



Evaluation of IgE reactivity of active and thermally inactivated actinidin, a biomarker of kiwifruit allergy

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ABSTRACT

Actinidin, an abundant cysteine protease from kiwifruit, is a specific biomarker of isolated allergy to kiwifruit. This study evaluates the IgE-binding properties of biologically active and thermally inactivated actinidin. Employing two different activity assays (caseinolytic assay and zymogram with gelatin) we showed that actinidin obtained from kiwifruit extract under native conditions represents a mixture of inactive and active enzyme. The structural integrity of actinidin was confirmed by SDS–PAGE, Edman degradation, mass fingerprint and Western blot with polyclonal antibodies. Although it was capable of inducing positive skin prick test reactions, we failed to detect IgE reactivity of active actinidin in Western blot with patient sera. Thermally inactivated actinidin exhibited IgE reactivity both *in vivo* and *in vitro*, indicating that heat processed kiwifruit products may induce clinical reactivity. These findings imply that apart from the allergenic epitopes on its surface, actinidin also contains hidden epitopes inside the protein which become accessible to IgE upon thermal treatment.

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1. Introduction

The popularity of kiwifruit and the availability of new varieties on the international market imposed an increasing interest in examination of its influence on human health. Kiwifruit has become very popular in the human diet due to its pleasant taste, high content of vitamin C, and its low caloric value. Several investigations on possible health-promoting effects of kiwifruit have been reported (Jung et al., 2005; Rush et al., 2002). However, kiwifruit has become one of the most common causes of food allergies with a wide range of symptoms, including oral allergy syndrome, urticaria, and anaphylaxis (Lucas et al., 2004; Aleman et al., 2004; Bublin et al., 2004; Palacin et al., 2008). Two predominant kiwifruit cultivars available on the international market are green-fleshed cultivar *Actinidia deliciosa* cv. Hayward and yellow-fleshed cultivar *Actinidia chinensis* cv. Hort16A. These cultivars differ in taste but also in the composition of kiwifruit proteins (Bublin et al., 2004; Lucas et al., 2005). Eleven IgE-reactive molecules from kiwifruit have been reported and registered by the WHO/IUIS Allergen Nomenclature Subcommittee (www.allergen.org). Actinidin (Act d 1) is the most abundant protein

and the major allergen from green kiwifruit (Aleman et al., 2004; Bublin et al., 2004; Pastorello et al., 1998). It is a kiwifruit-specific allergen since no cross-reactivity with birch or grass pollen has been observed (Pastorello et al., 1996). Even though actinidin undergoes irreversible unfolding when heated under neutral pH conditions, it preserves *in vitro* IgE reactivity (Bublin et al., 2008).

Actinidin belongs to the class of plant thiol proteinases (EC 3.4.22.14) which are widespread in nature (Carne and Moore, 1978). A number of allergens belonging to the papain-like cysteine proteinases have been identified, including inhalant allergens such as the major dust mite allergen Der p 1 and also plant food allergens such as Ana c 2 (bromelain) from pineapple, Car p 1 (papain) from papaya, and a major allergen from soybean seed storage vacuoles Gly m 1 (Hauser et al., 2008). Actinidin is encoded as a pre-protein with an endoplasmic reticulum (ER)-targeting signal peptide and N- and C-terminal propeptides (NTPP, CTPP). This enzyme is synthesized as a zymogen and processed into an active form after secretion or sequestration in the cell (Nieuwenhuizen et al., 2007). Ten different actinidin mRNAs for acidic and basic protein isoforms were identified which encode mature proteins. Extremely high levels of an acidic actinidin protein were detected in *A. deliciosa* fruit, but this acidic protein appeared to be at a very low level in Hort16A, the most important commercial cultivar of *A. chinensis* (Bublin et al., 2004; Nieuwenhuizen et al., 2007).

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Food processing, particularly thermal processing, was observed to reduce the allergenicity of some foods, and is therefore considered as an alternative management for the allergenic risk of foods (Wal, 2003; Fiocchi et al., 2004). At the molecular level thermal processing induces progressive loss of tertiary and secondary protein structures and the appearance of disorganized structures that adopt an unfolded, random-coil conformation. However, a decrease or increase of allergenicity can be caused by protein unfolding, misfolding, and aggregation, as well as by chemical modifications occurring during food processing (Marzban et al., 2009).

Along with skin prick testing, another widespread screening tool in the field of allergy diagnostics is immunoblotting by which a patient can be profiled against multiple allergen markers simultaneously, allowing insight into the integrity and IgE-binding epitopes of allergens. Therefore, one and two-dimensional Western blotting is a frequently employed method for determination of the sensitization patterns of atopic persons (Weghofer et al., 2005). In addition, alterations in IgE-binding affinity to separated proteins under different analytical conditions and eventual post-translational modifications can be elaborated (Herndl et al., 2007).

Inherent properties of a specific source material, for instance the presence of proteolytic enzymes, necessitate optimization of the extraction procedure for the preparation of plant-derived food allergen extracts. Therefore, to inhibit actinidin activity, kiwifruit extracts used in scientific research are often prepared in the presence of a thiol protease inhibitor E64 (Pastorelo et al., 1998) or a protease inhibitor cocktail (Bublin et al., 2004; Chen et al., 2006; Nieuwenhuizen et al., 2007).

In this study a protocol employed for actinidin purification under native conditions, without addition of protease inhibitors, revealed the presence of a mixture of active and inactive enzyme in *A. deliciosa* kiwifruit extract. In order to assess the allergenic properties of the active enzyme isolated under native conditions and to determine the effect of thermal processing on the allergenicity of kiwifruit, the actinidin preparation was either fully activated by incubation in L-cysteine buffer or inactivated by thermal treatment. *In vitro* and *in vivo* IgE-binding properties of proteolytically active and thermally inactivated Act d 1 were evaluated in Western immunoblot and skin prick test.

2. Materials and methods

2.1. Preparation of protein extract

Kiwifruit (*A. deliciosa* cv. Hayward) was purchased in a local store. The pulp (150 g) of the fresh fruit was homogenized in 300 mL of 100 mM sodium citrate buffer, pH 5.0. After extraction (2 h at 4 °C), the slurry was centrifuged (1000g, 5 min) and the obtained supernatant was extensively dialyzed against the extraction buffer. Protein concentration in the extract was 0.8 mg mL⁻¹ as determined by Bradford assay.

2.2. Actinidin purification

Actinidin (Act d 1) was purified from the kiwifruit extract (250 mL) which was subjected to ion-exchange chromatography on a DEAE-Sephadex A-50 column (145 mm × 27 mm) (GE Healthcare, Uppsala, Sweden), pre-equilibrated with 100 mM sodium citrate buffer, pH 5.0. The unbound protein fraction (300 mL, collected at a flow rate of 1 mL min⁻¹) was concentrated by ultrafiltration, and dialyzed against 20 mM TRIS-HCl buffer, pH 8.0 for 48 h. The protein sample (125 mL) was applied on a SP-Sephadex C-50 ion-exchange column (125 mm × 15 mm) (Pharmacia-Biotech, Uppsala, Sweden) equilibrated in the dialysis buffer and the column was eluted by a pH step gradient ranging from pH 5 to pH 2.5 with eight column volumes (CVs). The employed flow rate was 0.8 mL min⁻¹. Collected fractions (10 mL each) were analyzed by SDS-PAGE, and those containing actinidin were pooled and concentrated by ultrafiltration. About 20 mg of actinidin preparation (Act d 1) was isolated from 250 mL of the kiwifruit extract. The purity of the protein preparation was estimated to be >97% by SDS-PAGE and N-terminal amino acid sequencing.

2.3. Production of polyclonal antibodies

Purified Act d 1 was used for production of polyclonal antibodies. Two rabbits were immunized according to the protocol described by Harlow and Lane (1988). In brief, 0.25 mL Act d 1 (0.50 mg mL⁻¹) was mixed with 0.25 mL FCA (Freund's complete adjuvant) for the first immunization. Every 30 days, for 6 months, the rabbits were boosted with a mixture of 0.25 mL of Act d 1 and 0.25 mL of incomplete Freund' adjuvant. Each rabbit was subcutaneously immunized with 0.50 mL of the emulsion. After 6 months, sera were collected and stored at -20 °C before use. The immunization protocol was approved by the Ethics Committee of the Torlak Institute of Virology, Vaccines and Sera.

2.4. 1-D and 2-D PAGE

SDS-PAGE analysis of the actinidin preparation was performed on 4% stacking gel and 14% resolving gel under reducing conditions (Bollag et al., 1996). Before migration, the actinidin preparation (20 µg) was incubated in sample buffer (60 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.1% (v/v) bromophenol blue, 14.4 mM 2-mercaptoethanol) in a ratio 4:1 (v/v) (60 min at 45 °C) and centrifuged (13,400g for 1 min). After electrophoresis, proteins were stained with Coomassie Brilliant Blue (CBB-R250, Serva, Heidelberg, Germany).

In addition, the homogeneity of the actinidin preparation was analyzed by 2D-PAGE under reducing conditions. In brief, the sample (25 µg) was separated under native conditions by isoelectric focusing in 5% (w/v) polyacrylamide gel containing 2.4% (v/v) ampholytes (pH 3.5–10, Pharmacia). Following first dimension separation, the gel stripe containing actinidin was incubated in equilibration buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2.3% SDS, 10% glycerol) for 15 min and then run in the second dimension on SDS-PAGE (14%). Actinidin was visualized following staining with Coomassie Brilliant Blue.

2.5. Proteolytic activity assays

Proteolytic activity of the actinidin preparation was assessed by using a protease enzymatic assay with casein as a substrate, as previously described (Drapeau, 1976). In brief, a 2% casein solution was prepared in 100 mM potassium phosphate buffer, pH 7.6. A 400 µL aliquot of casein was added to 40 µL of 250 mM L-cysteine, 40 µL of 250 mM EDTA, 40 µL of 250 mM NaOH, 120 µL of 1 M KH₂PO₄, pH 7.0 and 80 µL of distilled water. The reaction mixture was equilibrated to 37 °C, after which 80 µL of enzyme preparation (1 mg mL⁻¹ of Act d 1) was added and the assay mixture was incubated for 1 h at 37 °C. The enzyme reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid (TCA) and the reaction mixture was incubated for an additional hour at 37 °C. Following centrifugation (13,000g for 15 min) absorption of the supernatant was measured at 280 nm against a blank. The blank was prepared by first mixing the enzyme with TCA and then adding the casein solution to the enzyme-TCA mixture. One unit of activity was determined as that amount of enzyme which releases acid soluble fragments equivalent to 0.001 A₂₈₀ per minute at 37 °C under the specified conditions.

For activation of the actinidin, samples were incubated in 20 mM potassium phosphate buffer, pH 6.5 containing 52 mM L-cysteine and 2 mM EDTA (1:1; v/v) for 1 h at 45 °C. Inactivation of the enzyme was achieved either by thermal treatment (95 °C for 5 min) or by addition of P2714 Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, USA) to the protein samples (1:1; v/v).

To detect proteolytic activity of actinidin after SDS-PAGE, zymograms were run according to Grobe et al. (2002) with some modifications, as described by Gavrovic-Jankulovic et al. (2005). Briefly, after incubation in reducing sample buffer (1 h at 45 °C), protein samples were applied onto a 14% resolving gel co-polymerized with 0.1% gelatin for SDS-PAGE analysis. After electrophoresis, the gel was incubated in a 100 mM potassium phosphate buffer, pH 6.5 with 26 mM L-cysteine and 1 mM EDTA for 16 h, followed by staining with Coomassie Blue (Serva).

2.6. MS analysis and N-terminal sequencing

The molecular mass of purified actinidin was determined by matrix assisted desorption/ionization mass spectrometry (MALDI TOF-MS) with a Reflex III (Bruker-Daltonik, Bremen) in the positive ion mode. The protein was dissolved in water at a concentration of 5 µg µL⁻¹ and further diluted in the ratio of 1:60 with freshly prepared matrix solution consisting of saturated 3,5-di-methoxy-4-hydroxycinnamic acid (sinapic acid, SA; Aldrich) in a 2:1 mixture of 0.1% trifluoroacetic acid/ acetonitrile. 0.5 µL aliquots were deposited on the sample holder and analyzed immediately after drying in a stream of air.

For N-terminal sequencing after SDS-PAGE protein samples were transferred by semidry blotting onto a PVDF membrane (Serva, Heidelberg, Germany) according to Gavrovic-Jankulovic et al. (2002). The membrane was washed in double-distilled water, stained with 0.1% Coomassie Blue (Serva) in 50% methanol and air-dried. The protein bands were excised and microsequenced using a Procise protein sequencer connected to an online PTH amino acid analyzer (PE Biosystems, Wittersdorf, Germany).

To verify the intactness of inactive and active actinidin mass spectrometric peptide mapping was performed. In brief, the protein sample was separated by SDS–PAGE electrophoresis, followed by Coomassie Blue staining of the gel. Protein bands corresponding to inactive and active actinidin were excised from the gel, destained and digested overnight with sequence grade trypsin from bovine pancreas (Roche Diagnostics GmbH, Mannheim, Germany) as described by Shevchenko and Shevchenko (2001). For MALDI TOF–MS analyses of the tryptic fragments α -cyano-4-hydroxycinnamic acid, CCA, was used as a matrix.

2.7. Patients sera

Sera from patients with allergic symptoms and positive skin-prick test to kiwifruit were used to check the *in vitro* IgE-binding capacity of thermally inactivated and active actinidin forms. Serum of a nonallergic subject was used as control. Demographic and clinical data of the studied patients are summarized in Table 1. Written informed consent was obtained from all patients and the study was approved by an Institutional Ethics Committee.

2.8. Western blot

Prior to electrotransfer samples of activated and thermally inactivated actinidin were prepared and run on SDS–PAGE as described above. After electrophoresis actinidin preparations were semi-dry transferred for 40 min (2 mA cm^{-2}) to a nitrocellulose membrane (pore size $0.45 \mu\text{m}$, Serva) as previously described by Gavrovic-Jankulovic et al. (2002). For IgE immunoblot development, following an 1 h incubation step with blocking solution consisting of 5% (w/v) nonfat dry milk in TTBS buffer (50 mM Tris–HCl, pH 7.4, and 150 mM NaCl containing 0.1% Tween 20), membranes were incubated with individual patient sera (dilution 1:2.5, (v:v) in TTBS buffer with 0.1% of nonfat dry milk) for 4 h at room temperature (RT). Afterwards the membrane strips were incubated for 1 h at RT with secondary antibody (goat anti-human IgE, dilution 1:10,000 in TTBS; Sigma–Aldrich) followed by incubation with rabbit anti-goat IgG labeled with alkaline phosphatase (dilution 1:30,000 in TTBS; Sigma–Aldrich) for 1 h at RT.

Additionally, samples of active and thermally inactivated Act d 1 were tested for IgG reactivity. After incubation with blocking solution, these membrane strips were incubated for 1 h at RT with polyclonal anti-Act d 1 antibodies raised in rabbits (dilution 1:20,000, v:v in TTBS buffer with 0.1% of nonfat dry milk) followed by incubation with secondary goat anti-rabbit IgG antibodies (dilution: 1:20,000, v:v in TTBS buffer with 0.1% of nonfat dry milk) for 1 h at RT and tertiary anti-goat IgG antibodies labeled with alkaline phosphatase (dilution 1:30,000 in TTBS; Sigma–Aldrich) for 1 h at RT. Immunoblots were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma–Aldrich) as substrates.

2.9. Skin tests

Skin prick tests (SPTs) with the kiwifruit extract ($500 \mu\text{g mL}^{-1}$) and serially diluted two concentrations of activated Act d 1 (500 , 50 and $5 \mu\text{g mL}^{-1}$) and thermally inactivated Act d 1 (500 , 50 and $5 \mu\text{g mL}^{-1}$) were performed as previously described (Gavrovic-Jankulovic et al., 2002). Histamine phosphate (10 mg mL^{-1}) and 0.9% saline were used as positive and negative controls, respectively. The mean wheal diameter of at least 3 mm or greater compared with that produced by the negative control 20 min after puncture was considered a positive response.

3. Results

3.1. Identification of actinidin preparation

Actinidin (Act d 1) was isolated from kiwifruit (*A. deliciosa*) protein extract, which was prepared without addition of protease inhibitors. A combination of DEAE–Sephadex and SP–Sephadex ion-exchange chromatographies were employed for the actinidin purification. Homogeneity of the isolated protein was assessed by SDS–PAGE and 2D–PAGE in reducing conditions. The purified protein sample revealed two protein bands of about 22 and 30 kDa (Fig. 1). Both bands revealed the same N-terminal sequence (LPSYVD) which corresponded to amino acids 127–132 of the complete sequence of actinidin (UniProt ID: A5HII1). The molecular mass of isolated actinidin was 23883.1 Da (Fig. 2).

To confirm that the 22 kDa Act d 1 did not derive from proteolytic processing of 30 kDa Act d 1, the 22 kDa band was excised from a SDS–gel, and after incubation with a cocktail of inhibitors (1 h incubation at 37°C), the 22 kDa protein was rerun by SDS–PAGE, showing electrophoretic mobility of a 30 kDa protein (Supplementary material Fig. 1S).

3.2. Detection of actinidin proteolytic activity

The caseinolytic digestion assay was performed to test proteolytic activity of actinidin (Act d 1). Four samples of Act d 1 were analyzed (a) Act d 1, (b) Act d 1 activated in the buffer with L-cysteine, (c) Act d 1 inactivated by thermal treatment and (d) Act d 1 inactivated by a cocktail of inhibitors. Relative proteolytic activity was calculated as % of proteolytic activity of the sample compared to activated actinidin (100% activity). Act d 1 showed 55% of caseinolytic activity when compared to activated Act d 1. 5.2% of caseinolytic activity was detected upon thermal treatment and 7.9% after treatment of Act d 1 with cocktail of inhibitors, respectively.

In addition, samples of Act d 1 were analyzed by SDS–PAGE and proteolytic activity was afterward visualized by zymogram with gelatin as substrate. All Act d 1 samples were run under reducing conditions in SDS–PAGE. Act d 1 inactivated by thermal treatment, or by protease cocktail inhibitors, revealed one strong band of about 30 kDa in reducing conditions (Fig. 3a). Act d 1 without additional treatment upon purification, as well as after activation with L-cysteine, revealed two bands that differed in electrophoretic mobility and intensity. The higher, faint band corresponded to inactive Act d 1, while the lower, stronger band of about 22 kDa seems to be active Act d 1. To confirm this assumption a zymogram with the same palette of Act d 1 samples was performed. Act d 1 samples which were inactivated either thermally or with the cocktail of protease inhibitors showed no proteolytic activity, while Act d 1 and activated Act d 1 were proteolytically active (Fig. 3b). In correlation with caseinolytic activity assay, activated Act d 1 revealed higher activity compared to Act d 1.

3.3. Mass analysis of actinidin preparation

Mass fingerprint of the 30 kDa band and the 22 kDa band of Act d 1 was performed after their excision from the SDS–gel and tryptic digestion. By Mascot analysis in both samples peptides $^1\text{LPSYVDWR}^8$ and $^{40}\text{IVTGVLISLSEQLIDCGRTQNTR}^{58}$ corresponding to the first part of mature actinidin were detected, as well as $^{182}\text{NSWDTTWGEEGYMR}^{195}$ and $^{199}\text{NVGGAGTCGIATMPSYPVK}^{217}$ peptides from the C-terminal part of the Act d 1 primary structure.

3.4. IgG reactivity of active and thermally inactivated Act d 1

Both active and thermally inactivated Act d 1 showed IgG reactivity in Western blot with anti-Act d 1 rabbit antibodies (Fig. 4). Thermally inactivated Act d 1 was detected as one intensive band of 30 kDa, while active Act d 1 showed one intensive band of about 22 kDa, and a faint band of 30 kDa, indicating that some inactivation of the enzyme had occurred.

3.5. *In vitro* and *in vivo* IgE reactivity of active and thermally inactivated Act d 1

Eight patients with clinical history of kiwifruit allergy were recruited for *in vivo* and *in vitro* studies. Their clinical characteristics, specific IgE levels, and SPT results are given in Table 1. All patients showed positive SPT with kiwifruit extract.

In vitro IgE reactivity of active and thermally inactivated Act d 1 was assessed in Western blot. As a source of specific IgE sera from seven patients were employed. IgE reactivity was detected only for inactivated Act d 1 in all tested patients sera (Table 1, supplementary material Fig. 2S), while active Act d 1 revealed no IgE binding.

Six out of eight patients were skin prick tested with serial dilutions of active and thermally inactivated Act d 1. Five (83%) out of six patients revealed a positive reaction to the thermally inactivated Act d 1 ($c = 500 \mu\text{g mL}^{-1}$), and four (67%) showed a positive

Table 1
Demographic and clinical data.

Subject No.	Age/ sex	Symptoms	Other food allergies	Specific IgE CAP (kU/L)	SPT(mm)		IgE-immunoblot						Histamine	IgE-immunoblot	
					Kiwi fruit extract	i Act d 1 ($\mu\text{g mL}^{-1}$)	a Act d 1 ($\mu\text{g mL}^{-1}$)			i Act d 1	a Act d 1			i Act d 1	a Act d 1
							500	50	5						
1. JU	41/M	OAS	Tropical fruit, celery	0.35	5	3	0	0	3	3	0	5	+	–	
2. AT	39/F	OAS	/	4.31	14	NT	NT	NT	NT	NT	NT	5	+	–	
3. IM	21/M	OAS, TT	Fish	0.67	8	10	10	0	12	5	3	4	+	–	
4. AM	2/F	OAS	Pork, milk, peanut, soy	NT	10	NT	NT	NT	NT	NT	NT	3	+	–	
5. MD	74/F	OAS, TT	/	0.35	6	5	0	0	7	3	0	3	+	–	
6. FP	10/M	AE	Banana, tomato	1.14	3	3	0	0	0	0	0	3	+	–	
7. JT	27/F	OAS	/	0.35	3	0	0	0	3	0	0	3	+	–	
8. LK	40/F	AE	/	0.35	3	3	0	0	0	0	0	4	NT	NT	

AE, Angioedem; OAS, oral allergy; TT, tightness of throat; NT (not tested); a Act d 1, active Act d 1; i Act d 1, inactivated Act d 1.

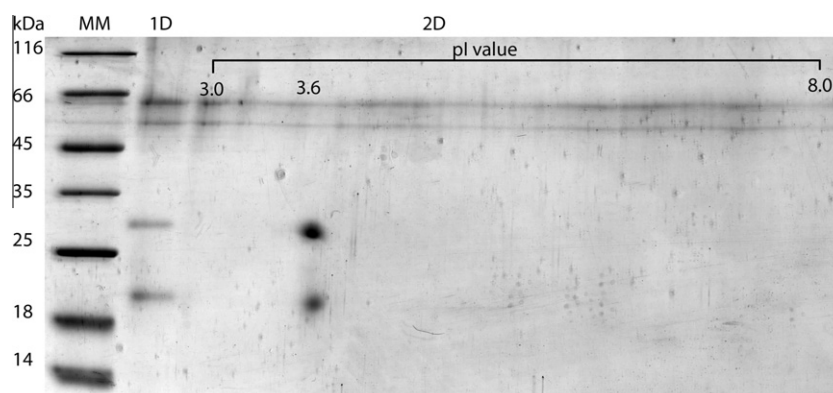


Fig. 1. Analysis of actinidin preparation (Act d 1) by (1) 1-D and (2) 2-D PAGE, MM) molecular markers. Proteins were stained by CBB.

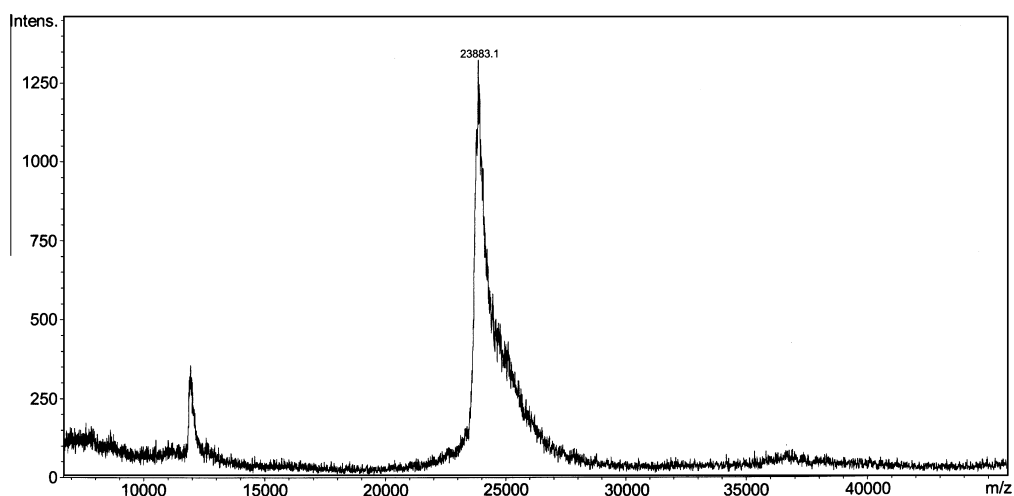


Fig. 2. Mass analysis of purified Act d 1.

reaction to the active Act d 1 ($c = 500 \mu\text{g mL}^{-1}$). However, three persons were more sensitive to active ($c = 50 \mu\text{g mL}^{-1}$) than to inactivated Act d 1. One person showed a positive reaction only to active actinidin and two showed a positive reaction only to the thermally inactivated Act d 1.

4. Discussion

Using proteomic tools and a combination of biochemical and immunochemical methods this study provides evidence that Act

d 1 isolated under native conditions from *A. deliciosa* kiwifruit extract represents a mixture of active and inactive enzyme. Proteolytic activity of the isolated Act d 1 was tested by caseinolytic assay and by zymography with gelatin. Under employed experimental conditions, without addition of protease inhibitors in the extraction buffer, the isolated Act d 1 preparation showed 55% of caseinolytic activity when compared to a preparation consisting of only activated Act d 1. Proteolytic activity was confirmed by zymography.

Mature Act d 1 contains seven cysteine residues, six of them being involved in disulphide bridges ($C^{22}-C^{65}$, $C^{56}-C^{98}$, $C^{156}-C^{206}$),

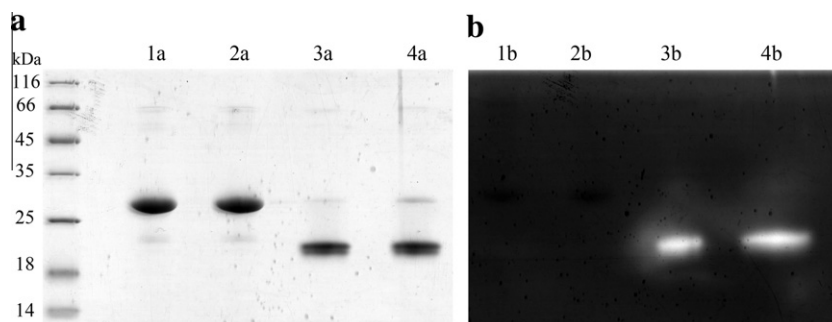


Fig. 3. SDS-PAGE analysis (a) and detection of proteolytic activity by zymogram (b) under reducing conditions: (1) Act d 1 inactivated by thermal treatment (95 °C, 5 min), (2) Act d 1 inactivated with cocktail of inhibitors, (3) Act d 1, (4) Act d 1 activated with L-cysteine, (M) molecular markers (from top to bottom: 116, 66, 45, 35, 25, 18, and 14 kDa).

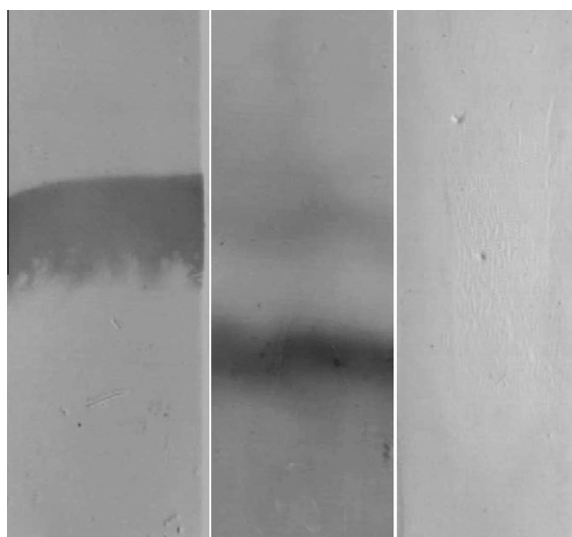


Fig. 4. Western blot with anti-Act d 1 rabbit antibodies: (1) thermally inactivated Act d 1, (2) active Act d 1, (3) negative control (buffer used instead of primary antibodies in immunoblot development).

and Cys²⁵ being involved in the active site (Baker, 1977). Unprotected active-site cysteine residues can undergo various covalent modifications which may regulate protein activity (Reddie and Carroll, 2008). In SDS-PAGE and 2D-PAGE under reducing conditions an extraordinary difference in electrophoretic mobility (30 kDa/22 kDa) between inactive and active Act d 1 was noticed. By using SDS-PAGE analysis of the kiwifruit extract treated with E64 protease inhibitor (Pastorelo et al., 1998) noticed a large increase in the 30 kDa actinidin band. Mass analysis of tryptic digests of inactive and active Act d 1 revealed that both bands preserved the primary structure of mature actinidin, suggesting that the difference in electrophoretic mobility between active and inactive actinidin is due to conformational changes in the protein structure. There are several reports in literature on anomalies in electrophoretic mobility of proteins with a high number of disulphide bonds. Thaumatin-like protein from apple (Mal d 2), with a 23 kDa molecular mass confirmed by mass analysis, migrated as a 31 kDa band in SDS-PAGE (Krebitz et al., 2003). Similar findings were reported for two kiwifruit allergens: thaumatin-like protein (Act d 2) (Gavrovic-Jankulovic et al., 2002) and kiwellin (Act d 5) (Tamburini et al., 2005).

Biological activity of Act d 1 was preserved upon thermal treatment (1 h at 45 °C), in the presence of SDS and 2-mercaptoethanol, indicating that S-S bridges in the protein remained intact, i.e. that the protein structure is very compact. Antigenicity of active and

thermally inactivated Act d 1 was confirmed by anti-Act d 1 IgG antibodies in Western blot.

In vitro IgE reactivity of thermally inactivated Act d 1 was shown in Western blot in seven kiwifruit allergic persons. None of them showed IgE reactivity to active Act d 1. A considerable difference in *in vitro* IgE reactivity due to environmental effects on the protein conformation have been reported for several food allergens. A significant increase in IgE-binding under reducing conditions was observed for Mal d 2, while none of the tested patients sera reacted with the unreduced protein in Western blot (Herndl et al., 2007). Enhanced IgE-reactivity of Mal d 2 was, therefore, found only after the breakage of disulphide bridges, suggesting that IgE-binding epitopes of Mal d 2 were hidden inside the molecule not easily accessible for the IgE molecules (Marzban et al., 2009). It has also been recently shown that the conformation, and consequently *in vitro* and *in vivo* IgE reactivity of Act d 5 (kiwellin), was affected by the physico-chemical characteristics of the environment (Bernardi et al., 2010), suggesting that the assay conditions influence the results of the diagnostic systems by modulating the pattern of exposed antigenic epitopes.

In vivo IgE reactivity of active and thermally inactivated Act d 1 was evaluated by skin prick test. These results show that *in vivo* the active actinidin is more IgE reactive. Since no protein precipitation was observed after the thermal inactivation (95 °C for 5 min) of Act d 1, it seems that monomolecular conformational scrambling brought to the inactivation of the enzyme. In line with results from *in vitro* IgE reactivity, data from *in vivo* testing indicates that besides conformational, hidden IgE binding epitopes, upon exposure, contribute to the allergenicity of Act d 1. It is believed that allergenicity of fresh fruits can be reduced rather easily by heat processing, allowing the production of hypoallergenic jams and juices (Soler-Rivas and Wichers, 2001). However, assessment of *in vivo* IgE reactivity of thermally inactivated Act d 1 suggest that thermally processed kiwifruit products could induce clinical symptoms in kiwifruit allergic persons. It has been shown that in a double-blind placebo-controlled food challenge (DBPCFC) steam-cooked kiwifruit preparation induced oral allergy syndrome in a child with kiwifruit allergy (Fiocchi et al., 2004). To the best of our knowledge this is the first evidence that thermally treated Act d 1 is capable of inducing clinical reactivity in kiwifruit allergic persons. However, our study has been performed on a rather small group of patients. In order to get a better insight into the IgE reactivity of biologically active and thermally inactivated actinidin a larger population of kiwifruit allergic subjects should be tested.

Even though our study was conducted in a small group of kiwifruit allergic patients, it indicates that compared to inactive 30 kDa actinidin, active 22 kDa actinidin under employed experimental conditions was not capable of binding specific IgE in Western blot. Such results could be misleading for allergy diagnosis as Act d 1 is regarded to be a selective biomarker of kiwifruit allergy.

IgE-binding assays with purified allergens should enable reliable detection/quantification of allergen-specific IgE, however deeper knowledge of the physico-chemical features of allergenic proteins is necessary for the improvement of diagnostic assays.

Conflict of Interest

The authors declare that there are no conflicts of interest

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fct.2011.12.030](https://doi.org/10.1016/j.fct.2011.12.030).

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