 2 belongs to the family of TLPs, also designated PR-5, which is one of the 17 plant families classified as pathogenesis-related proteins [9]. In addition to their important role in the plant's defense against pathogens, TLPs are of high interest for food allergy research. TLPs have been characterized as allergens of several fruits such as kiwi, apple, cherry, bell pepper, grape, and orange as well as of cypress, mountain, and Japanese cedar pollen [10, 11].

Food allergens have several biochemical features in common. These include abundance in the food and resistance to proteases and thermal treatment [12, 13]. The physicochemical properties of most food allergens confer stability to the proteolytic and acidic conditions in the digestive tract, which increases the probability of reaching the intesti-

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Abbreviations: CD, circular dichroism; SGF, simulated gastric fluid; SPT, skin-prick tests; TLP, thaumatin-like protein

nal mucosa, where absorption and interaction with the immune system can occur.

The aim of the present work was to investigate the physicochemical characteristics of Act d 1 and Act d 2 regarding their stability to heating and acidic conditions and to test the digestibility of these proteins. The digestion was performed using an *in vitro* digestion model employing two physiologically relevant stages to mimic the passage through the stomach into the duodenum including the addition of physiological surfactants such as phosphatidylcholine and bile salts [14–16]. Thermal stability was studied at neutral and acidic conditions in order to simulate the conditions of processing of kiwi containing food products.

2 Materials and methods

2.1 Purification of Act d 1 and Act d 2 from kiwifruit

Act d 1 was purified from kiwifruit extracts by covalent chromatography using activated thiopropyl sepharose 6B (Amersham Bioscience, Little Chalfont, UK), as described previously [17]. In order to purify Act d 2, we enriched the protein in a first step by 50% ammonium sulfate precipitation. The precipitate was dissolved in 20 mM Bis-Tris-HCl, pH 6.5, and desalted by dialysis. The sample was loaded onto a DEAE sepharose fast flow column (Amersham Biosciences). The flow-through fraction was adjusted to pH 7.8 and applied to an SP sepharose fast flow column (Amersham Biosciences). Bound proteins were eluted by a linear gradient from 0 to 50% of 20 mM Bis-Tris-HCl, pH 7.8, 1 M NaCl. Eluted fractions were dialyzed against 10 mM Bis-Tris-HCl, pH 7.0 buffer and loaded onto an Affi-Gel Blue column (BioRad Laboratories, Hercules, CA, USA). The adsorbed proteins were eluted with 2 M NaCl in the same buffer. The purified proteins were quantified by the BCA Protein Assay Kit (Pierce, Rockford, USA).

2.2 LC-ESI-MS

The molecular mass of purified Act d 2 was determined according to the method of Moreno *et al.* [15].

2.3 2-D PAGE

Gel strips (Immobiline DryStrip pH 3–10, 7 cm, Amersham Biosciences) were rehydrated overnight in 125 μ L IEF buffer (7 M urea, 2 M thiourea, 2% 3 (3-cholamidopropyl)dimethylammonio-propanesulfonic acid, 0.5% IPG buffer 3–10 (Amersham Biosciences) and 0.002% bromophenol blue) containing 4 μ g of protein sample. Strips were focused for 10 000 V $\cdot$ h with a maximum of 5000 V at 20°C using an Ettan IPGphor device (Amersham Biosciences). Prior to second dimension separation, strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 0.002% bromophenol blue)

with 65 mM DTT for 15 min and then with 250 mM iodoacetamide in the same buffer without DTT. Second dimension separation was performed by SDS-PAGE on 12% gels and the gels were stained with CBB R-250.

2.4 *In vitro* gastric and duodenal digestion

In vitro gastric and duodenal digestions were performed as described by Moreno *et al.* [14]. Digestions were performed either in presence or absence of phosphatidylcholine. Phospholipid vesicles were prepared by sonicating egg L-phosphatidylcholine (Lipid Products, Surrey, UK) with simulated gastric fluid (SGF).

In vitro gastric digestion (phase 1): Act d 1 and Act d 2 (5.55 mg/mL) were subjected to pepsin (porcine pepsin, enzymatic activity of 4230 U/mg protein, Sigma, Dorset, UK; product no. P6887) digestion using a physiological enzyme/substrate ratio of 1:20 w/w. The digestion was performed in SGF (0.15 M NaCl adjusted with 1 M HCl to pH 2) at 37°C and aliquots were taken at 0, 2, 5, 15, 30, 60, and 120 min. The digestion was stopped by raising the pH to 6.5 by addition of 1 M NaOH.

In vitro duodenal digestion (phase 2): phase 2 digestion was performed using 120 min gastric digesta as starting material. The following reagents were added: 0.125 M bile salt mixture containing sodium taurocholate and glyco-deoxycholic acid, 9.2 mM CaCl_2 , 25 mM Bis-Tris-HCl, pH 6.5, 0.1% w/v porcine pancreatic lipase (25 000 U/mg, Sigma–Aldrich, Poole, Dorset, UK) and 0.055% w/v porcine colipase (Sigma–Aldrich). Finally, solutions of trypsin and chymotrypsin were added at approximate physiological ratios of protein/trypsin/chymotrypsin = 1:400:100 by weight = 1 mg/34.5 U/0.44 U. The digestion was performed at 37°C and aliquots were taken at 2, 5, 15, 30, 60, and 120 min for further analysis. The digestion was stopped by adding a solution of a trypsin–chymotrypsin inhibitor (Sigma–Aldrich). Control samples with no enzymes added serving as negative controls were also analyzed. Digestions of α -lactalbumin and β -lactoglobulin, were included as positive controls for gastric and duodenal enzymes, respectively.

2.5 N-terminal amino acid sequence analysis

Samples were blotted to polyvinylidene difluoride membranes and stained with CBB R-250. Bands were excised and subjected to N-terminal amino acid sequencing using a Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA).

2.6 RP-HPLC

Prior to analysis, digested Act d 2 (50 μ L, 20 μ g) was subjected to microdialysis against 10 mM ammonium carbonate overnight at 4°C by using the Micro Dispodialyzer

Table 1. Clinical data of the patients used in this study

No.	Age (years)	Gender	Total IgE (kU/L)	P–P kiwi	CAP kiwi (kU/L)	IgE Act d 1 (ELISA)	IgE Act d 2 (ELISA)	Kiwi-related symptoms	Positive SPT to aero-allergens	Other food allergies	Atopic dermatitis
1	40	F	>2000	2+	n.d.	0.238	0.253	OAS, Dy	B, M, D, C	None	Yes
2	26	F	346	2+	<0.35	0.256	0.382	OAS	B, Mu, G, M, D, C	Peanut, nuts, RF, strawberry, AV, spinach, orange	Yes
3	37	F	564	2–3+	n.d.	0.073	0.271	OAS, GIS, Dy	B, G, M, D, C	Nuts, RF, AV	No
4	28	F	927	3+	<0.35	0.421	0.074	OAS	B, Mu, G, M, D, C	Peanut, nuts, RF	Yes
5	24	F	206	2+	n.d.	0.202	0.324	OAS	B, G, M, D, C	None	Yes
6	28	F	n.d.	3+	<0.35	0.420	0.069	OAS, RC, GIS	B, G, D, C	Peanut, nuts, RF	Yes
7	28	F	800	2–3+	5	0.189	0.267	OAS, Dy	B, Mu, G, M, D	Nuts, RF	No
8	48	F	202	2+	<0.35	0.232	0.081	OAS, RC		Peanut, nuts, RF sesame	No
9	58	M	58	2+	<0.35	0.165	0.079	OAS, Dy	B, A, G, M, D	Peanut, nuts, RF	No
10	65	F	n.d.	2+	<0.35	0.145	0.081	OAS	B, G, M, D	nuts, RF, melon	No
11	28	F	368	3+	n.d.	0.387	0.09	OAS	B, G, M, D, C	Peanut, nuts, RF	Yes
12	42	F	n.d.	4+	5	0.986	0.342	OAS, GIS	B, G, M, D, C	Peanut, RF, banana, melon, tomato	Yes
13	39	F	144	2–3+	n.d.	0.066	0.065	OAS	B, D	Plum, peach, chestnut, avocado, tomato	Yes
14	41	F	1634	4+	n.d.	0.589	0.251	OAS, Dy	B, G, M, D, C	Peanut, nuts, banana, pineapple	Yes
15	43	F	>2000	3+	1.2	0.710	0.450	OAS	B, G, M, D, C	Peanut, RF, mustard	Yes
NHS 1	37	F	n.d.	n.d.	n.d.	0.075	0.069	None	None	None	No
NHS 2	26	F	n.d.	n.d.	n.d.	0.072	0.076	None	None	None	No
NHS 3	23	F	n.d.	n.d.	n.d.	0.071	0.070	None	None	None	No

P–P, prick to prick test; OAS, oral allergy syndrome; Dy, dyspnea; RC, rhinoconjunctivitis; GIS, gastrointestinal symptoms; B, birch; Mu, mugwort; G, grass; M, house dust mites; D, dog; C, cat; RF, Rosaceae fruits; AV, Apiaceae vegetables; NHS, normal human serum; n. d., not done; f, female; m, male. The ELISA values above the threshold (0.078 for Act d 1 and 0.076 for Act d 2) are indicated in bold.

membrane cut-off 1000 Da (Harward Apparatus, Holliston, MA, USA). Act d 2 was reduced with 10 mM DTT (50 μ L), dissolved in 10 mM ammonium carbonate, and incubated at 65°C for 10 min. Act d 2 samples (reduced and nonreduced) were applied to a peptide column (Phenomenex Jupiter Proteo 90 Å pore size, 4 μ m particle size (250 $\times$ 4.6 mm id) attached to a Dionex HPLC system with a diode array detector. Samples were eluted using 0.1% w/v TFA in double-distilled water as solvent A and 0.085% w/v TFA in double-distilled water/acetonitrile (10:90 v/v) as solvent B. The column was equilibrated with 1% solvent B. The elution was performed as follows: 0–5 min, 1% solvent B, and then a linear gradient by increasing the concentration of solvent B from 15 to 50% in 55 min. The HPLC column temperature was maintained at 25°C and autoinjector at 4°C. Peptides were detected by their absorbance at 215/280 nm.

2.7 Circular dichroism (CD) spectroscopy

CD spectra of Act d 1 and Act d 2 at pH 7 and 3.5 (for Act d 1) and pH 2 (for Act d 2) were recorded with a J-710 spectrometer (Jasco Corporation, Tokyo, Japan) using a 0.2 cm path length quartz cell. Thermal denaturation was recorded from 20 to 90°C. The reversibility of the temperature effect

was monitored by measuring the restoration of the CD signal after the sample was cooled to 20°C. Spectra represent the average of four accumulations collected at 100 nm/min with a 2 s time constant, 0.5 nm resolution, and sensitivity of ± 100 mdeg. Spectra are represented as molar CD (with respect to moles of amide bonds).

2.8 Patients

Fifteen patients' sera were selected based on the presence of reported allergy and positive prick-to-prick test to green kiwifruit (Table 1). SPT were performed on the volar side of the arm, following the recommendations of the EAACI, with a standardized skin test lancet (ALK-ABELLÓ, Nieuwegein, The Netherlands) using commercial extracts or in case of kiwi with the prick-to-prick method. Histamine dihydrochloride (10 mg/mL) was used as a positive control, and the glycerol diluent of the SPT-extracts was used as a negative control (ALK-ABELLÓ). SPT were considered positive when the wheal reaction was 7 mm² (diameter 3 mm) and greater than the negative control. Specific IgE to green kiwifruit was determined by CAP-system FEIA (Phadia, Uppsala, Sweden). This study was approved by the Ethical Committee of the University Medical Centre Utrecht (Utrecht, The Netherlands). The blood samples

were taken between 1 and 11 months after the most recent reaction to kiwi. Specific IgE to Act d 1 and Act d 2 was determined by IgE ELISA using purified allergens (Table 1).

2.9 SDS-PAGE and immunoblotting

Reduced and nonreduced samples were subjected to 15% SDS-PAGE. Detection of IgE binding proteins by immunoblotting was performed as previously described [7]. IgE binding capacity of native and digested Act d 2 were tested using a pool of sera nos. 1, 2, and 3. For detection of TLPs, immunoblotting was performed using a rabbit antiserum raised against grape TLP (provided by Elizabeth Waters, Australian Wine Research Institute, Adelaide, Australia).

2.10 IgE ELISA and ELISA inhibition

IgE ELISA experiments with native and digested Act d 1 and Act d 2 as well as with native and heated Act d were performed as previously described [7]. For ELISA inhibition experiments, individual patients' sera preincubated with native or digested Act d 2 at final concentrations of 50 µg/mL were added to wells coated with native or digested Act d 2. Buffer and sera of three nonallergic subjects were used as negative controls and od values were counted positive when they exceeded the mean od of the negative controls by more than three SDs.

2.11 Preparation of extracts from kiwifruit containing food products

Four commercially available kiwifruit containing food products (nectar, iced tea, jam, whey, and yogurt) were purchased in a local store and the pH of each product was measured. Nectar, iced tea, and whey (1 mL of each) as well as kiwifruit pieces from joghurt were lyophilized and then dissolved in 100 µL of SDS-PAGE sample buffer. Jam (100 mg) was diluted with distilled water, homogenized, and centrifuged. Finally, the resulting supernatant was diluted with 0.5 volumes of SDS-PAGE sample buffer. Samples were subjected to 12% SDS-PAGE and immunoblotting. Immunoblotting was performed using sera containing Act d 1 or Act d 2 specific IgE and a rabbit antiserum raised against grape TLP.

3 Results

3.1 Purification and characterization of Act d 1 and Act d 2

Using the described procedures, 100 mg Act d 1 and 60 mg Act d 2 were obtained from 2 kg of kiwifruits. 2-D PAGE of Act d 1 showed that purified Act d 1 was separated into six isoforms with molecular masses of 27 kDa and p/s between

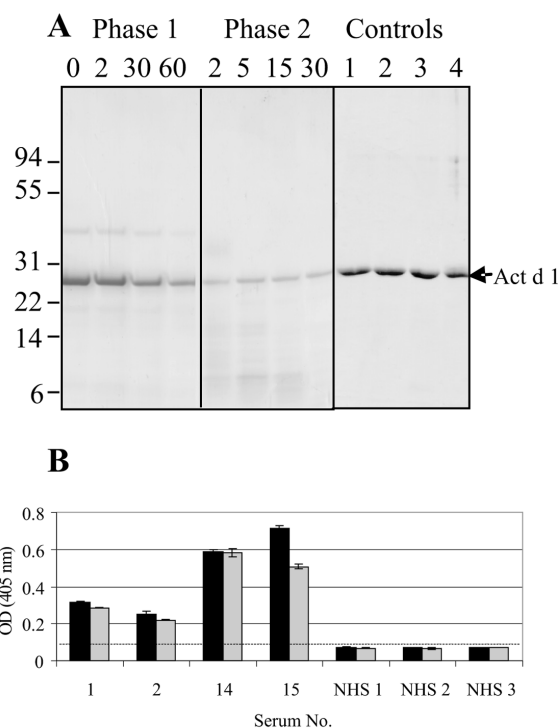


Figure 1. Gastric (phase 1) and duodenal (phase 2) digestion of Act d 1. Controls: samples without enzymes added (lanes 1, 2: 0 and 60 min phase 1; lanes 3, 4: 2 and 30 min phase 2). Samples taken at indicated times were analyzed by SDS-PAGE (A) and IgE ELISA (B). Black: native Act d 1, gray: digested Act d 1, NHS: normal human serum. The dashed line shows the highest od mean value (background) + 3 × SD value of the control sera.

3 and 4. 2-D PAGE of Act d 2 yielded a mixture of two proteins with molecular masses of 24 kDa and p/s of 8.5 and 9. Both isoforms yielded identical N-terminal sequences of ten residues, which matched the previously published N-terminal sequence of Act d 2 [5]. LC-ESI-MS analysis of purified kiwi-TLP gave two major components with masses of 21 563 and 21 592 Da.

3.2 *In vitro* digestibility of Act d 1

Act d 1 precipitated in SGF at pH 2. Digestion of the aggregated protein proceeded slowly. SDS-PAGE analysis showed that about 20% of Act d 1 remained intact after 1 h of gastric digestion. A pH dependent solubility study of Act d 1 showed that the precipitate could be resolubilized by increasing the pH to 6.5. Based on these observations, gastric digestion of Act d 1 for 1 h was followed by 30 min of duodenal digestion at pH 6.5 (Figs. 1A and B). SDS-PAGE analysis showed Act d 1 to be partially degraded by trypsin and chymotrypsin. However, intact protein and smaller fragments could be still detected after 30 min of duodenal digestion. Analysis of digesta by SDS-PAGE under nonre-

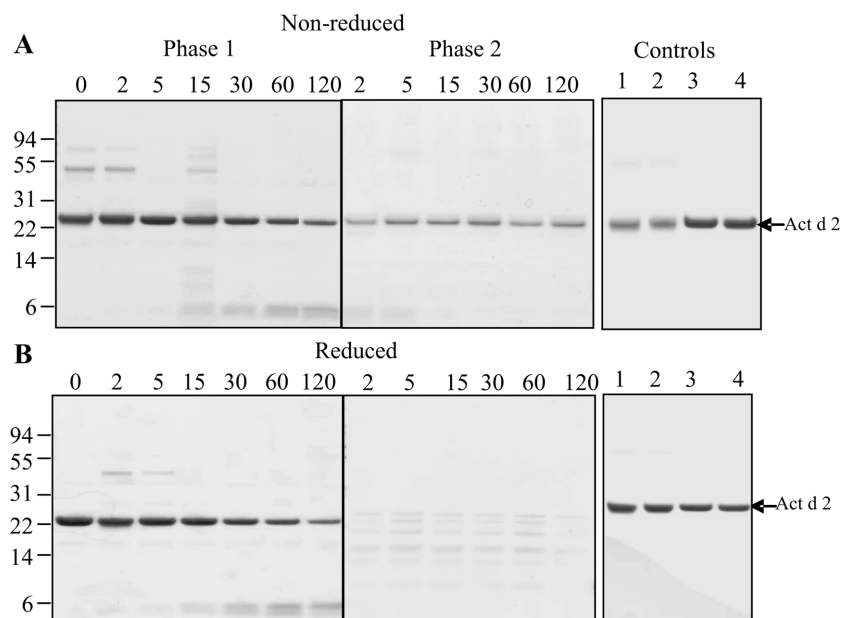


Figure 2. SDS-PAGE analysis under nonreducing (A) and reducing conditions (B) of Act d 2 subjected to gastric digestion for 120 min followed by duodenal digestion for 120 min. Samples with no enzymes added served as negative controls (lanes 1 and 2: 0 and 120 min phase 1; lanes 3 and 4: 2 and 120 min phase 2).

ducing conditions showed no significant differences compared to reducing conditions (data not shown).

3.3 IgE binding capacity of digested Act d 1

The digested Act d 1 was tested for IgE binding by ELISA using sera from four kiwi allergic patients (sera nos. 1, 2, 14, and 15). Compared to the untreated allergen, the digested protein exhibited only a small decrease in IgE binding (Fig. 1B).

3.4 *In vitro* digestibility of Act d 2

SDS-PAGE analysis under nonreducing (Fig. 2A) and reducing conditions (Fig. 2B) showed Act d 2 to be only slowly degraded by pepsin (phase 1) as even after 2 h about 40% of the allergen remained intact. This was accompanied by the appearance of lower molecular weight fragments of about 4 and 6 kDa running close to the gel dye front (Figs. 2A and B). RP-HPLC analysis showed that intact Act d 2 (eluting at 55 min) was present and that the 4 and 6 kDa fragments observed in SDS-PAGE were not homogenous but a mixture of peptides eluted between 20 and 45 min. After 2 h of gastric digestion, the pH was raised to 6.5, and the sample subjected to duodenal digestion (phase 2). The 4 and 6 kDa fragments remaining after pepsinolysis were rapidly broken down during duodenal digestion. The nonreduced Act d 2 sample showed little change in its migration on SDS-PAGE even after 2 h of duodenal digestion (Fig. 2A). However, when the sample was reduced prior to SDS-PAGE, the band of the intact protein disappeared and fragments of around 22, 20, 15, 13 kDa and <10 kDa were observed after a short period of digestion. The fragments

were resistant to subsequent digestion for the 2 h duration of the experiment (Fig. 2B). RP-HPLC profiles of Act d 2 after duodenal digestion showed the presence of intact Act d 2 under nonreducing, but only trace amounts of Act d 2 under reducing conditions, confirming the results obtained by SDS-PAGE analysis (Fig. 3). Preheating of Act d 2 at pH 2 or the presence of phosphatidylcholine did not affect its resistance to digestion (data not shown).

As positive control for gastric digestion, the stability of α -lactalbumin, which is known to be degraded by pepsin [18], was analyzed. α -Lactalbumin was quickly degraded by pepsin and after 2 min only a faint band of intact protein could be observed. This was accompanied by the appearance of small fragments of around 6 kDa, which disappeared after 15 min of pepsin digestion (data not shown). β -Lactoglobulin was used as positive control for duodenal digestion. In line with an earlier study, β -lactoglobulin was stable to gastric digestion but was rapidly broken down during duodenal digestion and after 30 min all traces of the protein were degraded (data not shown) [19].

3.5 IgE binding capacity of digested Act d 2

Reduced and nonreduced Act d 2 digests were subjected to SDS-PAGE, blotted to nitrocellulose membranes and probed either with a pool of sera from kiwi-allergic patients or with anti-TLP polyclonal antibodies. The fragments of the reduced sample were recognized by anti-TLP polyclonal antibodies, but no fragments were recognized by IgE from the serum pool (Figs. 4A and B, lane 4). In contrast, nonreduced digested Act d 2 was recognized by polyclonal antibodies as well as by IgE (Figs. 4A and B, lane 3). In order to confirm the presence of IgE binding epitopes on

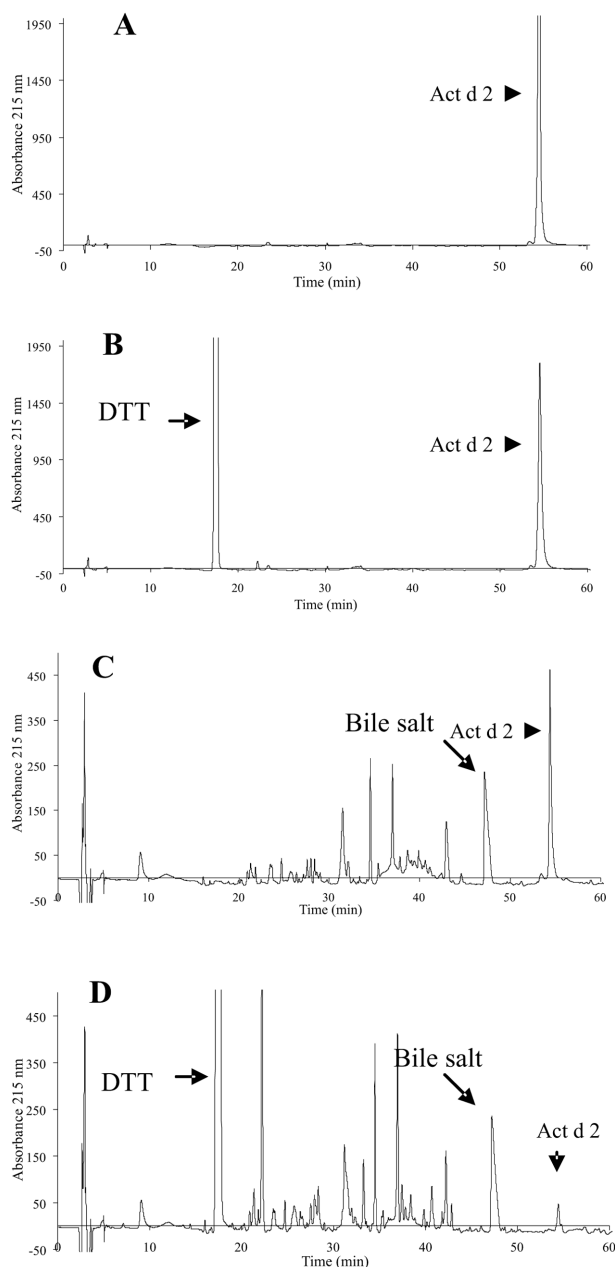


Figure 3. RP-HPLC patterns of (A) nonreduced (B) reduced Act d 2 subjected to gastric digestion for 120 min followed by duodenal digestion for 120 min analyzed in the absence (C) or in the presence of DTT.

digested Act d 2, IgE ELISA inhibition experiments with three sera (sera nos. 1, 2, 3) containing IgE specific for Act d 2 were performed. Native and digested Act d 2 showed similar inhibition of IgE binding to native Act d 2 with all tested sera (Fig. 4C).

3.6 Thermal stability of Act d 1

Thermal stabilities of Act d 1 and Act d 2 were evaluated using CD spectrometry by monitoring the ellipticity during

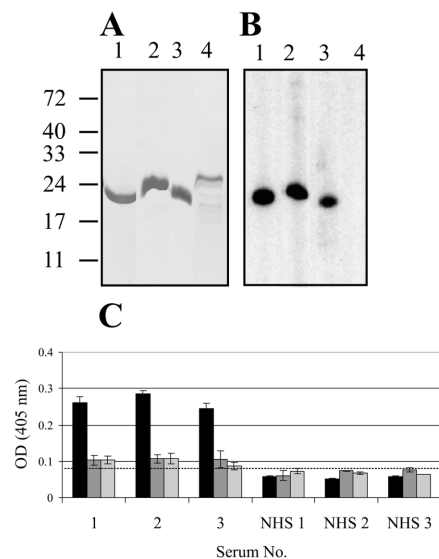


Figure 4. Immunoblot with rabbit anti-grape thaumatin antibodies (A) and a pool of patients' sera (B) of nonreduced and reduced Act d 2 before (1, 2) and after (3, 4) digestion. (C) ELISA inhibition with 3 sera: IgE binding to native Act d 2 (black) and after inhibition with native (dark gray) and digested Act d 2 (light gray). NHS: normal human serum. The dashed line shows the highest od mean value (background) + $3 \times$ SD value of the control sera.

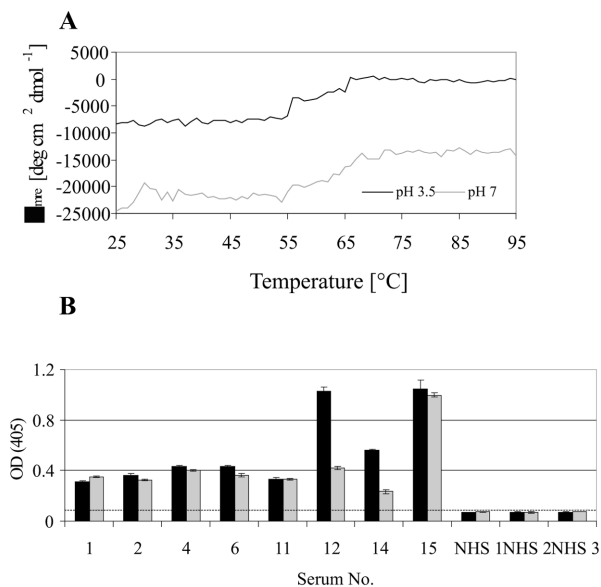


Figure 5. (A) Thermal unfolding curves of Act d 1 at pH 3.5 and 7 monitored by the CD signal at 208 nm. (B) Comparison of IgE binding of 12 individual kiwi allergic patients' sera to native Act d 1 (black bars) and to Act d 1 after heating at pH 7 (gray bars). NHS: normal human serum. The dashed line shows the highest od mean value (background) + $3 \times$ SD value of the control sera.

heating and recooling. The CD spectra of Act d 1 at pH 7 and 3.5 were different. At pH 7, the spectrum showed a positive peak at 186 nm and negative peaks at 208 and 222 nm.

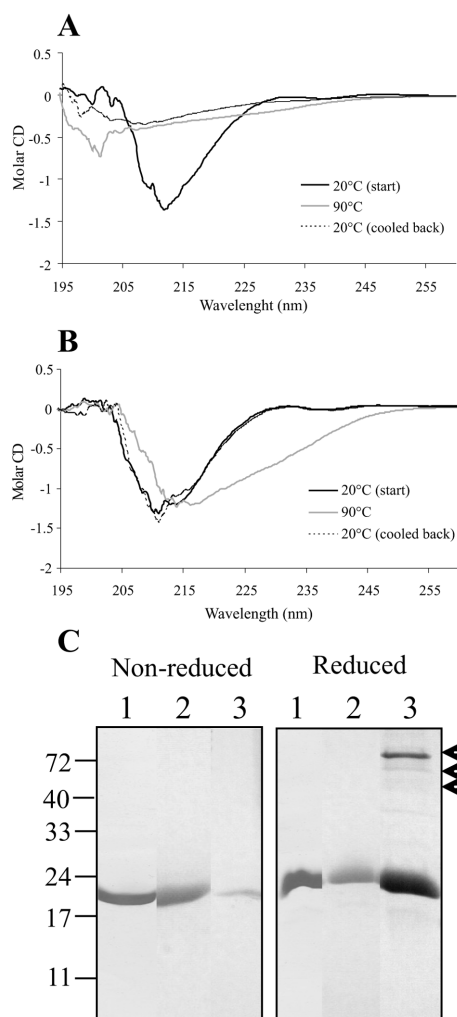


Figure 6. CD spectra of Act d 2 after heating and cooling at pH 7 (A) and pH 2 (B). (C) SDS-PAGE of Act d 2 heated at pH 7 under nonreducing and reducing conditions. Lane 1: non-heated Act d 2; lane 2: supernatant after heating; lane 3: precipitate. Bands of dimeric, trimeric, and tetrameric Act d 2 are indicated by arrows.

The spectrum at pH 3.5 displayed one positive peak at 196 nm which is absent in the CD curve at pH 7 and a decrease in ellipticity at 208 nm, indicating that significant conformational changes took place under these conditions (data not shown). Thermal denaturation profiles of Act d 1 monitored by ellipticity at 208 nm are shown in Fig. 5A. During heating at pH 7 and 3.5 the protein lost its secondary structure with a midpoint of unfolding at 61°C. The spectra of Act d 1 prior and after heating at pH 7 were different, showing that thermal unfolding was irreversible, whereas at pH 3.5 the protein irreversibly aggregated at temperatures exceeding 67°C. The heated preparation of Act d 1 (only at pH 7, because of aggregation at pH 3.5) was subjected to IgE binding analysis by ELISA using kiwi allergic patients sera (sera nos. 1, 2, 4, 6, 11, 12, 14, and 15) containing IgE specific for actinidin. The data from these experiments are

presented in Fig. 5B. The data indicate that IgE binding to heated Act d 1 varies from patient to patient. Heating of Act d 1 at pH 7 did not significantly decrease binding of IgE from most of the tested sera (serum nos. 1, 2, 4, 6, 11, and 15). However, a lowered capacity (>50%) to bind IgE was observed for two out of eight sera (serum no. 12 and 14).

3.7 Thermal stability of Act d 2

The effect of heating on the CD spectra of Act d 2 at pH 7 and 2 is shown in Fig. 6. Act d 2 showed a spectrum typical for proteins with structures dominated by β -strands with a characteristic negative peak at 213 nm and a positive peak at 197 nm. After heating from 20 to 90°C at pH 7 the CD signal changed from one characteristic for a folded protein to one resembling a random coil (Fig. 6A). The unfolding transition began at approximately 55°C with a melting temperature of 70°C. The protein was unable to refold after cooling to 20°C at pH 7. However, thermal unfolding of Act d 2 appeared reversible at pH 2.5 (Fig. 6B). Upon cooling to 20°C, the CD signal of native Act d 2 was fully recovered.

To further examine the cause of this difference, an Act d 2 solution was heated to 95°C at pH 7 for 15 min. After cooling, the resulting supernatant and precipitate fractions were analyzed by SDS-PAGE under nonreducing and reducing conditions. The supernatant fraction yielded a single band corresponding to unheated native Act d 2 irrespective of oxidation state (Fig. 6C, lane 2). The precipitate fraction yielded a weak band under nonreducing conditions (Fig. 6C, lane 3). Upon reduction, SDS-PAGE of the precipitate fraction revealed several bands. Although most of the protein were in a monomeric state, faint bands corresponding to dimeric, trimeric, and tetrameric Act d 2 were also observed.

3.8 Differences in Act d 1 and Act d 2 content in kiwifruit products

We analyzed the presence of Act d 1 and Act d 2 in selected kiwifruit food products. The pH of the products ranged from 2.5 for jam and nectar to 3 for yogurt, whey, and iced tea. Extracts from these products were subjected to SDS-PAGE and immunoblotting using purified allergens as controls. With the exception of kiwifruit pieces in yogurt, Act d 1 could not be detected in any of the tested products by immunoblotting using sera containing Act d 1 specific IgE. Act d 2 was recognized in extracts from jam, whey, and kiwifruit pieces in yogurt but not in iced tea and nectar by anti-TLP antibodies as well as by IgE from a serum pool containing Act d 2 specific IgE (data not shown).

4 Discussion

Digestibility, thermal stability, and abundance of proteins are considered to be of special relevance to understanding

the basis of allergenicity of food proteins [20]. Although many patients allergic to kiwifruit suffer from oral allergy syndrome, a significant number of individuals, particularly young children, show more severe reactions including angioedema, pharyngeal swelling, dyspnea, urticaria, vomiting, and even cardiovascular collapse [21]. Therefore, the aim of the present study was to gain insight into the biochemical properties of Act d 1 and Act d 2, previously described important kiwifruit allergens [4, 5]. Fifteen patients with positive case history of allergy to kiwi according to interviews with an allergist in an allergy outpatient clinic were selected for this study. All patients were positive to kiwifruit in prick-to-prick test, but only three out of nine tested patients were positive using the CAP system to measure kiwi specific IgE. Reports of sensitivity of measuring kiwi specific IgE vary between 40 [22] and 67% [5]. Only 8 out of 20 DBPCFC positive kiwi allergic patients were positive using the CAP system [22].

Oral allergy syndrome was the most common reported symptom. That is also consistent with reported observations that most kiwifruit allergic patients suffer from OAS when consuming the fruit, although some individuals, including more than half of our subjects, experience severe symptoms [21] (Table 1). Thirteen out of fifteen patients' sera showed IgE reactivity to Act d 1 and eight to Act d 2. However, the presence of IgE specific for Act d 1 or Act d 2 in serum could not be associated with the type of allergic symptoms to kiwifruit.

According to the overall yield of about 50 and 30 mg of pure protein *per* 1 kg of kiwifruit, Act d 1 and Act d 2 are abundant fruit proteins. Abundance is common property identified for many, although not for all food allergens. For example Pru av 2, an important cherry allergen, comprises up to 42% of total soluble proteins of cherry mesocarp [23]. Likewise, many allergens from cereals, legumes and tree nuts are abundant seed storage proteins [20].

In this study, the resistance of Act d 1 and Act d 2 to gastrointestinal digestion was assessed using an *in vitro* digestion system employing two stages to mimic the passage of food through the stomach into the duodenum according to the method of Moreno *et al.* [14, 15]. Surprisingly, Act d 1 aggregated after contact with SGF and slowly degraded within 2 h of gastric digestion (Fig. 1A). This indicates that aggregation impaired susceptibility of the protein to pepsin digestion. However, after increasing the pH to 6.5, Act d 1 completely redissolved and after 30 min of duodenal digestion showed only a small decrease in IgE binding compared to untreated Act d 1 (Fig. 1B). Similarly, it has been shown that digested Ara h 1, the major peanut allergen, retained the T-cell stimulatory and IgE binding properties of the intact protein [16].

SDS-PAGE as well as HPLC analysis of Act d 2 showed that 25% of the allergen remained intact following *in vitro* gastric digestion (Figs. 2 and 3). During duodenal digestion, residual intact Act d 2 was still present when analyzed by

SDS-PAGE under nonreducing conditions but disappeared after addition of a reducing agent. Moreover, native and digested Act d 2 showed similar IgE binding capacities and both inhibited IgE binding to native Act d 2 (Fig. 4). These results confirm that disulfide bonds are important in the resistance of this allergen to digestion with proteases commonly encountered in the gastrointestinal tract. Similar results have been obtained for milk and wheat allergens. When the structures of the major allergens of these foods were disrupted by reduction of disulfide bonds, the allergens were very sensitive to digestion and lost their ability to elicit allergic reactions in previously sensitised dogs [24, 25]. Recently, similar results were obtained for Pers a 1, the major avocado allergen involved in the latex-fruit syndrome [26].

Many of the proteolysis studies of allergens give little information regarding their resistance to digestion in the human gastrointestinal tract, because of the excess of pepsin used. It has been estimated that the daily pepsin secretion in adults sums up to between 20 and 30 kU of enzyme activity at 37°C [27] equivalent to around 10 mg, while a typical adult dietary intake of protein comprises around 75 g/24 h, giving a pepsin/protein ratio of 1:7500 [28]. Previously, Fu *et al.* [18] observed that the enzyme/protein ratio could greatly influence the observed period of stability. For instance, the stability of the major egg allergen ovalbumin changed from greater than 120–0 min as the ratio of pepsin/protein increased from 0.1 to 100 [29]. In contrast to our results, a rapid degradation of Act d 2 by pepsin within 1 min was observed following *in vitro* digestion [5]. In that study, the protein was tested in SGF with an extremely high enzyme/protein ratio of 16:1 compared to 1:20 in our study. The authors followed the method of Astwood *et al.* [30] which used enzyme/protein ratio even 12.3-fold higher than recommended by the FAO/WHO [31]. Along these lines it was recently demonstrated that after digestion of Act d 2 with an enzyme/protein ratio of 6.5:1 and in the presence of pectin, similar to our results, intact Act d 2 could be detected after 1 h of digestion [32].

The resistance of both allergens to gastrointestinal digestion in our *in vitro* system suggests a role in sensitization to kiwifruit *via* the gastrointestinal tract. While most kiwifruit-allergic patients are pollen-allergic and probably sensitized to still unidentified crossreactive pollen allergens, a subgroup of patients shows monosensitization to kiwifruit frequently accompanied by severe symptoms [33, 34]. Act d 1 seems to be a kiwi-specific allergen as no crossreactivity with birch or grass pollen has been observed [8]. Possible crossreactivity to tropical fruits (pineapple, papaya) containing similar thiol proteases have been proposed but never shown. Similarly, no crossreactivity between Act d 2 and other TLPs from fruits or Cupressaceae pollen has been described.

Thermal stability of Act d 1 and Act d 2 was investigated under neutral and acidic conditions. Heating at acidic pH

was performed to simulate the conditions encountered in kiwifruit products during thermal treatment. Our results demonstrated that the thermal stability of Act d 1 is strongly dependent on pH. The conformational instability followed by precipitation of Act d 1 prevented the analysis at pH values less than 3.5. The CD spectrum of Act d 1 at pH 3.5 showed an acid-induced unfolded state, which is quite unstable to thermal denaturation compared to the native state at pH 7 (Fig. 5). Examination of the literature revealed that several plant cysteine proteases form partially structured states known as molten globule at low pH [35, 36]. Thermal aggregation of an acid-induced unfolded state of Act d 1 could explain our findings about the absence of actinidin in processed products containing kiwifruit.

Act d 2 aggregated after heating at pH 7, but not at pH 2 (Fig. 6). Our results show that crosslinking of Act d 2 molecules at pH 7 was mediated by intermolecular disulfide bonds induced by heating. The cleavage of disulfide bonds at neutral pH and high temperature was previously observed for thaumatin [37], but also in unrelated proteins including ovalbumin and transferrin [38]. The most probable mechanism of this reaction is a β -elimination, where a base-catalyzed subtraction of a β -proton from a cysteine results in cleavage of disulfide bonds. This creates intermolecular disulfide linkages and induces aggregation. In contrast, thermal denaturation at acidic conditions (such as those found in juice) reversibly unfolded the protein. Consequently, immunoblotting of extracts prepared from kiwifruit jam and whey showed that Act d 2 was not removed and its allergenic activity was not decreased by technological treatments.

The physicochemical features of actinidin and kiwi TLP should provide new insights into their role as allergens. Their thermostability, stability to gastric pH and gastrointestinal digestion as well as their abundance are typical features of food allergens, implying that Act d 1 and Act d 2 play a role in sensitization of kiwi allergic patients via the gut immune system.

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