

## ORIGINAL PAPER

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## Isolation and characterization of a major allergen in kiwi fruit

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**Abstract** The isolation of an important allergen in kiwi fruit (*Actinidia chinensis*) by ion-exchange chromatography (IEC) and micropreparative SDS-PAGE followed by electroelution is reported. The purity of the allergen was analysed by SDS-PAGE and immunoblotting with sera from patients who have an allergy to kiwi. The allergen was shown to have a molecular weight of 43 kDa by SDS-PAGE/immunoblotting and an isoelectric point of approximately 6.9 as estimated by IEC. In accordance with World Health Organization nomenclature, this allergen is called Act c 2. By immunoblot inhibition it was shown that epitopes from different allergens in kiwi fruit are also located on Act c 2. N-terminal amino acid sequencing of 17 amino acid residues did not reveal homology with the major allergens in birch pollen (Bet v 1), apple (Mal d 1) or with other proteins of allergenic plant foods. In addition, the isoelectric point of a 67-kDa allergen in kiwi fruit was estimated to be 7.4 by IEC, but micropreparative isolation of this allergen failed because of its very low content in the fruit.

**Key words** Food allergens · Immunoblotting · Ion-exchange chromatography · Kiwi fruit · N-terminal microsequencing

### Introduction

Immediate-type allergic reactions to foodstuffs are almost all caused by water-soluble proteins, which are natural components of the food. These reactions, which usually

lead to local symptoms such as oral itching and swelling of the lips or throat, are mediated by human immunoglobulin E (IgE) antibodies specific to these allergens. Combined allergies to different food or respiratory allergens are caused by cross-reactivities of specific IgE. These cross-reactivities are based on common epitopes located on the allergens. Although some allergens in food and pollen have been identified, knowledge about the allergenic proteins and their chemical structure is almost nonexistent, especially in relation to tropical fruits.

Recently, two important allergens of 43 and 67 kDa in kiwi fruit were characterized by SDS-PAGE/immunoblotting using sera of patients allergic to kiwi [1]. These allergens were also responsible for the cross-reactions between kiwi fruit and birch pollen, which have been reported by several groups [1–3]. However, 12 allergens with molecular weights ranging from 12 to 64 kDa and a major allergen of 30 kDa have been characterized [3]. With the exception of the 30-kDa allergen, these IgE-binding components also showed cross-reactivity with allergens from birch pollen.

In addition, different investigations have revealed that there are cross-reactivities between kiwi fruit, latex, avocado and banana [4, 5]: it has been shown that the 43–67 kDa allergens in kiwi fruit also cross-react with allergens from avocado, banana and latex [6]. In the present study we intended to isolate these allergens from kiwi fruit by ion-exchange chromatography (IEC) and micropreparative SDS-PAGE followed by electroelution. Following this, the purified 43-kDa allergen was analysed by immunoblotting and immunoblot inhibition. In addition, the allergen was characterized by its isoelectric point and N-terminal amino acid sequencing.

### Materials and methods

#### Reagents

Ovalbumin, ovotransferrin and ovomucoid were purchased from Fluka, Buchs, Switzerland. Myoglobin from horse hearts and  $\alpha$ -lactalbumin

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were obtained from Sigma, Deisenhofen, Germany; ribonuclease was purchased from Serva, Heidelberg, Germany. If not otherwise mentioned, all chemicals were of analytical grade.

#### *Patients' sera*

Sera Bo 506 K and B 508 K from patients with an allergy to kiwi fruit were obtained from Hospital Borkum Riff, Borkum, Germany. Sera of kiwi-allergic patients J. A., K. B. and K. N. were collected at the University Hospital Eppendorf, Departments of Dermatology and Allergy and Experimental Dermatology and Allergology, Hamburg, Germany, and Serum Mast 103 was supplied by Mast Diagnostica, Reinhold, Germany. The diagnosis was based on EAST (enzyme allergo sorbent test) and anamnesis. The serum of a nonallergic person served as a negative control.

#### *Purification of the allergens from kiwi fruit*

**Protein extraction.** Allergenic extracts from kiwi fruit were prepared by a previously described low-temperature method with some minor modifications [1, 7].

Briefly, pieces of fresh kiwi fruit were homogenized in acetone at  $-40^{\circ}\text{C}$  (cooling by dry ice) and the mixture was stored overnight at  $-20^{\circ}\text{C}$ . The precipitates were washed twice with acetone ( $-20^{\circ}\text{C}$ ) and once with acetone/diethylether (1:1, v:v,  $-20^{\circ}\text{C}$ ), filtered and lyophilized.

Protein extracts were obtained by extracting 6 g of this acetone powder with 120 ml double-distilled water for 60 min by ice-cooling. The suspension was centrifuged ( $4^{\circ}\text{C}$ , 10,500 g, 60 min) and the supernatant filtered. The solution was divided into aliquots of 5 ml, lyophilized and stored at  $-20^{\circ}\text{C}$  until use.

As determined by the Lowry method, the protein content of this extract was approximately 1050  $\mu\text{g/ml}$  [8, 9].

**Ion-exchange chromatography (IEC).** Lyophilized protein extracts were dissolved in 5 ml buffer A (20 mM Tris, pH 8.0), filtered (Spartan 30/B, 0.45  $\mu\text{m}$ , Schleicher and Schüll, Dassel, Germany) and placed on to a Resource Q-column, 6 ml (Pharmacia, Uppsala, Sweden) using a 5-ml-sample loop. For elution of proteins a gradient program was used: 0–7 min buffer A, 7–60 min linear to 50% buffer B (= buffer A with additional 0.5 M sodium chloride), 60–70 min 100% buffer B, afterwards re-equilibration with buffer A for 10 min. This gradient was achieved using a 655A-12 liquid chromatography pump and an L-5000 LC controller (Merck, Darmstadt, Germany) at a flow rate of 2 ml/min. Detection was carried out at 280 nm using an L-4000 UV-detector (Merck). Individual fractions were collected using a Serva Linear II fraction collector (Serva).

To remove salt, individual fractions were passed through Centriplus microconcentrators (Amicon, Witten, Germany, cut off = 10 kDa) according to the manufacturer's instructions. The retentate was lyophilized and dissolved in sample buffer for SDS-PAGE [10]. The proteins were reduced for 3 min at  $90^{\circ}\text{C}$ .

**SDS-PAGE.** Discontinuous SDS-PAGE was performed according to Laemmli [11]. As previously described, 5% (w/v) acrylamide stacking gels and 13% (w/v) resolving gels (250 mm $\times$ 125 mm $\times$ 0.5 mm) were used [1]. Each 20  $\mu\text{l}$  (= 10  $\mu\text{g}$  protein) of individual fractions obtained by IEC was loaded onto the gels. After electrophoresis, proteins were visualized by silver staining [12]. Afterwards, those fractions which contained proteins with molecular weights ranging from 40 to 70 kDa were analysed by immunoblotting as described below.

For electroelution, approximately 40  $\mu\text{g}$  of total protein of the fraction containing an IgE-binding protein of 43 kDa (fraction 4) and 50  $\mu\text{l}$  of the fraction containing the 67-kDa allergen (fraction 3) were loaded onto the gel (250 mm $\times$ 125 mm $\times$ 1.0 mm). After separation, the gel was stained with Coomassie Blue R-250 [13].

**Electroelution.** A protein band at approximately 43 kDa, which was visualized in fraction 4 by Coomassie Blue staining, was cut out to form gel slices equal in size (2 mm $\times$ 2 mm). Gel slices were

equilibrated in 0.5% (w/v) SDS. Afterwards, electroelution of the protein was performed using Biotrap apparatus (Schleicher and Schüll). An elution buffer of 100 mM ammonium bicarbonate and 0.1% (w/v) SDS was used. The protein was electroeluted at 50 V and room temperature overnight into an elution chamber [14].

To remove Coomassie Blue dye, the solution (400  $\mu\text{l}$ ) and an additional 1 ml of methanol/20% acetic acid (50/50, v/v) were passed through a Centriplus microconcentrator (cut off = 10 kDa, Amicon, 15 min, 3000 g). The retentate was washed three times with 500  $\mu\text{l}$  of methanol/20% acetic acid (50/50, v/v) and twice with double-distilled water to remove acetic acid. Afterwards, the concentrate was transferred into the retentate vial at 2000 g for 3 min. The membrane was rinsed twice with 150  $\mu\text{l}$  double-distilled water and centrifuged again. The retentate was divided into aliquots and lyophilized.

#### *Immunoblotting*

For immunoblotting approximately 10  $\mu\text{g/cm}$  of protein from IEC fractions containing proteins with molecular weights ranging from 40 to 70 kDa or 0.8  $\mu\text{g/cm}$  of the electroeluted protein was loaded onto the gels. After separation, protein transfer was carried out as described previously [1, 6]. The dried nitrocellulose membranes were blocked twice (15 min) with blocking buffer consisting of 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween 20 in phosphate-buffered saline [PBS (0.01 M potassium phosphate buffer, pH 7.4, 0.13 M sodium chloride)]. Nitrocellulose strips were incubated overnight with 100  $\mu\text{l}$  of patients' serum in 1400  $\mu\text{l}$  incubation buffer [0.3% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Tween 20 in PBS]. Incubation steps were carried out as described previously [1, 10].

For total protein staining one strip of the transferred extract and one strip of low-molecular-weight markers (Pharmacia) were stained with Indian Ink [15].

#### *Immunoblot inhibition*

Approximately 10  $\mu\text{g/cm}$  total protein from kiwi fruit extract was separated by SDS-PAGE, transferred to nitrocellulose membranes and blocked as described above. Then, 100  $\mu\text{l}$  of pooled serum from patients Bo 506 K, Bo 508 K, J. A., K. B. and K. N. was preincubated with 100  $\mu\text{g}$  kiwi fruit protein and 2  $\mu\text{g}$  electroeluted protein in a total volume of 1.5 ml incubation buffer. Afterwards, immunostaining was performed as described above. Ovalbumin was used to check non-specific inhibition and serum from nonallergic individual was used to check nonspecific binding.

#### *Amino acid sequencing*

Approximately 40  $\mu\text{g}$  protein of fraction 4 was separated by SDS-PAGE using a gel (250 mm $\times$ 125 mm $\times$ 1.0 mm) as described above. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an electrotransfer buffer [10 mM (3-cyclohexylamino)-1-propane sulphonic acid (CAPS), pH 11.0, 10% (v/v) methanol] for 75 min at 0.8 mA/cm<sup>2</sup>. Prior to use, membranes were rinsed in methanol and water (5 min each) and electrotransfer buffer (15 min). Proteins were visualized by staining with 0.1% (w/v) Coomassie Blue R-250 in methanol/20% acetic acid (50/50, v/v) for 20 min and destained with methanol/20% acetic acid (50/50, v/v). After staining, PVDF membranes were washed with double-distilled water (5 min) and air-dried [13].

The protein of approximately 43 kDa was cut out of the membrane. Microsequencing of the N-terminal amino acids was performed by Edman degradation with a 473A pulsed liquid protein sequencer with an on-line phenylthiohydantoin amino acid analyser (Applied Biosystems, Weiterstadt, Germany).

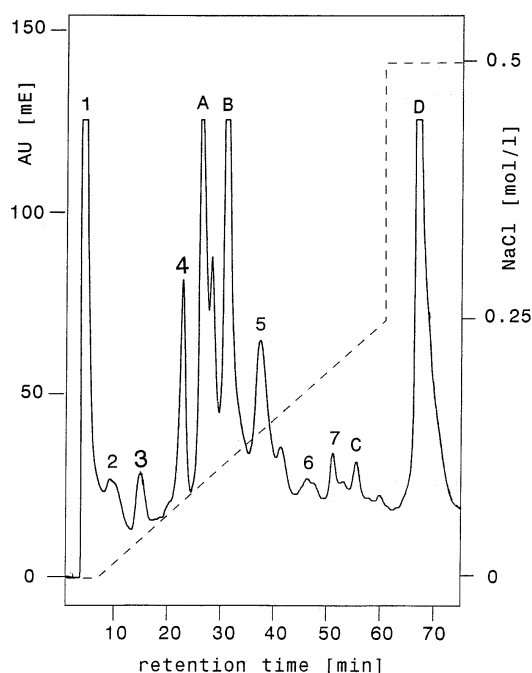


**Fig. 1** Separation of kiwi fruit extract by ion-exchange chromatography. The absorbance at 280 nm is reported as a function of retention time

## Results

### Fractionation by IEC

Fractionation of kiwi fruit proteins by IEC revealed a good separation, as shown in Fig. 1. The components causing these peaks were collected and analysed by SDS-PAGE followed by silver staining.

As shown in Fig. 2, fractions 1–7 contained proteins with molecular weights ranging from 40 to 70 kDa, whereas fractions A–D only contained proteins with molecular

weights below 30 kDa, which were seen to be predominant proteins, but not relevant allergens [1]. Fractions 1–7 were analysed by immunoblotting using patients' sera J. A. and Mast 103. Fractions 5 and 6 were combined, as were fractions 1 and 2, because of their similar spectrum of protein bands visualized by silver staining. The 43-kDa allergen was detected in fraction 4, whereas fraction 3 contained the allergen of approximately 67 kDa. In addition, small amounts of the 67-kDa allergen were also detected in fractions 1 + 2 as well as in fraction 4 (Fig. 3). Fractions 5 + 6 and 7 did not contain IgE-binding components.

### Isolation of the allergens

For micropreparative isolation of the allergens 40 ml kiwi fruit extract (= 42 mg kiwi fruit protein) was separated by IEC and fractions 3 and 4 (Fig. 1) were separately collected. The lyophilized fractions were desalted using microconcentrators and the lyophilized concentrate dissolved in 100 µl sample buffer for SDS-PAGE. After reduction of proteins, each 50 µl (= 40 µg protein of fraction 4) of these solutions was loaded onto the gel (1.0 mm in diameter). After electrophoresis the gel was stained with Coomassie Blue.

In fraction 4 protein bands at 43, 47 and 70 kDa were detected. The protein band at 43 kDa was excised and the protein electroeluted. Coomassie Blue was removed from the solution using microconcentrators. Finally the solution was divided into aliquots and lyophilized. A determination of the total protein content using one of these aliquots revealed that approximately 38 µg of the 43-kDa allergen was extracted from 42 mg crude kiwi fruit protein [8].

SDS-PAGE of fraction 3 did not reveal protein bands in Coomassie Blue staining of the gel, indicating that the amount of the 67-kDa allergen was below the detection limit.

**Fig. 2** SDS-PAGE followed by silver staining of the fractions obtained by ion-exchange chromatography. The number of the fractions corresponds to numbers of peaks in Fig. 1

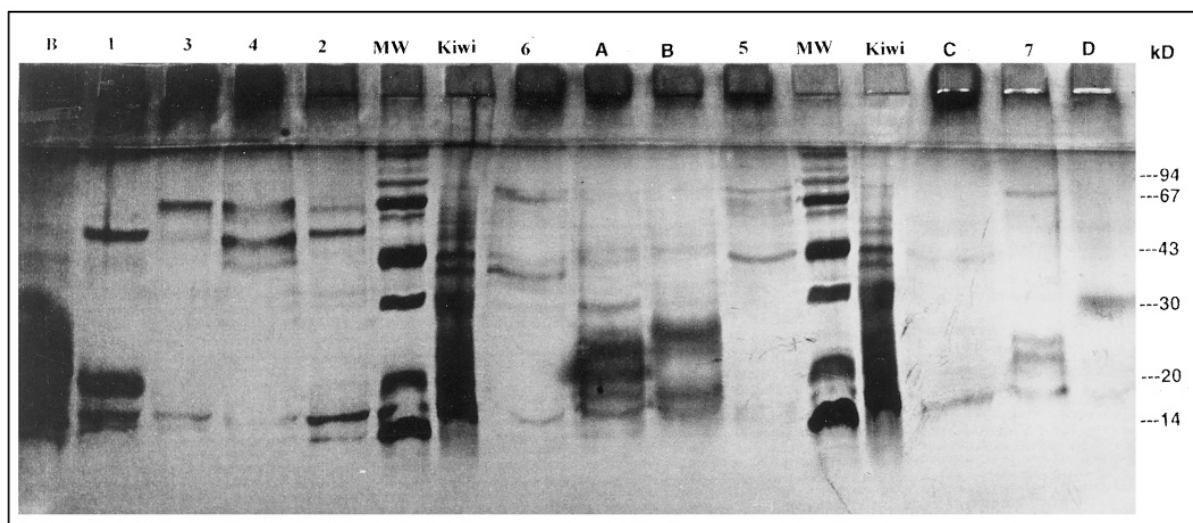


**Fig. 3** Immunoblotting of fractions 1–7 obtained by ion-exchange chromatography. Fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes. Patients' sera: 1, 4, 7, 10, 13: J. A.; 2, 5, 8, 11, 14: Mast 103; 3, 6, 9, 12, 15: control serum of a nonallergic individual

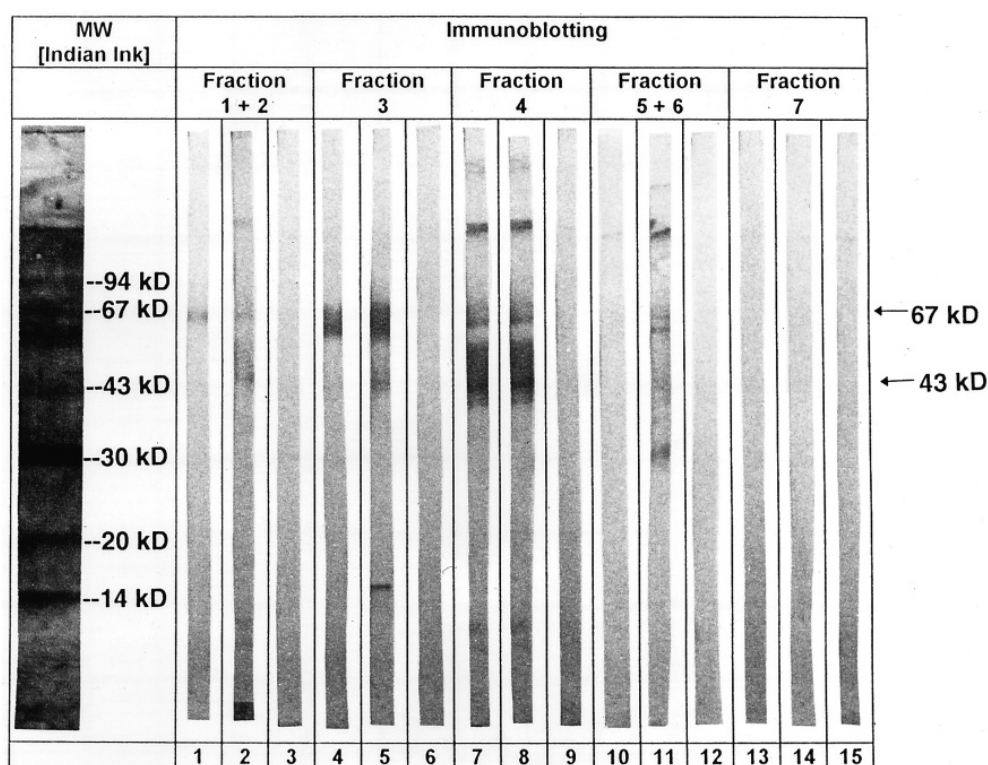


**Table 1** Calibration of ion-exchange chromatography (IEC) column using proteins of known isoelectric points (1 mg/ml of individual standards, conditions as given in Materials and methods)

| Protein               | Isoelectric point | Retention time (min) |
|-----------------------|-------------------|----------------------|
| Ribonuclease          | 8.8               | 4.4                  |
| Myoglobin             | 7.4               | 16.3                 |
| Ovotransferrin        | 6.6–6.1           | 26.4–33.8            |
| $\alpha$ -Lactalbumin | 4.8               | 36.1                 |
| Ovalbumin             | 4.6               | 39.6                 |
| Ovomucoid             | 4.1               | 44.8                 |

#### Characterization of the isolated allergen

##### Immunoblotting

An aliquot of the isolated protein was dissolved in sample buffer, reduced and analysed by SDS-PAGE/immunoblotting for a determination of the IgE-binding properties.

Staining of the transferred protein with Indian Ink showed a single band at 43 kDa. Because of the very low intensity of staining, the purity of the isolated protein could not be precisely determined using this method.

It was previously shown that many proteins in kiwi fruit extracts could be detected by Mast 103 in immunoblotting [1]; however, lane 1 in Fig. 4 shows that only one single band in the solution of the isolated protein was detected by serum Mast 103, thus, it could be concluded that the isolated protein was pure (Fig. 4). In addition, the protein revealed strong IgE binding using five of the six sera tested here, whereas nonspecific staining with the control serum of a nonallergic individual was not observed. So, it was concluded that the isolated protein was an allergen.

##### Immunoblot inhibition

Indian Ink staining of kiwi fruit extract visualized proteins ranging from 20 to 30 kDa, but not one of 43 kDa.

In immunoblotting the dominant allergens of 67 and 43 kDa were strongly detected by the pooled serum (Bo 506 K, Bo 508 K, J. A., K. B. and K. N.). In addition, slight antibody binding to a protein of approximately 22 kDa was observed. IgE binding was totally inhibited by preincubation with kiwi fruit extract and the isolated 43-kDa allergen (Fig. 5). Neither nonspecific inhibition by ovalbumin nor nonspecific binding by the control serum of a nonallergic individual could be observed.

##### Determination of the isoelectric point of the isolated allergen

For a determination of the isoelectric point, the IEC column was calibrated with proteins of known isoelectric points (Table 1). Using this calibration the isoelectric point of the 43-kDa allergen (retention time 21.5 min) was estimated to be 6.9, whereas that of the 67-kDa allergen (retention time 16.0 min) was estimated to be 7.4.

##### Microsequencing analysis

In total, 17 N-terminal amino acid residues were analysed by microsequencing of the Coomassie-Blue-stained protein spot of the 43-kDa allergen. At position 6 proline and glycine were simultaneously detected, whereas at position 7 no amino acid residue was detectable (Table 2).

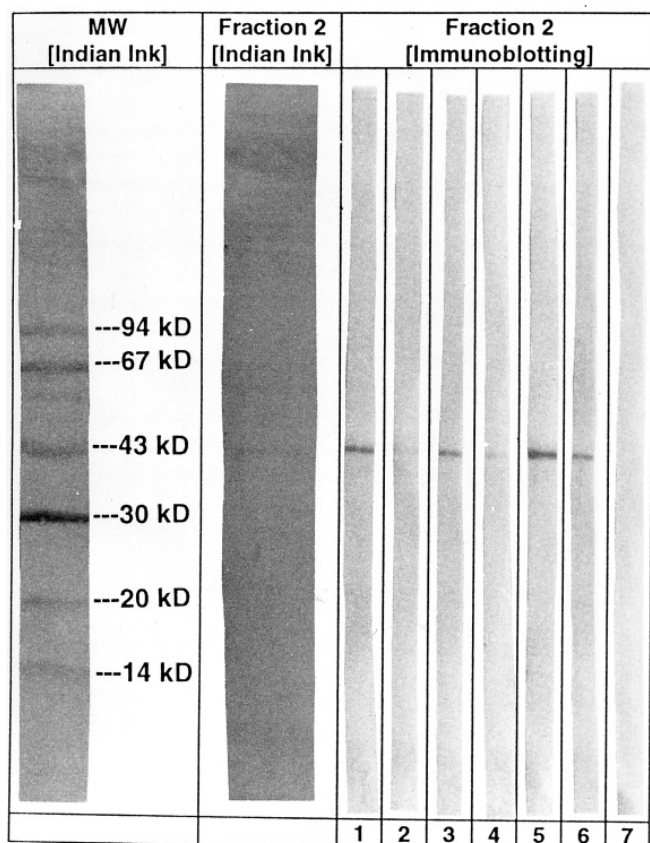


**Fig. 4** Immunoblotting of the isolated protein separated by SDS-PAGE and transferred to nitrocellulose membranes. Patients' sera: 1: Mast 103, 2: J. A., 3: K. B., 4: K. N., 5: Bo 506 K, 6: Bo 508 K, 7: control serum of a nonallergic individual

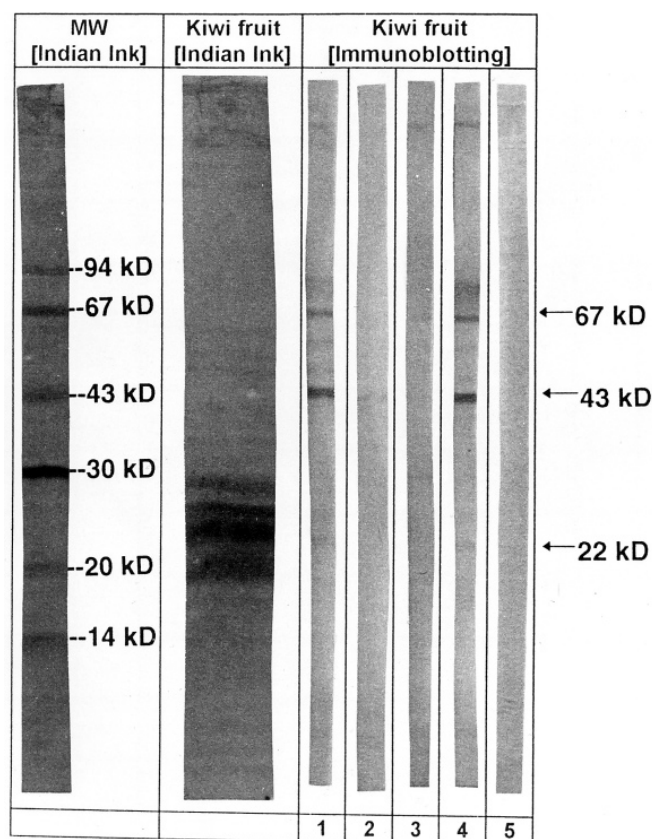


**Fig. 5** Inhibition of immunoglobulin E binding of pooled serum to kiwi fruit extract separated by SDS-PAGE and transferred to a nitrocellulose membrane. [1 Noninhibited, 2 inhibited with kiwi fruit extract (100 µg/inhibition), 3 inhibited with the isolated allergen (5 µg/inhibition), 4 inhibited with ovalbumin (100 µg/inhibition) to check for nonspecific inhibition, 5 control serum to check nonspecific binding]

## Discussion

In the present investigation an important allergen from kiwi fruit was isolated from an aqueous extract by IEC and micropreparative SDS-PAGE followed by electroelution. To our knowledge there is no information about the purification of allergens in kiwi fruit, but IEC has already been used for purification of a pectin-methylesterase inhibitor from a kiwi fruit extract [16]. The application of electroelution to isolate allergenic proteins has been described by different groups [17, 18].

In this investigation proteins were extracted from acetone powder by double-distilled water instead of PBS, which is commonly used for extraction of proteins from crude fruit material. The protein contents in aqueous (approximately 1050 µg/ml) were below those of PBS extracts (approximately 1600 µg/ml) but, because of the high salt content of PBS extracts (0.14 M sodium chloride, 0.03 M

potassium chloride), separation by IEC was impossible. In addition, the difference between protein contents in PBS extracts and aqueous extracts may be explained by an increasing extraction of proteins with a very low isoelectric point by PBS, as shown by an increasing peak eluting at a very low isoelectric point. Separation of kiwi fruit extract by size-exclusion chromatography (SEC) in combination with IEC revealed that these proteins have molecular weights <30 kDa (unpublished results). Recently, it has been shown that these proteins are not potent allergens [1]. SEC was performed with a Sephacryl S 100 HR-column (850×26 mm, Pharmacia) and an elution buffer of 0.01 M potassium phosphate buffer (pH 7.0) containing 0.03 M sodium chloride. Using these conditions there was insufficient resolution of proteins with molecular weights ranging from 40 to 70 kDa. Initial experiments for further purification by SEC failed, but subsequent electroelution resulted in

**Table 2** N-terminal amino acid sequence of the 43-kDa allergen of kiwi fruit

| Position   | 1   | 2   | 3   | 4   | 5   | 6        | 7 | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | – |
|------------|-----|-----|-----|-----|-----|----------|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
| Amino acid | Ala | Lys | Glu | Asp | Pro | Gly, Pro | ? | Asn | Lys | Phe | Ala | Arg | Ile | Gly | Ala | Leu | Val | – |

a highly pure fraction which was virtually free of allergenic and nonallergenic protein contamination (Fig. 4).

The isolated 43-kDa allergen was characterized by purity, molecular weight, isoelectric point and by N-terminal microsequencing. According to World Health Organization nomenclature, this allergen is called Act c 2 [19]. The name Act c 1 had already been postulated for a 30-kDa allergen in kiwi fruit by another group, although this allergen was only characterized by its molecular weight [3]. The quantity of Act c 2 as a percentage of the total protein in kiwi fruit was estimated to be approximately 0.1%. The isolation of the 67-kDa allergen failed, because no protein bands were visualized in SDS-PAGE/Coomassie Blue staining after separation of the IEC fraction containing this allergen, so electroelution could not be performed. These facts indicate that the amount of this allergen is less than that of Act c 2. The very low content of these allergens in relation to the total protein content in kiwi fruit had already been indicated by Indian Ink staining of the transferred extract and SDS-PAGE followed by silver staining. In these cases proteins with molecular weights ranging from 20 to 30 kDa were strongly detected whereas proteins with molecular weights of 43 and 67 kDa were not observed.

The isoelectric points of the allergens were estimated to be 6.9 for Act c 2 and 7.4 for the 67-kDa allergen by IEC. An attempt to determine the isoelectric point by isoelectric focusing failed because of a precipitation of Act c 2. The relationship between these allergens, which is indicated by their similar isoelectric points, is confirmed in immunoblot inhibition. Preincubation of the pooled serum with Act c 2 led to an inhibition of IgE binding to Act c 2, the 67-kDa allergen and the slightly detected allergen of approximately 22 kDa; thus, all epitopes of the 67- and the 22-kDa allergens are also located on Act c 2. In the purification procedure Act c 2 was stained with Coomassie Blue and destained with methanol/20% acetic acid (50/50, v/v). These treatments will influence the conformation of the proteins, but, because of the strong IgE-binding potency of the isolated allergen, sequence epitopes seem to be located on Act c 2.

N-terminal microsequencing revealed a sequence of 15 amino acid residues. The residue in position 7 was not identified and it was not possible to distinguish whether glycine or proline was at position 6, because both amino acids were detected simultaneously. The N-terminal amino acid sequence did not show similarities to major allergens from birch pollen (Bet v 1) and apple (Mal d 1) [20, 21], which corresponds with the lack of cross-reactivity to Bet v 1 observed in a previous study [1]. Using protein data banks no significant homologies between the N-terminal amino acid sequences of Act c 2 and other proteins were found.

In further investigations we intend to characterize the epitopes of Act c 2 by specific cleavage of the allergen. In addition, we intend to determine the amino acid sequences of these fragments in order to obtain information about the biological function of this protein. These investigations require higher amounts of Act c 2 which may be obtained by polymerase chain reaction (PCR).

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