

Research Article

Quantification of the thaumatin-like kiwi allergen by a monoclonal antibody-based ELISA

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Thaumatococcus-like proteins (TLPs) have been established as a new family of fruit and pollen allergens. The aim of this study was to develop a two-site ELISA for the quantification of the thaumatococcus-like kiwi allergen (Act d 2) in kiwifruit extracts and kiwifruit-containing food products. Genomic DNA (gDNA) of Act d 2 was amplified and the deduced amino acid sequence was determined to obtain a primary structure. Act d 2 purified from kiwifruit extract by HPLC was identified by Edman degradation and MS. Balb/c mice were immunized with Act d 2 for the production of mAbs by hybridoma technology. The optimized ELISA measured Act d 2 concentrations ranging from 0.2 to 9.0 ng/mL, with intra- and interassay coefficients of variation of 3.65 and 10.44%, respectively. The developed ELISA is a useful method for the quantification of the thaumatococcus-like kiwi allergen in kiwifruit extracts as well as the allergen level in kiwifruit-containing food products. It may be a helpful analytical tool for the evaluation of the stability (integrity) of fruit allergen extracts for *in vitro* diagnosis.

Keywords: Act d 2 / Food allergy / Immunoassay / Kiwifruit / Thaumatin-like protein

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1 Introduction

Type I hypersensitivity to food is one of the most common chronic disorders in modern society [1], affecting approximately 3.5% of the adult population and up to 6% of the pediatric population [2]. The quality of allergen products is a key issue for allergy diagnosis and therapy [3], although standard immunotherapeutic approaches in food allergy are not adequate due to their unacceptably high risk-to-benefit ratio [4]. In spite of the enormous progress that has been made in the standardization of allergen extracts, its accomplishment may represent the most difficult endeavor in the field of biological medical products [5]. Diagnostic tests for food allergy frequently have poor specificity and sensitivity [6], as the quality of allergen extracts from fruits and

other plant foods can vary due to the inherent presence of proteolytic enzymes, the ripening stage and/or storage conditions of the allergenic source materials [7, 8]. In this regard, many commercial food extracts that are used in the diagnosis of food hypersensitivity still lack appropriate biological standardization or precise adjustment of the content of individual allergens [1]. Sensitivity as low as 20% in the ImmunoCAP assay and the skin prick test for cherry has been reported [9]. Only 50% of the patients with a clinical history to kiwi allergy had a positive IgE result to the kiwi extract determined by ELISA or CAP [10].

A prerequisite for the improvement of predictive and prognostic values of *in vitro* diagnostic results in food allergy is the development of reliable allergen reagents. The EuroPrevall consortium within the Sixth Framework Program of the EU [11] has taken up the challenge of developing novel diagnostic tools for food allergy, aiming to provide a better correlation of *in vitro* results with the clinical reactivity. One approach toward improvement of the quality of allergen products is to measure the individual major allergens in mass units using mAb-based ELISAs [12].

Kiwifruit is a frequent cause of fruit allergy, which can result in severe, life-threatening reactions, particularly in

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Abbreviations: mAb, monoclonal antibody; PR, pathogenesis-related; TLP, thaumatococcus-like protein

young children [13]. Recently conducted studies considerably highlighted the molecular basis of kiwifruit allergy nominating five IUIS kiwifruit allergens (www.allergen.org) [10, 13–16]. Nevertheless, a correlation (an algorithm) between the clinical profile and the allergen-specific sensitization pattern has not been revealed. Act d 1, the major and the most abundant allergen of green kiwifruit, seems not to be present in the gold cultivar (*Actinidia chinensis*) [15]. In contrast to Act d 1, Act d 2 and Act d 4, are found in both cultivars. Although it shows properties related to class 2 food allergens in terms of digestion stability [14], recent studies revealed Act d 2 among the sensitization profile in patients with pollen–fruit sensitization as well as in mono-sensitized individuals with anaphylaxis to kiwifruit [10]. Due to its biological activity [14] Act d 2 is a good candidate for component-resolved diagnosis of patients with kiwifruit allergy.

Although the clinical relevance of TLPs has not yet been established [17], thaumatin-like proteins (TLPs) are a new family of fruit and pollen allergens [18], as they are found in apple Mal d 2 [19], cherry Pru av 2 [20], bell pepper Cap a 1 [21], grape [22] and orange fruit [23], and may serve as quality markers for the fruit extract preparations. Allergenic TLPs have also been described in pollen grains of the mountain cedar (Jun a 3; *Juniperus ashei*) [24], in *Cupressus arizonica* pollen (Cup a 3) [25], and recently in Japanese cedar (Cry j 3; *Cryptomeria japonica*) [26].

The aim of this work was to establish a monoclonally based sandwich ELISA for quantification of the TLP in kiwifruit extract and other processed kiwifruit-containing food products.

2 Materials and methods

2.1 Genomic DNA (gDNA) extraction and PCR

gDNA was extracted from freeze-dried kiwifruit (*Actinidia deliciosa*) using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sense and antisense-specific primers with *Nco* I and *Bam* H I restriction sites (5'-CCATGGCCACTTTTAATATCA-TAAACAAGTGGCC-3' and 5'-GGATCCAGGGCAAAA-CACCACCTTATAGTTGG-3') were used for the nucleotide sequence amplification. The amplified Act d 2-encoding fragment was ligated by TA cloning into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α (Invitrogen, Paisley, UK). The construct was verified by DNA sequencing. Multiple sequence alignment was performed using the Vector NTI® program (Invitrogen).

2.2 Preparation of allergen extracts

Protein extracts of green (*A. deliciosa*) and gold (*A. chinensis*) kiwifruits, bell pepper (*Capsicum annuum*), and banana

(*Musa acuminata*) were prepared according to Bjorksten *et al.* [27] with replacement of 10 mM potassium phosphate with 100 mM ammonium bicarbonate solution (pH 8.0). Protein was extracted from apple (*Malus domestica*) and cherry (*Prunus avium*) according to Vieths *et al.* [28]. All protein extracts were dialyzed (MWCO 6000–8000 Da, Serva) overnight at 4°C and, after centrifugation at 10000 × *g* for 20 min, they were stored at –20°C before use. Kiwi-containing food products (jam, juices) obtained from local stores were homogenized with 10 mM PBS at a 30% w/v ratio and extracted for 1 h at room temperature (RT) and after centrifugation (10000 × *g*, 20 min) were stored at –20°C. The protein concentration was determined using the bicinchoninic acid (BCA) assay [29] with BSA as the standard.

2.3 Isolation of Act d 2

A three-step protocol was developed for the isolation of the thaumatin-like allergen Act d 2. The kiwi protein extract was fractionated on a QAE-Sephadex A-50 column pre-equilibrated with 100 mM ammonium bicarbonate (pH 8.0). The unbound protein fraction was concentrated by ultrafiltration, and dialyzed against buffer A (20 mM sodium citrate, pH 3.4). The sample was loaded on a Mini S column (Pharmacia Amersham, Uppsala, Sweden) connected with an HPLC system (ÅKTA Purifier, Pharmacia Amersham). Bound proteins were eluted with a linear gradient of 0–400 mM NaCl in buffer A. The Act d 2 enriched fractions were pooled, dialyzed against distilled water, and rechromatographed on a µRPC column (Amersham Pharmacia) pre-equilibrated with solution B (0.1% TFA). Act d 2 was eluted with a linear gradient of solution C (0–90% ACN in 0.1% TFA). The purity of isolated Act d 2 validated by 2D-PAGE as well as by Edman degradation [14] was above 97%. The molecular mass of purified Act d 2 was determined using a high-resolution ESI FT-MS. For experimental details see ref. [30]. The Act d 2 concentration was determined by the molar extinction coefficient (1.12), which was calculated from the obtained amino acid sequence, according to the following formula: $A_{280} (1 \text{ mg/mL}) = (5690 n_w + 1280 n_y + 120 n_c) \times M^{-1}$, where n_w , n_y , and n_c are the numbers of Trp, Tyr, and Cys residues [31] in the Act d 2 polypeptide; M was the average molecular mass of Act d 2 determined by ESI FT-MS.

2.4 Production of anti-Act d 2 mAbs

MABs to Act d 2 were produced by the hybridoma technology [32]. Balb/c mice were immunized subcutaneously with a 50 µg/dose of Act d 2 in complete Freund's adjuvant, incomplete Freund's adjuvant and PBS at 3 wk intervals. Three days after the final immunization, spleen cells (2×10^8) from each mouse were prepared and fused to Sp2/mIL-6 cell myeloma cells (1×10^8) in the presence of 50%

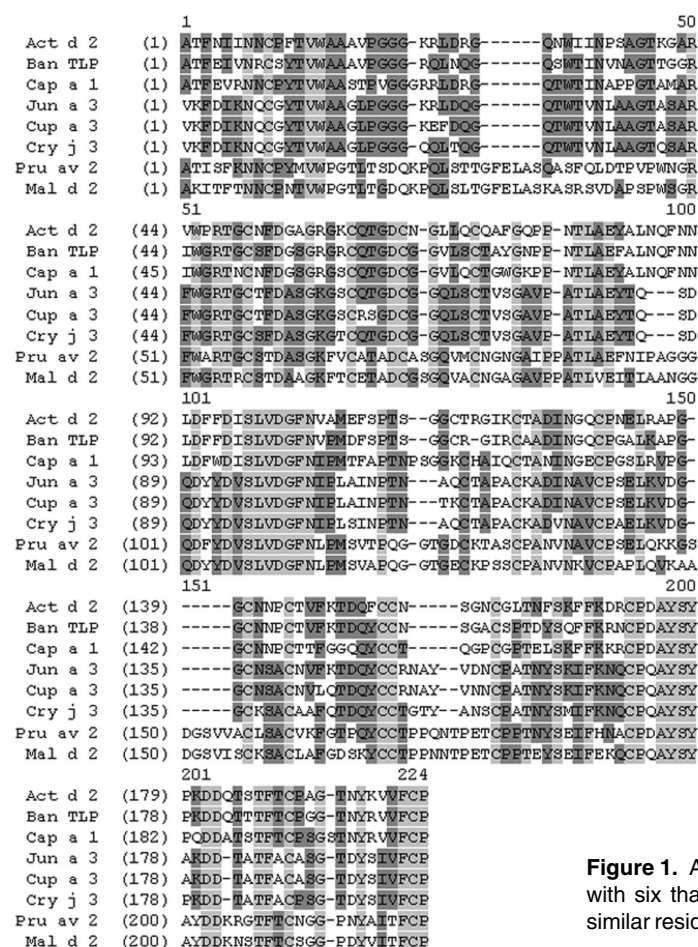


Figure 1. Alignment of the amino acid sequences of Act d 2 (EF417825) with six thaumatin-like allergens, and banana TLP: dark grey shading, similar residues; light grey shading, conserved residues.

w/v PEG 1000. Act d 2 positive hybridomas were subcloned by the limiting dilution method and finally propagated in CELLline CL 350 (INTEGRA Bioscience AG, Chur, Switzerland). Specificity and isotypes of the mAbs were determined by indirect and direct ELISA, respectively. Two selected IgG₁ mAbs, BG148 and BG7, were affinity purified on a Protein A-Sepharose 6B (Pharmacia Amersham) column according to the manufacturer's instructions. The additivity index (AI) for these mAbs was determined by indirect ELISA [33]. For sandwich ELISA, the purified mAb BG7 was biotinylated with *N*-hydroxysuccinimide biotin (Sigma–Aldrich, Steinheim, Germany) [32].

2.5 Immunodetection of TLPs in food extracts

Fruit protein extracts were separated under nonreducing conditions in a discontinuous buffer system in SDS-PA gel (12%) with a stacking gel (4%) in a Hoefer vertical electrophoresis slab system according to Laemmli. The amount of 2.5 µg/mm of the proteins was applied on the gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45 µm, Serva, Heidelberg, Germany) by semidry transfer at 0.8 A for 60 min. The membrane was cut into strips and, after blocking with 1% BSA in TTBS

(0.01% Tween, 20 mM TBS, pH 7.6) for 60 min at RT, the strips were incubated with the mAbs (dilution 1:100) for 180 min at RT or in TTBS buffer (control). After a washing step (3 × 10 min, TTBS), they were incubated in alkaline phosphatase-labeled anti-mouse IgG (1:10000, Jackson ImmunoResearch Laboratories, Suffolk, UK) or streptavidin-AP (Serva). Bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Serva) was used as the substrate for the detection of antigen–antibody interactions [32].

2.6 Quantification of kiwi TLP by sandwich ELISA

MaxiSorp ELISA plates (Sigma–Aldrich) were coated with affinity purified mAb BG148 (5 µg/mL, 100 µL per well) in 15 mM Na₂CO₃/35 mM NaHCO₃, pH 9.5 overnight at 4°C. Plates were washed (4 × 10 min) with PBS containing 0.01% v/v Tween 20 (TPBS). After blocking for 1 h at 37°C with 1% BSA in TPBS, ten-fold dilutions of Act d 2 (100 ng/mL), or the fruit extracts (1 µg/mL) in 0.1% w/v BSA in TPBS were added to the plate and incubated for 2 h at 37°C. The bound Act d 2 (or TLP homolog) was detected using biotin-labeled capturing mAb BG7 (5 µg/mL, 100 µL/well). After 2 h at 37°C, the plate was subsequently incubated with 100 µL streptavidin–alkaline phosphatase

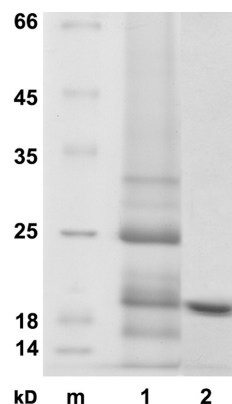


Figure 2. SDS-PAGE of Act d 2 purified by HPLC: (m), molecular mass markers; (1) green kiwifruit extract; (2) Act d 2.

(1:5000, Sigma–Aldrich) for 1 h. After incubation with a substrate solution (1 mg/mL *p*-nitro-phenylphosphate, Sigma–Aldrich), the absorbance was measured at 405 nm (reference wavelength 620 nm) [32]. The protein amount was converted to milligram *per* gram of fresh kiwifruit tissue, or kiwifruit-containing food.

3 Results

3.1 Genomic cloning of Act d 2

In order to obtain a defined standard for the calibration of the ELISA test, we used DNA cloning to determine a construct which refers to a 201 amino acid TLP proteins (GenBank accession no. EF417825). The protein revealed a theoretical *pI* of 7.90 and molecular mass of 21 627 Da (reduced form). One potential *N*-glycosylation site was identified in the N¹⁶³FS position. The encoded Act d 2 had an amino acid identity of 99% to AJ871175, with replacements at two positions G³⁶ → S³⁶ and Y¹⁵² → F¹⁵². Comparison of the amino acid sequence of Act d 2 with other thau-

matin-like allergens: Mal d 2 (AJ243427), Pru av 2 (2AHNA), Cap a 1 (CAC34055), Cup a 3 (CAC05258), Cry j 3 (AB254807), Jun a 3 (P81295), and banana TLP (1Z3QA) is presented in Fig. 1. The sequence similarity between Act d 2 and other TLPs was 78% to banana TLP, 72% to Cap a 1, 59% to Jun a 3, 56% to Cup a 3, 56% to Cry j 3, 38% to Pru av 2, and 34% to Mal d 2.

3.2 Purification of Act d 2

Act d 2 used for the production of mAbs and ELISA development was purified by HPLC. Anion- and cation-exchange chromatography were combined with RP chromatography (Fig. 2). The N-terminal amino acid sequence of the purified protein was ATFNIINNCPTV, corresponding to the N-terminal sequence of Act d 2. The purified Act d 2 analyzed by means of ESI FT-MS, provided two molecular ions (Fig. 3) with charge states of 10+, 11+ corresponding to average molecular masses of 21 570.6 Da and 21 600.7 Da, suggesting the presence of two isoforms of the mature protein. Although the Act d 2 sequence possesses one putative *N*-glycosylation site, mass analysis of the natural Act d 2 ruled out the presence of carbohydrate residues when compared to the theoretical mass.

3.3 Immunodetection of food TLPs

Protein profiles of six self-prepared food extracts obtained by SDS-PAGE are shown in Fig. 4A. Nine mAbs were developed with specificity for Act d 2 by anti-Act d 2 hybridoma technology. Based on their reactivity and the AI, two mAbs were selected for further studies. Anti-Act d 2 mAbs were used for the detection of TLPs in green and gold kiwifruit extracts in Western blot (Fig. 4B). Reactivity to TLP homologs in the fruit extracts was checked with the whole protein extracts of apple, banana, cherry and bell pepper. Both mAbs reacted with protein bands of about 21–23 kDa in gold and green kiwifruit, bell pepper,

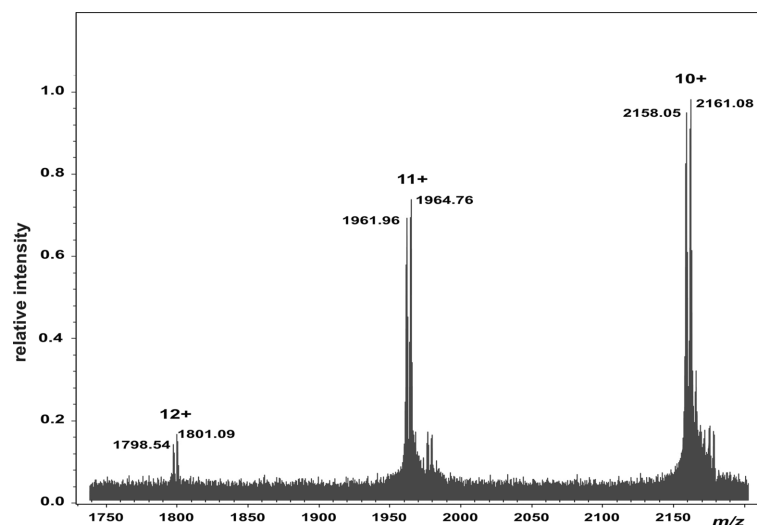


Figure 3. Positive ion ESI FT-MS mass spectrum of nonreduced Act d 2.

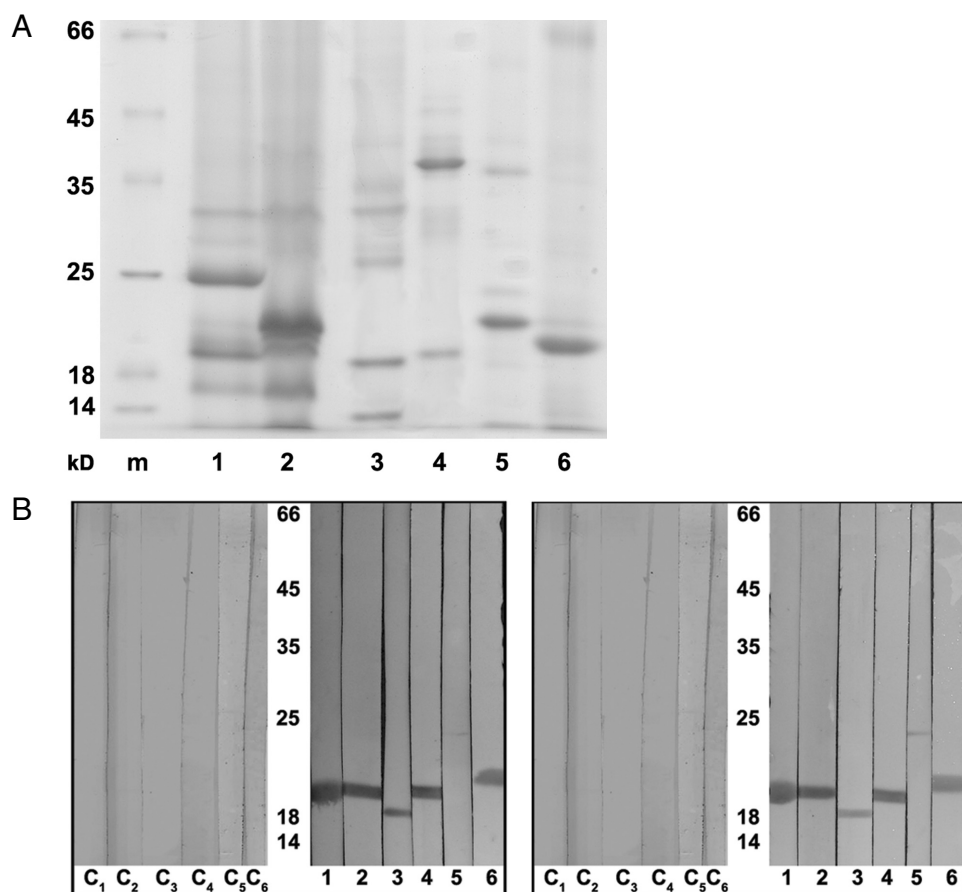


Figure 4. SDS-PAGE (A) and immunodetection of TLPs (B): (1) green kiwifruit, (2) gold kiwifruit, (3) banana, (4) bell pepper, (5) apple, and (6) cherry extract, with mAb BG148 (left) and mAb BG7 (right); (m), molecular mass markers; (c), control.

banana, apple and cherry, corresponding to Act d 2, Act c 2, Cap a 1, banana TLP, Mal d 2, and Pru av 2, respectively.

3.4 Act d 2 sandwich ELISA

A high AI was determined for the pair of selected mAbs (AI 85), suggesting their simultaneous binding to the antigen, *i.e.* different epitope specificity for native Act d 2, hence their applicability in sandwich ELISA. Figure 5A shows parallel dose–response curves for purified Act d 2 and a self-prepared green kiwifruit extract indicating that the Act d 2 epitopes had not altered during the purification process. The detection limit of 0.1 ng/mL has been assessed by determining the mean of 15 blanks plus three times the SD on five consecutive days. The working range of the assay was 0.2–9.0 ng/mL (range of linearity). The precision was determined by analyzing triplicates of the same sample in eighth independent assays. The mean intra assay CV in these assays was 3.65%, and the interassay CV was 10.44%.

TLPs in green and gold kiwifruit extracts represented 31% and 27% of the total protein content, respectively. Several kiwi-containing food products were tested for Act d 2 content. The detected concentrations are summarized in Table 1.

The selectivity of the assay was analyzed with various fruit extracts. The positive responses obtained for green and

Table 1. Quantification of kiwi TLP in self-prepared kiwifruit extracts and kiwi-containing food products

Sample	Protein content (mg/g)	Kiwi TLP (mg/g)
1 Green kiwifruit	3	0.936
2 Gold kiwifruit	3	0.820
3 Kiwifruit jam	27	4.320
4 Juice 1 ^a	2	0.135
5 Juice 2 ^a	3	0.390
6 Juice 3 ^a	3	0.264

a) Numbers indicate different manufacturers.

gold kiwifruits, banana, bell pepper, apple, and cherry (Fig. 5B), indicated certain degree of epitope homology between Act d 2 and respective TLPs, which was detected by two mAbs. However, for the accurate quantification the corresponding purified TLP for each fruit extract should be used as the standard in the dose–response curve.

4 Discussion

Consistent quality of plant food allergen extracts is compromised by biovariability of the allergenic source materials, interfering compounds (polysaccharides, phenolic compounds), inherent presence of proteolytic enzymes, but also

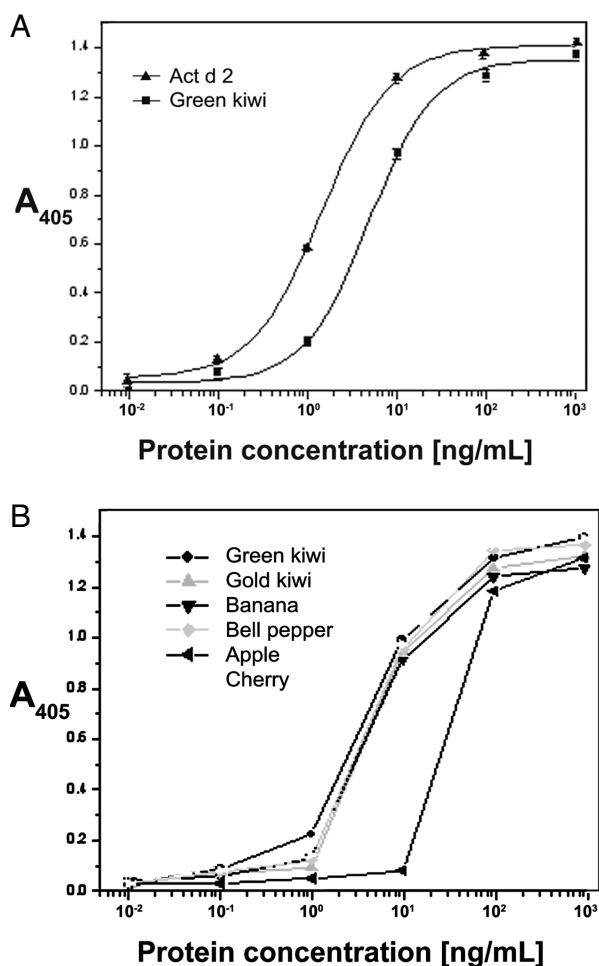


Figure 5. Development of the mAb-based ELISA. (A) Act d 2 standard curve (▲), green kiwifruit (■). (B) Dose–response curves in the TLP ELISA: green kiwifruit (●); gold kiwifruit (▲); banana (▼); bell pepper (◆); apple (◄); cherry extract (►).

instability of the allergens during the extraction procedure. Due to their presence in various fruits, TLPs may be good quality markers of fruit allergen extract preparations for *in vitro* diagnosis. In this study, a sensitive ELISA test based on two specific mAbs has been developed to measure thaumatin-like kiwi allergen. Accordingly, the Act d 2 ELISA may find an application in the quality control of kiwifruit extracts. In addition, a recombinantly produced Act d 2 may serve as an excellent reference material in similar quantitative assays.

We have previously identified and partially characterized the Act d 2 allergen [14]. In this study, structural characterization of Act d 2 was accomplished. Two molecular species of Act d 2 with a slight mass difference, identified by high-resolution ESI-MS, confirmed the presence of two protein isoforms, which had already been found by 2D-PAGE (pI 9.4 and 9.5) [14]. The Act d 2 sequence obtained from the gDNA of green kiwifruit encoded a 201 amino acid protein. Unlike Mal d 2 [34], no intron was found in the

genomic sequence of Act d 2. As shown for the plant expressed rMal d 2 [35], mature Act d 2 does not seem to be glycosylated.

Pathogenesis-related (PR) proteins represent a very important, structurally heterogeneous group of plant allergens [36]. Family 5 of PR proteins consists of TLPs, present in food as well as in tree pollens, which exist as acidic or basic isoforms. Among food allergens registered so far, Mal d 2 and Pru av 2 represent acidic TLPs, while Cap a 1 and Act d 2 are basic isoforms. With the selected anti-Act d 2 MAbs, we were able to detect Mal d 2, Pru av 2, Cap a 1, and banana TLP in the fruit extracts besides Act d 2 and its homolog in gold kiwifruit. The relative abundance of the protein in food is a factor to be considered in the evaluation of its allergenicity [37]. TLPs were important constituents of the analyzed kiwifruit extracts representing 27–31% of total proteins. TLPs abundance in the fruits together with the structural features (eight conserved disulphide bridges), and the complex plant-specific tissue matrix [38, 39] may contribute to their survival in the gastrointestinal tract and the process of allergic sensitization.

This is the first mAb-based sandwich ELISA developed for the quantification of the kiwi TLP allergen. It may be a useful tool for the evaluation of the stability (integrity) of kiwifruit allergen extracts, hence contributing to the quality control and improvement of *in vitro* diagnosis.

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The authors have declared no conflict of interest.

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