RESEARCH ARTICLE

Cysteine proteinase inhibitor Act d 4 is a functional allergen contributing to the clinical symptoms of kiwifruit allergy

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Kiwifruit has become a frequent cause of fruit allergy in the recent years. The molecular basis of type I hypersensitivity to kiwifruit is attributed to 11 IUIS allergens, with Act d 1, Act d 2 and Act d 5 characterized *in extenso*. Evaluation of the allergenic properties of Act d 4, a cysteine proteinase inhibitor from green kiwifruit (*Actinidia deliciosa*) was performed in this study. Identity of the purified glycoprotein was determined by Edman degradation and by mass fingerprint whereby more than 90% of the primary structure of the mature kiwifruit cystatin was confirmed. Using MALDI TOF analysis, molecular masses of 10902.5 and 11055.2 Da were detected for Act d 4, respectively. Positive skin prick reactivity with Act d 4 was induced in three kiwifruit allergic patients, as well as the upregulation of CD63 and CD203c molecules in the basophile activation assay. The IgE reactivity was detected in dot blot analysis while Western blot analysis was negative using sera from six kiwifruit patients, suggesting the presence of conformational IgE epitopes on the Act d 4 molecule. As activator of effector cells in type I hypersensitivity Act d 4 is a functional allergen contributing to the clinical symptoms of kiwifruit allergy.

Received: January 21, 2009
Revised: April 30, 2009
Accepted: June 5, 2009

Keywords:
Act d 4 / Allergen / Cysteine proteinase inhibitor / Kiwifruit / Phytocystatin

1 Introduction

Kiwifruit (*Actinidia deliciosa*) has been recognized as a food allergen source for almost 30 years [1]. Since early 1960s, when kiwifruit became available, its growing popularity has been accompanied with an increasing number of reports of allergic reactions to kiwifruit [2–6]. The symptoms of kiwifruit allergy are usually localized to the oral mucosa [7], but severe symptoms have been reported, particularly in the pediatric population [8]. Kiwifruit allergy is sometimes associated with the latex-fruit syndrome, including allergy to avocado, banana and chestnut. The outcome of the clinical studies revealed that kiwifruit allergy is not a homogenous disorder because several clinical subgroups can be established [8–10]. In addition, diverse IgE recognition patterns [11] seem to be related to different geographical populations in kiwifruit allergy.

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Abbreviations: AAL, *Aleuria aurantia* lectin; **CPI,** cysteine proteinase inhibitor; **RT,** room temperature; **SPT,** skin prick test

The molecular basis of kiwifruit allergy has been ascribed to eleven IUIS nominated kiwifruit allergens (www.allergen.org). Act d 1 (actinidin, a cysteine proteinase) has been established as a major kiwifruit allergen [4], and a link between IgE level to Act d 1 and anaphylaxis has been revealed in a group of Spanish kiwifruit allergic patients [10]. However, according to Lucas *et al.* [11], the *in vitro* IgE reactivity to Act d 1 is lacking in the population of patients allergic to kiwifruit in UK. Act d 2 (a thaumatin-like protein) [12] seems to be a relevant kiwifruit allergen in the Mediterranean region [9, 10, 13]. Act d 3, a 40 kD protein with a high sequence identity (72%) to a hypothetical hydrolase from *Ricinus communis* [10], is a clinically relevant allergen in the Spanish population of kiwifruit allergic subjects. Clinical relevance of other identified kiwifruit allergens such as Act d 4 (cysteine proteinase inhibitor, CPI) [13], Act d 5 (kiwellin) [14], Act d 6 (a pectin methylesterase inhibitor), Act d 7 (a pectin methylesterase) [15], Act d 8 (a Bet v 1-homologue) [16], Act d 9 (profilin), Act d 10 (nsLTP1, www.allergen.org), and recently registered Act d 11 (major latex protein, www.allergen.org) remains to be determined in a larger population of patients allergic to kiwifruit.

Due to the inconsistent quality of plant-food allergen extracts, current *in vitro* diagnosis to kiwifruit allergy is limited, with less than 50% of the patients with a clinical history to kiwifruit allergy having a positive IgE result to the kiwi extract used for skin prick test (SPT) and in the ELISA or CAP system [9]. The replacement of fruit allergen extracts with component-resolved allergens seems to be a promising concept, as shown for cherry. The panel of three cherry recombinant allergens was superior to diagnostic methods based on the cherry extract [17]. In this regard, the evaluation of allergenic properties of a particular allergen candidate for component-resolved diagnosis needs to be performed. By proper evaluation of a candidate allergen it is possible to differentiate between clinically relevant IgE reactivity in terms of its capability of cross-linking FcεRI receptors and IgE reactivity not accompanied by clinical symptoms [18].

The cystatin superfamily consists of proteins that specifically inhibit cysteine proteinases, which is of fundamental functional importance in cells. Cystatins have been identified in various organisms such as plants, vertebrates, invertebrates [19], and have also been found in tissues and body fluids of animals and humans [20, 21]. Those from plants, referred to as phytocystatins, comprise of more than 80 members (Pfam databank) [22], and they cluster in a major evolutionary tree branch of the cystatin superfamily of proteins [23]. Most phytocystatins are small proteins ranging from 12 to 16 kDa and contain neither disulphide bonds nor putative glycosylation sites. The kiwifruit cystatin, with the consensus sequence characteristic for phytocystatins, was subsequently isolated from cortex and seeds [24], and the first report on its IgE-binding activity from sera of a group of kiwifruit allergic patients was presented [13]. The first report on IgE reactivity to cystatins refers to short ragweed (*Ambrosia artemisifolia*) cystatin Amb CPI [25]. IgE-reactive cystatins have been reported as a cat cystatin Fel d 3 [26] and more recently, Ani s 4, from parasite *Anisakis simplex* [27].

The aim of this study was to characterize biochemically the isolated CPI Act d 4 from green kiwifruit and to explore its allergenic potential for *in vivo* and *in vitro* diagnosis of kiwifruit allergy.

2 Material and methods

2.1 Patients

Kiwifruit allergic patients were selected for the study according to a positive SPT to kiwifruit extract, and/or

Table 1. Demographic and clinical data

Patient no.	Age/sex	Symptoms	Other food allergies	Specific IgE (kU/L)	SPT	
					Kiwi fruit extract	Act d 4
1. AŠ	58/F	AE	–	0.87	+	+
2. K6	33/M	OAS, TT	Apple, cherrypear, melon	NT	+	NT
3. MD	73/F	OAS, TT	–	<0.35	+	+
4. K17	33/F	OAS, TT, DP	Peanut, hazelnut, walnut, pear, banana, tomato, bell pepper, cucumber, soybean, almond	<0.35	+	NT
5. MJ	52/F	AE, TT	–	<0.35	+	NT
6. DD	42/F	SA	–	<0.35	+	+
7. K18	22/F	OAS, U, A, TT	Peanut, hazelnut, apple, peach, cherry, pear, pineapple, banana, melon	2.3	–	NT

A, asthma; AE, angioedema; OAS, oral allergy; DP, dyspnoea; SA, systemic anaphylaxis, TT, tightness of throat; U, urticaria; NT (not tested).

specific IgE to kiwifruit. Demographic and clinical data of the studied patients are summarized in Table 1. Written informed consent was obtained from all patients. Pool of sera from two non-atopic persons was included as control.

2.2 Preparaton of papain affinity matrix

For the Act d 4 isolation from kiwifruit (*A. deliciosa*) a papain affinity matrix was prepared according to Harlow and Lane [28]. In brief, BioGel P-100 (6 mL, BioRad) was mixed with 25% glutaraldehyde solution (Fischer Chemicals AG, Zurich, CH) and incubated at 37°C with shaking overnight. Activated beads were extensively washed with 500 mM phosphate buffer pH 7.4. Dissolved papain (1.05 mg/mL) in 500 mM phosphate buffer pH 7.4 containing 1 mM PMSF was added to the glutaraldehyde-activated BioGel beads and incubated overnight with shaking at room temperature (RT). After washing with 500 mM phosphate buffer (ten column volumes), remaining active groups were quenched with 100 mM ethanolamine pH 7.0 for 4 h at RT. The gel was stored in PBS at 4°C until use. The efficacy of protein coupling determined by substracting the concentration of papain in solution after coupling from the initial concentration was 86%.

2.3 Purification of Act d 4

Isolation of kiwifruit cystatin was done according to Ryan *et al.*, [29] with a slight modification. In brief, fresh kiwifruit cortex was homogenized and suspended (1:3 v:v) in 100 mM sodium citrate, pH 3.0, containing 4 mM EDTA, 1% L-cysteine, 0.1% PEG 400, 0.6 mM PMSF (Sigma-Aldrich, Steinheim, Germany) and 10 mg/mL PVP (Sigma-Aldrich). The extract was filtered through cheesecloth and centrifuged ($5000 \times g$, 30 min) at 4°C. Actinidin was inactivated by incubation of supernatant at 60°C for 10 min. After cooling and centrifugation ($5000 \times g$, 20 min), supernatant was neutralized with 1 M NaOH and centrifuged again. Total proteins were precipitated using ammonium sulphate to achieve 100% saturation. Following centrifugation ($5000 \times g$, 10 min), protein precipitate was resuspended in minimal volume of deionized water and additionally centrifugated ($13\,400 \times g$, 10 min) before application onto the papain affinity column pre-equilibrated earlier with PBS buffer pH 7.4. Unbound proteins were eluted with ten column volumes of the starting buffer and bound fractions with 20 mM glycine-HCl buffer pH 2.5, which were immediately adjusted to pH 7.0 with 1.5 M Tris-HCl buffer, pH 8.8. Fractions containing Act d 4 were pooled and after lyophilization additionally resolved on a μRPC C2/C18 column (GE Healthcare), which was pre-equillibrated with 2% acetonitrile, 0.065% TFA in water and eluted using a linear gradient 10–80% of acetonitrile with 0.05% TFA in water. The homogeneity of isolated Act d 4 was analyzed by isoelectric focusing [30], SDS-PAGE [30] and 2-D PAGE [12].

2.4 MS analysis and N-terminal sequencing

The molecular mass of purified CPI Act d 4 was determined by MALDI TOF-MS with a Reflex III (Bruker-Daltonik, Bremen, Germany) in the positive ion mode. The protein was dissolved in water at a concentration of 5 μg/μL 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, Sigma-Aldrich) in a 2:1 mixture of 0.1% TFA /acetonitrile. In total 0.5 μL aliquots were deposited on the sample holder and analyzed immediately after drying in a stream of air. For N-terminal sequencing kiwifruit cystatin was transferred by semidry blotting onto a PVDF membrane (Serva, Heidelberg, Germany) according to Harlow and Lane [28]. The membrane was washed in double-distilled water, stained with 0.1% Coomassie Blue (Serva) in 50% methanol, destained in 50% methanol and air-dried. The protein band was excised and microsequenced using a Procise protein sequencer connected to an online PTH amino acid analyzer (PE Biosystems, Weiterstadt, Germany).

The amino acid sequence of isolated kiwi protein was verified by mass fingerprint. The protein sample was separated by SDS-PAGE, and after Coomassie Blue staining the protein band was 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 [31]. For MALDI TOF-MS analyses of the tryptic fragments, CHCA, were used as matrix. The mass spectra of the measured monoisotopic peaks were compared with the masses of the tryptic peptides of the phytocystatin sequence GenBank AAR92223.

2.5 Lectin binding to Act d 4

Presence of carbohydrate residues in the Act d 4 molecule was examined by two mannose-binding lectins, Concanavalin A (Con A) and recombinant BanLec (rBanLec), and fucose-binding *Aleuria aurantia* lectin (AAL) according to the previously described procedure [32]. In brief, 0.5 μg/mm of Act d 4 protein was loaded onto the SDS-gel. After gel electrophoresis, the protein was subsequently transferred to a nitrocellulose membrane (NC, 0.45 μm Serva), and the membrane was blocked with 1% BSA in 20 mM Tris buffered saline, 0.05% Tween 20 v/v pH 7.5. For glycoprotein detection, strips of the membrane were stained with biotin-labeled rBanLec, biotin-labeled Con A lectin (1:10 000, Sigma-Aldrich), or alkaline phosphatase conjugated AAL (1:2000, Vector Laboratories, Servion, Switzerland).

rBanLec and Con A lectin binding were detected with streptavidin-labeled alkaline phosphatase (1:5000, Sigma-Aldrich). Binding patterns were visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (0.033%/0.017% w/v, BCIP/NBT, Sigma-Aldrich) in 100 mM Tris buffer, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5.

2.6 Proteinase inhibition assay

Biological activity of purified kiwifruit cystatin was tested in the papain inhibition assay, performed in a 96-well microtitre plate (Sarstedt, Nümbrecht, Germany). Phosphate buffer (40 μL, 100 mM pH 6.8 with 2.5 mM DTT and 5 mM EDTA) was mixed with aqueous papain solution (10 μL, concentration 1 IU/mL, Sigma P-4762) and 10 μL of varying amounts of cystatin (from 8.33 μg/mL to 83.3 μg/mL) and incubated for 15 min at 25°C. The reaction was initiated by addition of 10 μL of substrate (Nα-benzoyl-L-arginine ethyl ester hydrochloride in DMSO) at three different concentrations (10, 25 and 50 mM). After 60 min the reaction was stopped by the addition of glacial acetic acid and the absorbance was measured at 405 nm was measured. The rates were calculated from linear regression data.

2.7 Antisera production

Antibodies against kiwifruit (*A. deliciosa*) extract [33] were raised in rabbits according to Harboe and Ingild [34]. The animals were injected with 0.5 mL of an emulsion (1:2 v:v) of kiwifruit protein extract (1 mg/mL) in complete Freund's adjuvant. Bleeding was performed 50 days after the first immunization and every two weeks thereafter. The serum was partially purified by ammonium sulfate fractionation (50% saturation). For immunodetection in immunoprint and Western blot a dilution of polyclonal antibodies (1:5000) was used. Bound antibodies were detected by alkaline phosphatase-labeled anti-rabbit IgG (dilution 1:2000, Sigma-Aldrich) and finally with a BCIP/NBT solution.

2.8 IgE immunodetection

For dot blot analysis 1 μg of purified Act d 4 was applied onto nitrocellulose membrane (0.45 μm, Serva), which was cut into strips and blocked with 30 mM TBS, pH 7.4, containing 1% w/v BSA and 0.5% v/v Tween 20 (Serva) for 1 h at RT. For visualization of IgE binding, strips were incubated overnight with patient's sera (dilution 1:2 v:v) at 4°C and following washing step, membranes were incubated with mouse anti-human IgE (1:3000) for 4 h at RT, and finally with alkaline phosphatase-labeled anti-mouse IgG (1:10 000, Jackson ImmunoResearch Laboratories, Suffolk, UK) for 4 h at RT. Visualization of IgE binding was done using BCIP/NBT solution. For Western blot analysis purified Act d 4 was separated under reducing conditions in a discontinuous buffer system in a SDS-PA gel (16%) with a stacking gel (4%) in a Hoefer vertical electrophoresis slab system according to Laemmli. The amount of 2.5 μg/mm of the protein was applied on the gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane (0.45 μm, Serva) by semidry transfer at 0.8 mA/cm^2 for 45 min. Further analysis was performed as described for dot blot assay.

2.9 Basophile activation assay

Aliquots of 100 μL heparinized whole blood of kiwifruit allergic patients were stimulated with the purified Act d 4 (50 μg/mL). Non-stimulated cells and stimulation with anti-human IgE served as controls. Samples were incubated for 20 min at 37°C. Afterwards, 5 μL FITC-conjugated anti-CD63 and 5 μL phycoerythrin-conjugated anti-CD203c (Immunotech, Marselle, France) were added to each sample and incubated for 20 min at 4°C. After the erythrocyte lysis and centrifugation (400 g for 5 min), the cells were resuspended in PBS (400 μL) and measurements were performed on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA).

2.10 SPT

SPTs with the kiwifruit extract (500 μg/mL) and Act d 4 (50 μg/mL) in PBS buffer/glycerol solution (1:2 v:v) were performed according to the standard procedure [35] in the volar side of the forearm. Histamine phosphate (10 mg/mL, Sigma-Aldrich, Buchs, Switzerland) and PBS were used as positive and negative controls, respectively. A diameter ≥ 3 mm compared with that produced by the negative control (20 min after puncture) was considered a positive response. As a control three healthy subjects were skin prick tested with the extract and the allergen.

3 Results

3.1 Purification of CPI Act d 4 from kiwifruit extract

For the isolation of cystein protease inhibitor from kiwifruit extract affinity chromatography was employed. The affinity matrix prepared by the immobilization of papain to a glutaraldehyde-activated BioGel P-60 was stable, and no protein leakage was observed. Following separation on papain affinity column, the Act d 4 preparation was additionally resolved by the reversed-phase chromatography. The major protein peak was eluted with 14.5% of buffer B, which, according to SDS-PAGE, provided homogenous protein species (Fig. 1), and was employed for further analysis. The yield of Act d 4 was about 6 mg from 1000 g of

fresh kiwifruit. The purified Act d 4 showed a single spot of about 11 kDa and p*I* of about 6.9 in 2D-Page immunoblot under reducing conditions, which was detected by rabbit polyclonal antibodies (Fig. 2). Biological activity of purified Act d 4 was confirmed with papain cysteine proteinase inhibition showing IC_{50} of 2.47 nM.

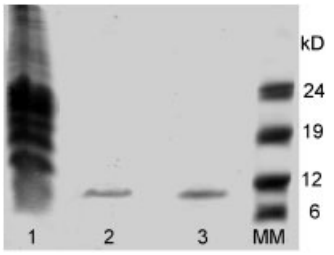

Figure 1. SDS-PAGE analysis of kiwifruit extract (lane 1), purified Act d 4 under reducing (lane 2) and non-reducing conditions (lane 3); MM, molecular mass markers.

Figure 2. IEF immunoprint and 2-D PAGE immunoblot of purified kiwifruit cystatin Act d 2 identified by rabbit polyclonal antibodies.

3.2 MS analysis and N-terminal sequencing

To confirm the identity of the purified protein PVDF-blotted Act d 4 was analyzed by Edman degradation. Fourteen amino acids identified as VAAGGXRPIESLNSA confirmed the sequence of mature kiwifruit cystatin (NCBI AAR92223). The signal sequence consisting of 26 amino acids, predicted to import the nascent polypeptide to the endoplasmatic reticulum, was cleaved off in the mature inhibitor. Although plant cystatins are regarded as proteins lacking post-translational modifications, one potential *N*-glycosylation site ($N^{83}LT$) was present in the Act d 4 amino acid sequence. Theoretically calculated molecular mass for the mature cystatin is 10052.46 Da with a p*I* 6.94, while experimentally determined molecular mass of the Act d 4 was 10 902.5 and 11 055.2 Da (Fig. 3). By mass analysis of in-gel tryptic digest more than 90% of the primary structure of Act d 4 was confirmed. Only the ^{83}NLTSFRKV^{90} peptide, containing the potential N-glycosylation site, was not identified, opening a possibility for the presence of carbohydrate residues (Fig. 4). Detection of carbohydrate residues on Act d 4 molecule was achieved with two mannose-specific lectins Con A and rBanLec and fucose-specific AAL. All three lectins bound to Act d 4 in Western blot, suggesting carbohydrate presence (Fig. 5).

3.3 IgE reactivity of Act d 4

Purified Act d 4 showed IgE reactivity in dot blot analysis with six from seven kiwifruit allergic subjects (Fig. 6). However, in Western blot analysis no IgE binding to Act d 4 was detected with any of these patients' sera, implying presence of conformational IgE-binding epitopes on Act d 4. Biological activity of the purified Act d 4 was evaluated by

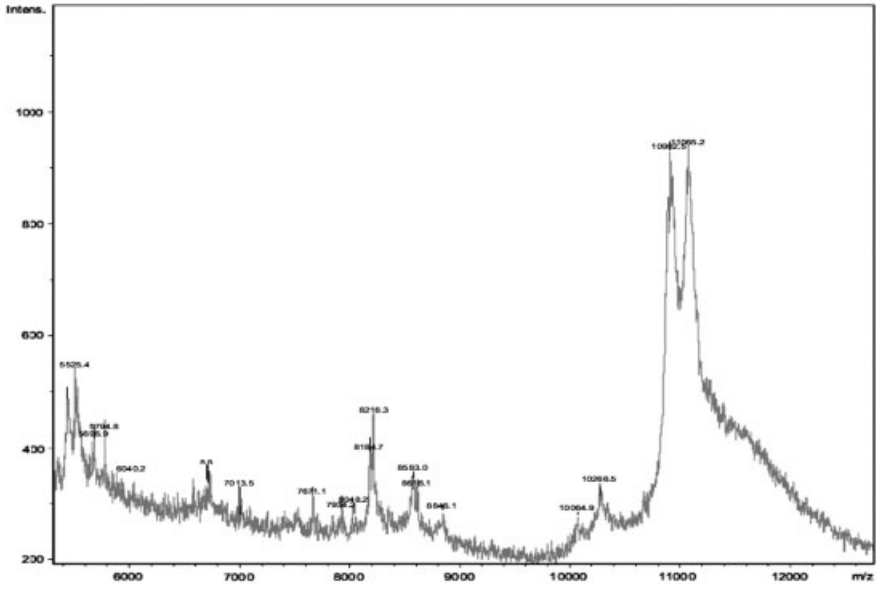

Figure 3. MALDI TOF analysis of Act d 4.


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                1                                                  50
  Act d 4     (1) --VAAGGWRPIESLNSAEVQDVAQFAVSEHNKQANDELQYQSVVRGYTQV
Amb a CPI     (1) MSILGGITEVKDNDNSVDFDELAKFAIAEHNKKENAALEFGKVIEKKQQA
  Ani s 4     (1) -GMLGGSSDVDVNDPEIKELAGKSIAKISAMINDGKPHELVKVVSAKKQV
  Fel d 3     (1) -MIPGGLSEAKPATP--EIQEIANEVKPQLEEKTNETYQKFEAIEYKTQV
                51                                                 100
  Act d 4    (49) VAGTNYRLVIAAKDGAVVGNYEAVVWDKPWMHFRNLTSFRKV--------
Amb a CPI    (51) VQGTMYIKVEANDGGEKKTYEAKVWVKLWENFKELQELKLV--------
  Ani s 4    (50) VAGDKYTLEILVKDGDHQDLCTVTIWQKKWENFEEVKVLKCDHQ------
  Fel d 3    (48) VAGINYYIKVQVDDNRYIHIKVFKGLPVQDSSLTLTGYQTGKSEDDELTGF
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Figure 4. Alignment of the amino acid sequences of Act d 4 with three IgE- reactive cystatins: Amb a CPI (Q38678), Ani s 4 (Q14QT4), and Fel d 3 (Q8WNR9). Conserved residues are highlighted; putative N-glycosylation consensus sequence in Act d 4 is shown in bold letters.

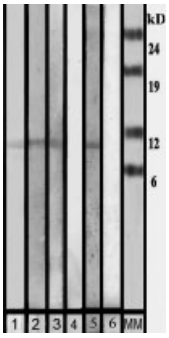

Figure 5. Lectin binding to Act d 4 in Western blot: NC incubated with (1) Ponceau S, (2) Con A lectin, (3) rBanLec, (4) streptavidin-alkaline phosphatase, (5) alkaline phosphatase conjugated AA lectin, (6) buffer; (MM) molecular markers.

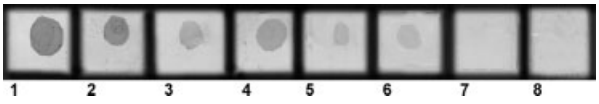

Figure 6. IgE reactivitiy of purified kiwifruit cystatin Act d 4 detected in dot blot: (1–7) patient sera; (8) control non-atopic serum.

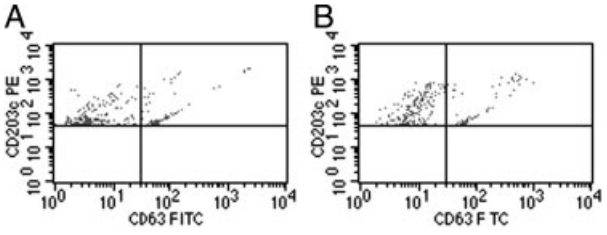

Figure 7. Basophil activation with: (A) Act d 4, (B) positive control.

SPT (Table 1). Positive response was elicited in three monosensitized patients to kiwifruit, suggesting that Act d 4 can induce clinical symptoms of allergy by triggering release of biologically active mediators. In addition, Act d 4 was able to activate basophils from a kiwi-allergic patient (no. 1, Table 1) by upregulation of CD63 and CD203c molecules (Fig. 7). More than 20% of basophils were activated with 50 μg/mL Act d 4, compared with 28 and 2% of activated cells with positive and negative controls, respectively.

4 Discussion

This study describes characterization of kiwifruit CPI Act d 4 in terms of its structural and allergenic properties. The Act d 4 protein, isolated from kiwifruit extract by ammonium sulphate precipitation, papain affinity chromatography and reversed-phase chromatography, had a molecular mass of 11 kDa and p*I* 6.9, and exhibited inhibitory activity against papain. Kiwifruit cystatin is synthesized as a 116 amino-acids pre-protein with a theoretical molecular mass of 12 755.7 Da and p*I* of 9.40. The isolated Act d 4 was a mature form of CPI with its first 26 amino acids, representing a signal sequence, cleaved off. Although the molecular mass of isolated Act d 4 showed about 1 kDa mass difference compared with theoretical mass, these findings are in agreement with the data reported by Rassam and Laing [24], which also employed MALDI TOF analysis to assess the molecular mass of Act d 4. The discrepancy between theoretical and experimentally determined mass of about 1 kDa suggests the presence of post-translational modifications and possibly, different isoforms. Besides Con A lectin, the presence of carbohydrate residues on the isolated Act d 4 was detected by mannose-specific BanLec which is specific for internal α1,3 linkages as well as β1,3 linkages in branched trisaccharides and branched pentamannose oligosaccharides [36], both of which are components of the ''core'' region of N-linked glycoproteins. Binding of AA lectin to Act d 4 indicates a complex glycane structure, with fucose linked to N-acetylglucosamine.

The cystatin superfamily has been subdivided into three families based on their sequence homology, the presence and position of intrachain disulphide bonds, and the molecular mass of the protein. The stefin family consists of proteins lacking disulphide bonds and carbohydrates, having molecular mass of approximately 11 kDa. The second family, called cystatin, consists of proteins of about 115 amino acids, which contain four conserved cysteine residues forming two disulphide bonds and may be glycosylated and/or phosphorylated. The third family is composed of larger glycoproteins of 60–120 kDa [19, 23]. The primary sequence of plant cystatins has a high degree of homology with the members of the cystatin family, but they resemble stefins by the absence of cysteine residues. Kiwifruit cystatin Act d 4 is an 11 kDa protein having no cysteine residues, however, the presence of one N-glycosylation site with the attached carbohydrates is an exception from the typical structural features of the phytocystatins.

Allergenic properties of Act d 4 were evaluated by employing *in vitro* and *in vivo* assays. Act d 4 showed IgE reactivity under native conditions, while heat treatment and SDS denaturation resulted in diminished IgE reactivity in Western blotting with the employed detection system. These data suggest presence of conformational IgE-binding epitopes on the molecule. However, Bublin *et al.* [13] were able to detect IgE reactivity of Act d 4 in an immunoblot, which could be due to the engagement of a more sensitive detection system based on radioactive labeling. Clinical evaluations of IgE reactivity of kiwifruit proteins following immunoblotting within large populations of patients usually employ enzyme-based detection assays [9, 11]. Our results suggest that the relevance of some kiwifruit allergens such as Act d 4 might have been underestimated under such experimental conditions. In addition, the presence and the amount of fruit allergens in allergen extracts largely depend on the stage of fruit ripening [37, 38] and can compromise the reliability of applied diagnostic procedure [39] and explain why routine tests like SPT and CAP are positive in less than 50% of the patients [9]. Instead of kiwifruit extracts highly purified individual natural or recombinant kiwifruit proteins should be employed for the evaluation of their clinical relevance [40].

IgE-reactive cystatins besides Act d 4 and Act c 4, have been identified in weed pollen *A. artemisifolia* as Amb a CPI and in animals *e.g.* Fel d 3 from cat dander and Ani s 4 from parasitic nematode *A. simplex*. The sequence similarity between Act d 4 and other cystatins was 40% to Amb a CPI, 27% to Ani s 4 and 13% to Fel d 3. All analyzed cystatins contain a conserved glycine residue in the N-terminal region and a highly conserved Q-X-V-X-G motif in the central loop of the cystatins, which seems to make the major contribution to the formation of the complex with a susceptible proteinase.

Further experimental evidence is necessary to explore the existence of cross-reactivity on the molecular level, between Act d 4 and Amb a CPI due to the sequence similarity, since kiwifruit allergy has been reported as pollen-associated type I hypersensitivity, developed due to presence of cross-reactive antigens in pollens and kiwifruit [3, 5, 6]. Nevertheless, in our study clinical relevance of Act d 4 has been revealed in three monosensitised kiwifruit patients implying its relevance in non-pollen related food allergy. Although the prevalence of kiwifruit cystatin remains to be determined in a large group of patients, Act d 4 is a clinically relevant kiwifruit allergen, which should be involved in the component-resolved diagnosis of kiwifruit allergy.

This work is supported by the Ministry of Science and Technological Development, Republic of Serbia, Grant No. 142020. The authors thank Daniela Warneke for her excellent technical assistance.

The authors have declared no conflict of interest.

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