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Allergenic potency of kiwi fruit during fruit development

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(Act c 2) change with fruit ripening. These findings should be taken into account during preparation of extracts for allergy diagnosis.

Keywords:

Kiwi fruit Actinidia deliciosa food allergy allergen actinidin thaumatin-like protein development
ripening

Abbreviations

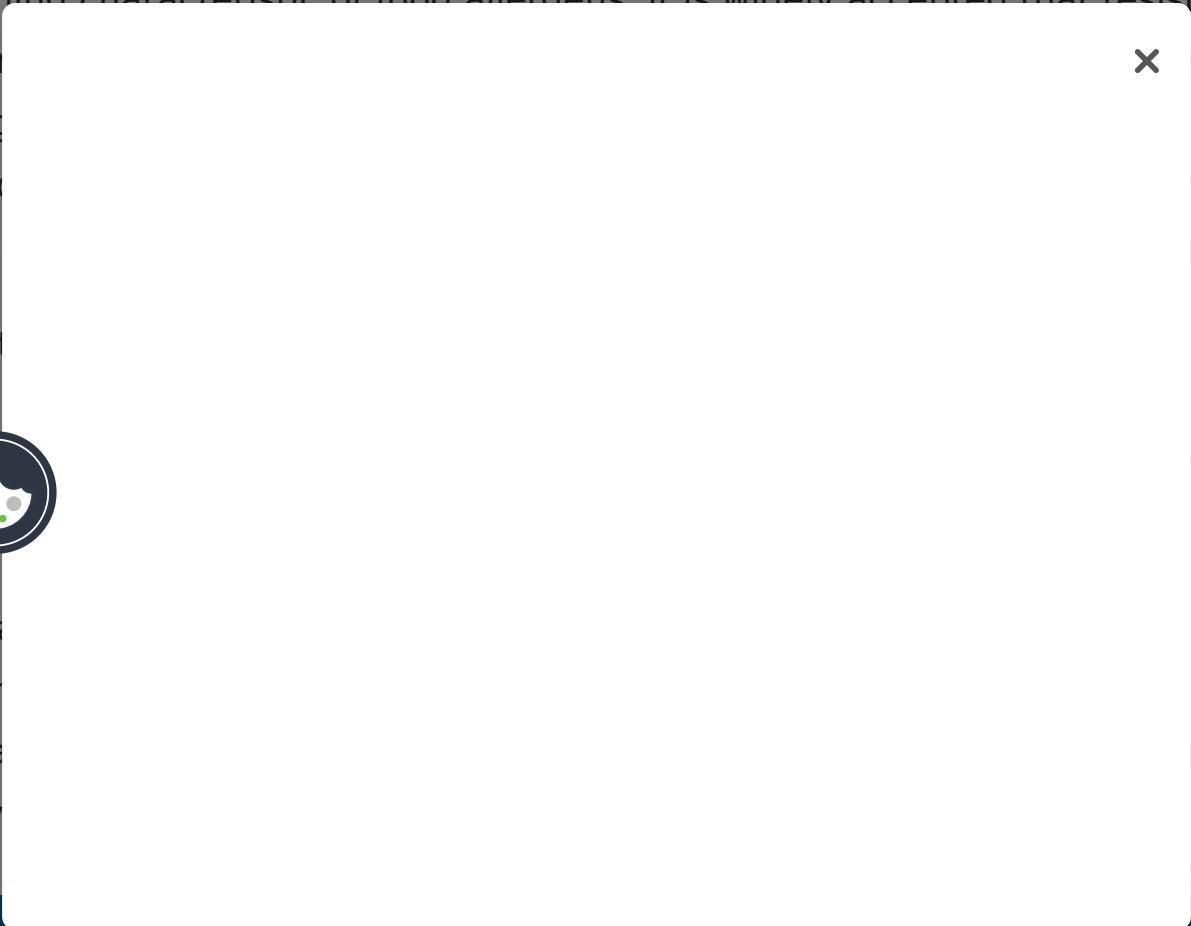
OAS	=	Oral-allergy syndrome
SPT	=	skin prick testing
SDS-PAGE	=	sodium dodecylsulfate-polyacrylamide gel electrophoresis
BSA		
phosph (PBS)		
TLP		
PVPP		
CBB		



SGF	=	simulated gastric fluid
RIE	=	Rocket immunoelectrophoresis

Introduction

Food allergy is an important health problem nowadays. Clinical reactions to food, including cutaneous, gastrointestinal or respiratory disorders or systemic anaphylactic reactions (Sampson [1999](#)) are demonstrated by 8% of children and 2% of adults (Helm & Burks [2000](#)). It is likely that both the incidence and prevalence of food allergies are increasing in line with other forms of allergic diseases (Kimber & Dearman [2001](#)). Food allergy can develop as an isolated reaction to food (fruit, fish, milk) or a secondary reaction after sensitization to pollen (oral-allergy syndrome, OAS) or latex (latex-fruit syndrome) when food allergens cross-react with allergens present in pollen or latex. It is generally believed that food allergens are proteins resistant to digestion because only intact proteins (or their larger fragments) are required for processing by antigen-presenting cells located in gut mucosa. Although stability under digestion may not be the defining characteristic of food allergens, it is widely accepted that resistance to



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major allergens of kiwi fruit have been isolated and characterized. Act c 1, a protein of molecular mass about 30 kDa, commonly known as actinidin (Pastorello et al. [1998](#)) is a cysteine protease related to papain (Varughese et al. [1992](#)). Act c 2 is a thaumatin-like protein, the plant defense protein with proven antifungal activity (Gavrovic-Jankulovic et al. [2002b](#), Wang et al. [2002](#)) that, in kiwi extract, exists in two isoforms differing slightly in pl value (9.4 and 9.5) with molecular mass of about 24 kDa (Gavrovic-Jankulovic et al. [2002b](#)).

Allergenicity of fruit during development depends on the expression of IgE-binding proteins. Vieths et al. ([1994](#)) demonstrated that the severity of symptoms in patients allergic to apples was highly correlated with the appearance of 18 kDa apple allergen during storage and suggested that this was caused by ripening. Additionally, Paschke et al. ([2001](#)) have correlated the number and intensity of bands in immunoblots with ripening stages of mango fruit and observed no difference caused by ripening.

Allergenic extracts, made by different manufacturers, for the use in diagnosis and therapy of allergic diseases usually show variations in allergenic potency. Especially allergen extracts from fruits, vegetables and other plant foods often lack sufficient biological activity due to the presence of proteolytic enzymes, carbohydrates, and phenol components (Vieths et al. [2001](#)). That is the consequence of applying different extraction methods and source materials. In the last few years several in vivo (such as

skin prick test) have been performed to evaluate the allergenic content of the extracts. In a study by Yunging et al. (2001) the allergenicity of the extracts proposed for use in diagnosis and therapy of allergic diseases was evaluated.

The purpose of this study was to evaluate the allergenic potency of the extracts proposed for use in diagnosis and therapy of allergic diseases. The allergenicity of the extracts was evaluated in vivo by skin prick test. The results of the study showed that the allergenicity of the extracts was significantly different from the control.

Material

Fruit c



Kiwi fruits (*Actinidia deliciosa*, Monti cultivar) were collected monthly from September to December in the period from 2000 to 2002, from the same tree in Bar, Montenegro. The fruits were stored at -20°C without specific treatment until use. The extracts were made according to previously published protocol (Gavrovic-Jankulovic et al. [2002b](#)). Briefly, the fruits were homogenized 1: 2 (w/v) in 100 mM sodium bicarbonate buffer, pH 9.3 containing 2% polyvinylpoly-pyrrolidone (PVPP) and 0.02% NaN_3 in a blender for 1 min. After extraction and centrifugation, ammonium sulfate was dissolved in the supernatants to achieve 90% saturation. After overnight standing at 4°C the solutions were centrifuged for 20 min at $10000\times g$. The obtained pellets were dissolved in a minimal volume of a starting buffer and dialyzed extensively against the same buffer. Protein concentration in the extract was determined by the Bradford assay (Bradford [1976](#)).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 4% stacking gel and 10% or 12% resolving gel according to the method of Laemmli (Laemmli [1970](#)) under reducing and non-reducing conditions. Twelve micrograms of proteins per lane were applied on the gel for Coomassie Brilliant Blue (CBB) staining or semidry transfer (0.8mAcm^{-2}) to a nitrocellulose membrane (Serva) for further examination. The content of major allergen Act c 1 in kiwi fruit extracts was determined by densitometric analysis after SDS-PAGE under reducing conditions.

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TBS containing 0.05% Tween 20, pH 7.8 for 5 hours. The blots were washed extensively with TBS containing 0.05% Tween 20, pH 7.8 and incubated for 2h with 1: 1000 diluted monoclonal anti-human IgE antibody labeled with alkaline phosphatase (Abcam Ltd., Cambridge Science Park) and, after washing, were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St Louis, MO, USA) as substrates as described (Gavrovic-Jankulovic et al. [2002a](#)). A serum pool of non-allergic persons was used as the negative control.

Digestion with simulated gastric fluid

The digestibility of kiwi proteins in simulated gastric fluid (SGF) was examined according to the method of Yagami et al. ([2000](#)). Briefly, 140 μg of kiwi extract proteins was dissolved in 40 μL of prewarmed SGF (US Pharmacopoeia) containing 0.32% w/v of pepsin A (Sigma Chemical Co). Digestion proceeded at 37°C with continuous shaking and an aliquot (8 μL) of the digest was periodically withdrawn (at 8 and 30 minutes). The digestion was stopped with 2.4 μL 0.2 M Na_2CO_3 , and samples were mixed with a sample buffer for SDS-PAGE analysis.

Determination of proteolytic activity of actinidin

Proteolytic activity of actinidin in different kiwi fruit samples towards 0.5 mM $\text{N}\alpha$ -Benzoyl-DL-Arginine 4-nitroanilide (Sigma) in 0.1 M potassium phosphate buffer, pH

6.5, containing 0.1 M NaCl was evaluated. The activity was calculated using the following formula: Relative proteolytic activity = (absorbance of sample) / (absorbance of standard) \times 100 (set up a standard curve). After preparing the samples, the absorbance was read after 15 minutes of incubation.

A zymogram was prepared. Briefly, 12 μg of kiwi extract was applied to each lane. After electrophoresis, the gel was stained with 10 mM Ca^{2+} , 5 mM Mg^{2+} , 10 mM Zn^{2+} and 10 mM Mn^{2+} for 15 minutes. The gel was stained with CBB.



Antibodies against the December kiwi extract were raised in rabbits according to Harboe and Ingild ([1983](#)). The animals were injected with 0.5 mL of a 1:1 emulsion of ripe kiwi extract (1 mg mL^{-1}) in complete Freund's adjuvant. Bleeding was performed 50 days after the first immunization and every two weeks thereafter. The presence of antibodies to kiwi proteins was detected by immunodiffusion. The serum was partially purified by ammonium sulfate fractionation (50% saturation).

Rocket immunoelectrophoresis (RIE)

The kiwi extract proteins ($5.5 \mu\text{g}$) were applied to 1% agarose gel containing 13% rabbit antibodies on a glass plate ($12 \times 7 \text{ cm}$). Electrophoresis was carried out in a buffer containing 5 mM barbital, 25 mM Na-barbital, pH 8.6, for 12 h at 2 V cm^{-1} . In order to compare the amount of the major allergen Act c 1 in different kiwi fruit samples, we applied $2.75 \mu\text{g}$ Act c 1 isolated according to Carne and Moore ([1978](#)) without derivatization with S-sulphenyl thiosulphate on the same agarose gel.

Patient sera

Five sera from patients allergic to kiwi fruit were used in this study (1, 2, 3, 4 and 5). All the patients showed OAS and one of them had severe contact dermatitis and anaphylaxis when touching the fruit (case No. 4 in [Table I](#)).

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Results

Kiwi fruit extracts

Kiwi fruit extracts (gathered in 2000, 2001 and 2002) were compared using SDS-PAGE, immunoblot and rocket immunoelectrophoresis. The same protein/antigen/allergen patterns were observed in all three years investigated. The concentration of proteins in kiwi fruit extracts increased with fruit development. The results presented here were all obtained using samples from the year 2001.

Protein patterns and actinidin activity in the zymogram

At least 10 protein bands with molecular weights between 67 and 10 kDa were detected by CBB staining in extracts of kiwi fruit from September to December. SDS-PAGE patterns under reducing (see [Figure 1a](#)) and non-reducing ([Figure 1b](#)) conditions were quite different. The most noticeable difference was in the mobility of the 24 and 30 kDa proteins in reducing conditions (most likely a thaumatin like protein and actinidin, respectively) compared to non-reducing conditions, when they exhibited 20 and 27 kDa molecular weights, respectively. The protein of 29 kDa was constitutively expressed. We also noticed three protein bands of 10, 12 and 14 kDa with similar appearance and in similar amounts from September to December. Several more protein bands of about 42, 56 and 62 kDa were also present in the extracts of fruits from

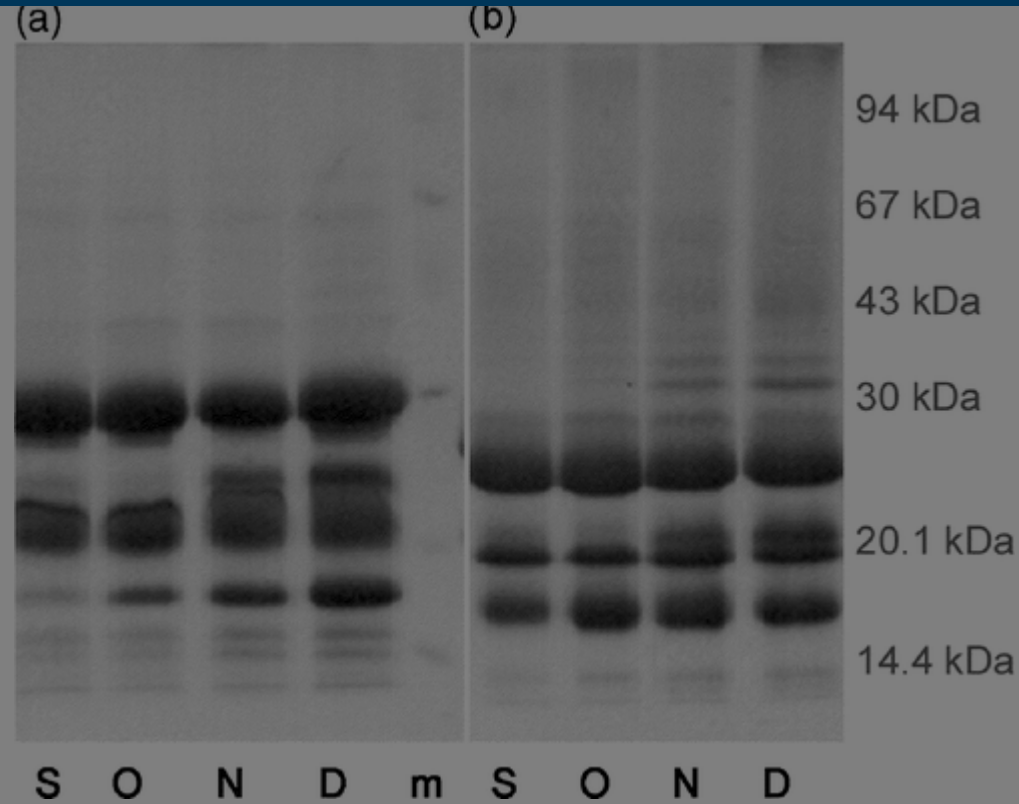
September and reached 67 kDa and 67 kDa appeared

Figure 1.

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Two-dimensional PAGE (Figure 2) showed fragmentation of the 30 kDa protein (actinidin), pI 3.6 into a 26 kDa protein with a pI value of 3.2. Both the protein and protein fragment showed proteolytic activity in zymogram (Figure 3). We observed two more proteins of 39 and 17 kDa with pI values of 5.0 and 4.8, respectively.

Figure 2. Two-dimensional PAGE of kiwi fruit extracts

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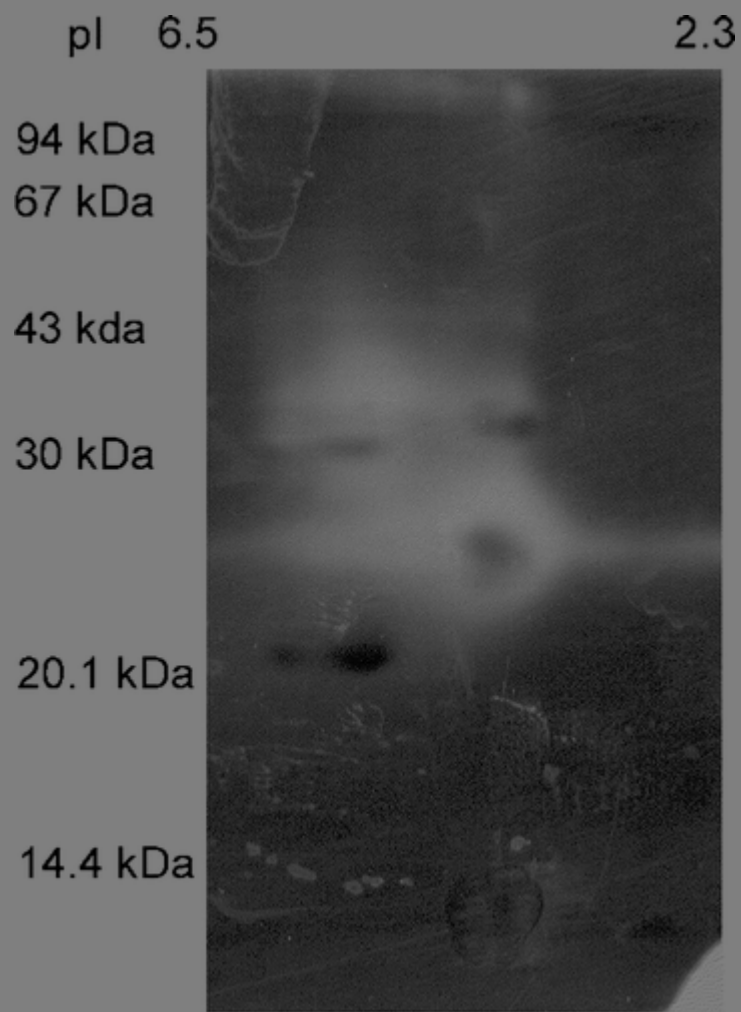
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Figure 3. Proteolytic activity of actinidin fragments from the November kiwi fruit extract after 2D PAGE.



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The major isoforms of actinidin were identified with isoelectric focusing (IEF) and SDS-PAGE. Jankulovic et al. (2008) reported that the 26 kDa and 17 kDa isoforms were the most abundant in the November kiwi fruit extract.

Hur et al. (2008) reported that the 26 kDa isoform was the most abundant in the November kiwi fruit extract.

In immunoblotting analysis, the 26 kDa isoform was detected in the November kiwi fruit extract. The 26 kDa isoform was also detected in the November kiwi fruit extract.

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of isoforms of actinidin were identified with isoelectric focusing (IEF) and SDS-PAGE. The amount of actinidin protein of November kiwi fruit extract was determined by immunoblotting analysis.

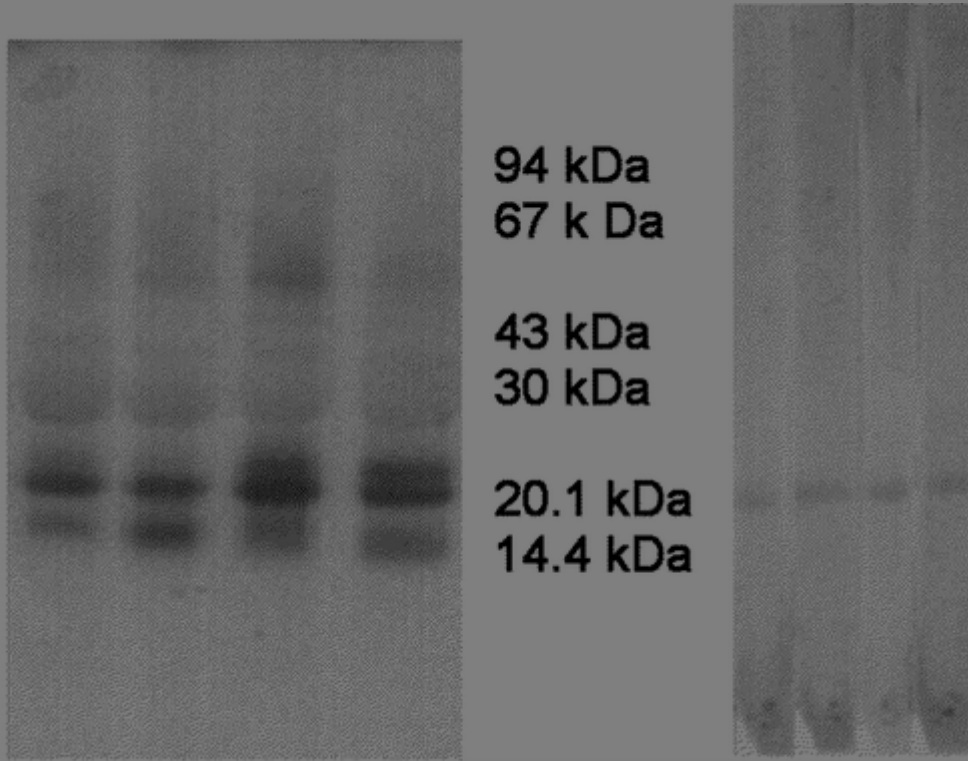
patients sera were analyzed by immunoblotting analysis. The 24 kDa isoform of actinidin was detected in the November kiwi fruit extract. The 29 kDa isoform of actinidin was also detected in the November kiwi fruit extract.



abundant in later months. Allergens of 42 and 68 kDa were present in the October and November samples. The allergen of 22 kDa was most pronounced in the November sample.

Figure 4. Binding patterns of patients' IgE to kiwi fruit extracts after Western blotting. a) immunoblot with serum pool; b) immunoblot with serum from patient 2.

S, September; O, October; N, November and D, December kiwi fruit extract.



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The quantity of actinidin in our samples (see [Table II](#)) was measured using three different methods: rocket immunoelectrophoresis, densitometry and determination of proteolytic activity. Rocket immunoelectrophoresis was run according to Hudson and Hay ([1989](#)). According to all three methods used in this study, the quantity of Act c 1 was the most pronounced in November kiwi fruit extract ([Table II](#)). The most noticeable difference in Act c 1 content between the November kiwi fruit extract and the other three kiwi fruit extract was obtained by determination of proteolytic activity.

Table II. Quantity of major allergen Act c 1 determined by three different methods.

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Digestibility of kiwi proteins

All kiwi proteins in September, October, November and December kiwi fruit extracts were decomposed completely within 30 minutes of exposure to simulated gastric fluid (see [Figure 5](#)), but the 24 kDa protein (presumably a thaumatin-like protein (TLP), Act c 2) appeared to be more resistant to digestion by gut enzymes in unripe fruit extracts.

Figure 5. Digestibility of kiwi fruit extracts in SGF. Lane m, Molecular weight markers; S,

September kiwi fruit extract; O, October kiwi fruit extract; N, November kiwi fruit extract; D, December kiwi fruit extract; C, control; S, September kiwi fruit extract; O, October kiwi fruit extract; N, November kiwi fruit extract; D, December kiwi fruit extract; C, control.

94 kDa

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The results of SPT and patients' clinical characteristics are shown in [Table I](#). All patients exhibited the most pronounced reaction to the November kiwi fruit extract. The intensity of the reaction to November kiwi fruit extract was approximately twice as strong as the response to the other kiwi fruit extracts (September, October and December).

Discussion

The aim of this study was to correlate the allergen profile and content in kiwi fruit during ripening and to answer the question whether the allergenicity and the digestibility of the fruit depend on its developmental stage or not.

We analysed kiwi fruit extracts from three consecutive years (2000, 2001 and 2002) by SDS-PAGE, 2D-PAGE, immunoblot and rocket immunoelectrophoresis. These extracts exhibited the same allergen and protein profile, ruling out the possibility that our results may depend on specific conditions of the year selected.

The allergenic potential in vivo of the different developmental stages of kiwi fruit (September, October, November and December extracts) investigated by skin prick tests was the highest in extract of fruit collected in November. The amount of Act c 1

(actinidin) in kiwi fruit extract was the highest in November. The allergenicity of kiwi fruit extract was determined by the activity of actinidin. The activity of actinidin in kiwi fruit extract was the highest in November.

decrease in actinidin activity was observed in kiwi fruit extract collected in September and October. The decrease in actinidin activity was observed in kiwi fruit extract collected in September and October.

densitometry was used to compare the actinidin activity in kiwi fruit extract collected in September, October, November and December. The densitometry was used to compare the actinidin activity in kiwi fruit extract collected in September, October, November and December.

unripe kiwi fruit. According to our results, the allergenicity of kiwi fruit extract seems to be related to the developmental stage of the fruit. The allergenicity of kiwi fruit extract seems to be related to the developmental stage of the fruit.

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with a presumably higher allergenic potential, appeared to be more prone to digestion in simulated gastric conditions (see [Figure 5](#)). Additionally, we noticed that the expression of some until now uncharacterized allergens, started in September and intensified to reach the highest value in November and December (17, 22, 26, 39 and 67 kDa molecular weights). These allergens may also contribute to higher allergenicity of the November extract. Also, it would be of interest to examine the role that these proteins may play in the process of kiwi fruit development.

A previous study by Paschke et al. ([2001](#)) showed no difference between allergenic potency of mango fruit during ripening but cannot be directly compared with our results due to the different time-scale of food collection (different fruit developmental stage samples). Kiwi fruits used in our study were harvested in September and October as unripe, while November and December kiwi fruits were ripe and edible. The fruit extracts were made immediately after harvesting. Paschke et al. ([2001](#)) performed their experiments only on extracts of consumable and fully developed mango fruit made five to 40 days after harvesting.

According to our results, one of the factors that contribute to the difficulties in proposing well-defined and standardized fruit extracts should also be time (developmental stage) of fruit harvesting. In this particular case, when the kiwi fruit was edible throughout November and December, we clearly showed strong

discrepancies in quantitative terms. It is less clear if the allergenicity of the group of allergens should be different and development should be prepared natural complex

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Acknowledgements

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Additional information

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