

Characterization of Allergens in Mango Fruit and Ripening Dependence of the Allergenic Potency

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The topic of the present paper is the detection of IgE-related allergens in mango fruit. Furthermore the occurrence of allergens during ripening of mango fruit is investigated. In order to identify the molecular weights of IgE-binding allergens SDS-PAGE/immunoblotting was performed. Fifty-two sera from patients with IgE-mediated sensitization to mango fruit were used. Forty-six of the 52 patient sera detected two allergens with molecular weights of approximately 40 and 30 kDa. These can be described as the so-called major allergens. Other IgE-binding patterns were detected for fewer than 50% of the assigned sera. By 2D-electrophoresis it could be proved that the major allergen with a molecular weight of approximately 40 kDa exists as an isoallergen with isoelectric points of 4.6 and 4.8. The allergen with a molecular weight of approximately 30 kDa had an isoelectric point of 4.9. According to international rules, the 40 kDa allergen was named Mangifera indica 1 (Man i 1) and the 30 kDa allergen, Mangifera indica 2 (Man i 2). By means of SDS-PAGE/immunoblotting, immunoblot inhibition and EAST-inhibition it could be shown that there is no significant difference in the allergenic potency during fruit ripening.

Keywords: Mango, allergy, ripening, characterization, SDS-PAGE, IEF, immunoblot, 2D-electrophoresis, EAST-inhibition

INTRODUCTION

The globalization of the world market leads to an increased supply of food. Thereby the consumption of tropical fruits has risen substantially during the last few years, and allergies to tropical fruits have also increased. After banana, the mango (*Mangifera indica*), with a world annual production of 14.7 million tons, is the most frequently cultivated tropical fruit. It is cultivated worldwide in more than 1000 varieties, but in Europe many strains are not accepted by the consumer because of their taste.

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Tommy Atkins, which is cultivated particularly in South America, is the variety most frequently sold. Further well-known strains are Osteen (Spain), Eden (Israel) and Ngowe (Kenya) (Franke, 1997).

Allergic reactions to mango have become increasingly important. Rubin *et al.* (1965) reported for the first time a type I reaction against mango fruits. Since then this reaction has been mentioned several times (Dang & Bell, 1967; Wüthrich & Hofer, 1984; Miell *et al.*, 1988; Jansen *et al.*, 1992). Sensitizations to mango pollen (Vargas Correa *et al.*, 1991), seed (Fernandez *et al.*, 1995) and fruit have been described.

In a French study, Andre *et al.* (1994) were able to show that in a collective of 580 patients with proven food allergy 6% were sensitive against mango fruit by enzyme allergosorbent test (EAST). As a reason for this frequent sensitization, cross-reaction between mango fruit and latex (Brehler *et al.*, 1997), mugwort pollen (Wüthrich & Hofer, 1984), peanut (Fernandez *et al.*, 1995), and birch pollen (Wellhausen *et al.*, 1996) was assumed.

In some fruits a dependence on variety of the allergenic potency is described. For example, apple-allergic patients frequently report that some apple strains are usually highly allergenic (i.e. Granny Smith, Golden Delicious) whereas others (i.e. Jamba, Gloster, Boskop) are tolerated without or with moderate symptoms (Vieths *et al.*, 1993, 1994; Li-Shan *et al.*, 1995). Additionally these authors were able to show that the occurrence of the major allergen Mal d 1 increased during ripening. As already described, there is no difference in the allergenic potency between different mango strains (Kinder *et al.*, 1999).

The aim of the present investigation was to characterize the allergens of mango fruit by their molecular weights and their isoelectric points using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting, isoelectric focussing (IEF)/immunoblotting and two dimensional electrophoresis (2D)/immunoblotting. In addition, the inhibition of an EAST was used to establish whether there are difference in the IgE-binding patterns during maturation of the mango fruit.

PATIENTS, MATERIALS AND METHODS

Patient Sera

Sera (52) were obtained from patients with RAST classes ≥ 2 for specific IgE to mango fruit by CAP-RAST (Pharmacia, Uppsala, Sweden). All patients were adults (25 female, 27 male), from 18 to 63 years of age. The patient selection was based solely on enzyme allergosorbent test and medical history. Skin prick test or challenge test results were not available. The clinical symptoms and the IgE contents of 16 sera are listed as examples in Table 1. The sera were collected at the University Hospital Eppendorf, Department of Dermatology and Allergy, Hamburg, Germany. For the examination by IEF/immunoblotting, 2D-electrophoresis/immunoblotting, EAST-inhibition, and SDS-PAGE/immuno-blotting pooled patient sera were used. They were obtained from patients with RAST classes ≥ 3 . The pooled serum contained equal aliquots of the sera 1, 4, 11, 12, 14, 15 and 16 (see Table 1).

Mango Extracts

The mango variety Tommy Atkins was purchased from Inter company, Großmarkt Hamburg, Germany. All fruits were in a consumable condition and harvested five days before preparing the allergen extracts. A possible change in the occurrence of IgE-binding patterns during ripening was examined using the mango fruit variety Tommy Atkins. The protein extraction was performed 5, 12, 21, 35 and 40 days after harvesting the mango fruits. The extracts were prepared by a low-temperature extraction method, as described previously (Möller *et al.*, 1997a, b).

TABLE 1. Characterization of patients' sera

Patient no. sex, age	Allergen	Clinical symptoms	Specific IgE (Uml—1)
1 f, 35	mugwort	R	4.8
	birch	R	> 17.5
	celery	IM, IT	<0.35
	carrot	SL, IM	8.2
	mango	SL, IT	1.6
2 f, 33	mugwort	R	14.2
	birch	R	> 17.5
	celery	SL	2.8
	carrot	IM	2.1
	mango	IM, IT	2.4
3 m, 50	mugwort	R	0.5
	birch	R	1.8
	celery	IM, IT	0.5
	carrot	IM, IT	<0.35
	mango	IM; IT	0.7
4 f, 61	mugwort	R	> 17.5
	birch	R	> 17.5
	celery	SL, DIA	11.7
	carrot	SL	0.7
	mango	IM, SL	3.9
5 f, 33	mugwort	R	8.3
	birch	R	2.1
	celery	IM	6.4
	carrot	SL	0.5
	mango	NE	0.7
6 f, 32	mugwort	R, U, D	3.4
	birch		<0.35
	celery		<0.35
	carrot		<0.35
	mango	SL, IM	0.9
7 m, 19	mugwort	R	3.4
	birch	R, U	1.6
	celery	IM, IT	3.4
	carrot		<0.35
	mango	IT, SL	2.0
8 f, 53	mugwort	R, SE, A	> 17.5
	birch	R, SE, A	> 17.5
	celery	DIA	1.4
	carrot	—	<0.35
	mango	DIA	4.9
9 f, 42	mugwort	R	> 17.5
	birch	R, U	> 17.5
	celery	IT	> 17.5
	carrot		0.7
	mango	IM, SL	12.1
10 m, 66	mugwort	R	> 17.5
	birch	R	2.9
	celery	IT,D	7.3
	carrot	IT	0.9
	mango	IT	1.2
11 m, 55	mugwort	R, SE, A	> 17.5
	birch	R, SE, A	> 17.5
	celery	IT, IM,	> 17.5
	carrot	DIA	11.5
	mango	IT, IM, DIA	> 17.5

TABLE 1. (Continued)

Patient no. sex, age	Allergen	Clinical symptoms	Specific IgE (Uml—1)
12 f, 43	mugwort	R, SE	3.3
	birch	R, SE	> 17.5
	celery	IT, IM	0.55
	carrot	IT, IM	1.8
	mango	IT, IM	4.8
13 f, 23	mugwort	R	> 17.5
	birch	R, A	> 17.5
	celery	IT, IM	> 17.5
	carrot	IT, IM	0.9
	mango	IT, IM	3.6
14 m, 64	mugwort	R	14.2
	birch	R	5.3
	celery	IT	7.4
	carrot	IT	1.1
	mango	SL	3.1
15 m, 18	mugwort	R, A	5.9
	birch	R, A	7.3
	celery	—	1.8
	carrot	—	0.7
	mango	NE	1.1
16 m, 59	mugwort	R, A	> 17.5
	birch	R	< 0.35
	celery	DIA	3.5
	carrot	—	1.2
	mango	NE	5.9

A: allergic asthma, DIA: diarrhea, D: dermatitis, IM: itching in mouth, IT: itching in throat, NE: never eaten before, R: rhinitis, SL: swelling of lips, SE: swelling of eyes, U: urticaria.

Chemicals

If not otherwise stated, all chemicals were of analytical grade.

SDS-Page

Discontinuous SDS-PAGE was performed according to the method of Lämmli (1970) with a 5% (w/v) acrylamide stacking gel and a 13% (w/v) acrylamide resolving gel. Protein samples were reduced with beta-mercaptoethanol (sample buffer according to Vieths *et al.*, 1992) and loaded onto the gel at a concentration of approximately 10 µgcm⁻¹. The gels (250 × 120 × 0.5 mm) were developed in a horizontal slab gel apparatus (Desaphor HF, Desaga, Heidelberg, Germany).

IEF

IEF was performed with a 5% (w/v) acrylamide gel, containing 1.7% (v/v) Servalyte™ pH 2–4 and 3.9% (v/v) Servalyte™ pH 2–11 (Serva, Heidelberg, Germany) according to the method of Westermeier (1990) under native conditions. Protein samples were desalted by membrane filtration (1 h, 2500 g, 6°C, Ultrafree™, exclusion size 5 kDa, Millipore, Bedford, USA) and loaded onto the gel in a concentration of approximately 5 µg. For the increase of the electrophoretic mobility after the IEF the gels were loaded with SDS, by laying them for 20 min in a solution containing 0.1% SDS (w/v).

2D-Electrophoresis

2D-Electrophoresis was examined by combination of IEF and SDS-PAGE as described above. After execution of the IEF, the protein samples were denatured by treating with an equilibration buffer (2.0 g SDS, 36 g urea, 3 mg ethylenedinitrilo tetraacetic acid (EDTA), 10 mg bromophenol blue, 10 mg pyronin-y, 35 ml glycerine, made up with 100 ml double-distilled water) for 3 min at 90°C.

Immunoblotting

Immunoblotting took place following the electrophoretic methods described above. Protein transfer was carried out as described previously (Möller *et al.*, 1997a, b). Immunostaining was performed according to Vieths *et al.* (1992) with minor modifications. The dried nitrocellulose membranes were blocked twice (15 min) with blocking buffer consisting of 5% skimmed milk powder, and 0.1% 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 µl of patient sera in 1400 µl incubation buffer [0.3% bovine serum albumin (BSA), 0.1% Tween 20 in PBS]. After incubation with rabbit anti-human IgE (1:4000, 60 min), biotinylated goat anti-rabbit IgG (1:6000, 60 min; both reagents from Dako, Copenhagen, Denmark) and streptavidin-horseradish peroxidase conjugate (1:20 000, 20 min, Medac, Hamburg, Germany), the blots were stained with 3, 3',5,5'-tetramethylbenzidine(TMB)-dioctylsodiumsulfosuccinate.

One strip was incubated with serum from a non-allergic patient to check non-specific binding. To check the efficiency of protein transfer, one strip with separated extract and one strip with low molecular weight marker proteins (Pharmacia, Uppsala, Sweden) were stained with colloidal gold (Bio Rad, Munich, Germany).

Immunoblot Inhibition

A total of 100 µl of the pooled serum was incubated with 100 µg of protein of the protein extracts in a total volume of 1.5 ml of incubation buffer for 60 min at room temperature. Afterwards, the pre-incubated solution was immunostained as previously described (Möller *et al.* 1997a, b).

Enzyme Allergosorbent Test (EAST) Inhibition

Protein extracts were diluted to total protein concentration of 10 mg l⁻¹ using a sodium carbonate-buffer (50 mM-NaCO₃, pH 9.6). 300 µl were added into each well of a Maxisorb-Microtiter plate (Nunc, Roskilde, Denmark) and coupled overnight at room temperature. Free binding sites were blocked with 5% skimmed milk powder and 0.1% Tween 20 in PBS for 1 h. Pooled serum was diluted 1:2 in incubation buffer (0.3% BSA, 0.1% Tween 20 in PBS). A dilution series of the inhibitor-extracts was prepared in six steps using the same incubation buffer (undiluted, 1:10, 1:100, 1:500, 1:1000, 1:10000). Ovalbumin was used to check non-specific inhibition. A total of 120 µl of diluted pooled serum was pre-incubated with 120 µl of the inhibitor solution for 1 h. 50 µl of this solution were put into the cavities of the microtiter plate. It was incubated overnight at room temperature in the dark. After three washes with 1% Tween 20 in PBS, 50 µl of anti-human IgE alkaline phosphatase conjugate (Allergopharma, Reinbek, Germany), diluted 1:200 in incubation buffer, were added and incubated overnight. The plates were washed again and the bound enzyme activity was stained with *p*-nitrophenylphosphate (PNPP) for 60 min at 37°C using an alkaline phosphatase staining kit (Allergopharma, Reinbek, Germany). Absorbance was measured at 405 nm.

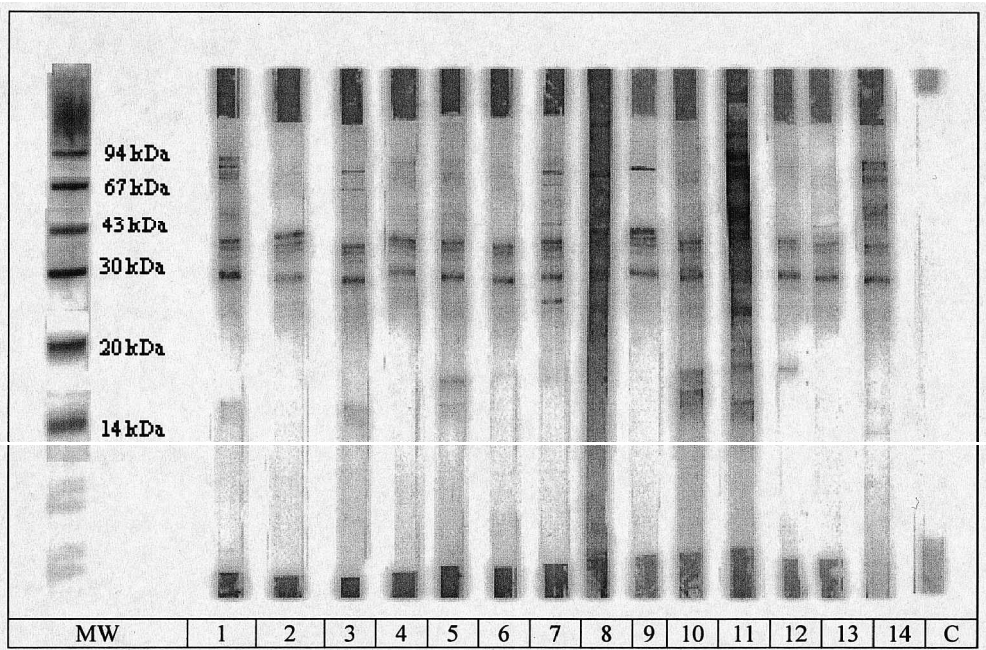


FIG. 1. Immunoblotting of mango fruit extract (variety Tommy Atkins) separated by SDS-PAGE and transferred to a NC-membrane; 1–14: patient sera; C: control serum of a non-allergic individual; MW: molecular weight marker proteins, colloidal gold staining

RESULTS

Characterization of the Allergens

SDS-Page/immunoblotting. The sera of 52 patients were examined for immunoblotting. Two allergens with molecular weights of approximately 40 and 30 kDa were both detected by 46 of the 52 sera. In Figure 1, 14 of these 52 patient sera are presented as examples. The clinical symptoms and the IgE contents of these sera are listed in Table 1. Moreover, IgE-binding proteins were seen at approximately 67 kDa (detected by 21 sera), 43 kDa (17 sera), 50 kDa (12 sera), 14 kDa (8 sera), 25 kDa (6 sera), and 16 kDa (6 sera). No IgE-binding proteins were detected by the control serum of the non-allergic patient.

IEF/immunoblotting. IEF/immunoblotting was performed under native conditions. For the increase of the electrophoretic mobility the gel strip was loaded with SDS and afterwards subjected during a period of 75 min to semidry electroblotting. For IEF/immunoblotting, the pooled serum was used as described above. It was shown that two allergens with isoelectric points of approximately 4.55 and 5.20 were detected (Figure 2). The control serum of the non-allergic patient detected no proteins.

2D-electrophoresis/immunoblotting. The final identification of the allergens in mango fruit was accomplished by 2D-PAGE followed by immunoblotting (Figure 3). The isoelectric focusing (first dimension) was performed as described above. The SDS-PAGE (second dimension) was carried out under reducing conditions. The pooled serum used in IEF/immunoblotting was also used for 2D-electrophoresis/immunoblotting. It could be proved

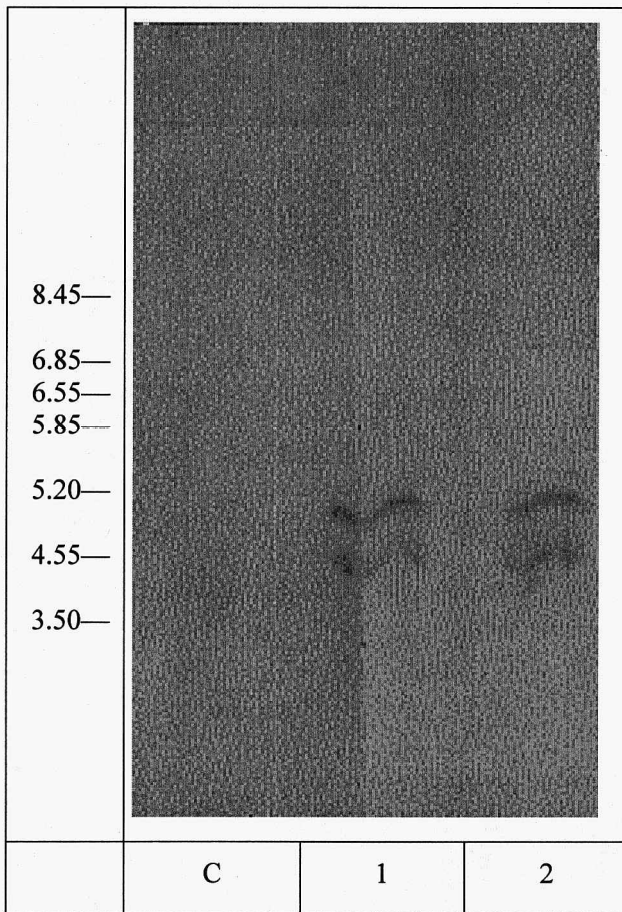


FIG. 2. Immunoblotting of mango fruit extract (variety Tommy Atkins) separated by IEF and transferred to a NC-membrane; C: control serum of a non-allergic individual; 1 and 2: pooled serum.

that the allergen with a molecular weight of approximately 40 kDa exists as two isoallergens with isoelectric points of 4.6 and 4.8. The allergen with a molecular weight of approximately 30 kDa has, like the protein with a molecular weight of approximately 67 kDa, an isoelectric point of 4.9. The protein with a molecular weight of approximately 43 kDa has an isoelectric point of 6.7. These results could be confirmed by means of ion exchange chromatography and subsequent SDS-PAGE/immunoblotting (not shown). Other IgE-binding proteins were not detectable.

Investigation of the Occurrence of Allergen Concentration During Ripening

SDS-PAGE/immunoblotting. Investigation of the occurrence of allergen concentration during ripening was examined in extracts of the mango variety Tommy Atkins first by SDS-PAGE/immunoblotting. The allergens were detected using the pooled serum described above. Allergen extraction was performed 5, 12, 21, 35 and 40 days after harvest. Allergens with molecular weights of approximately 14, 30, 40, 43 and 67 kDa were visible in each sample (Figure 4).

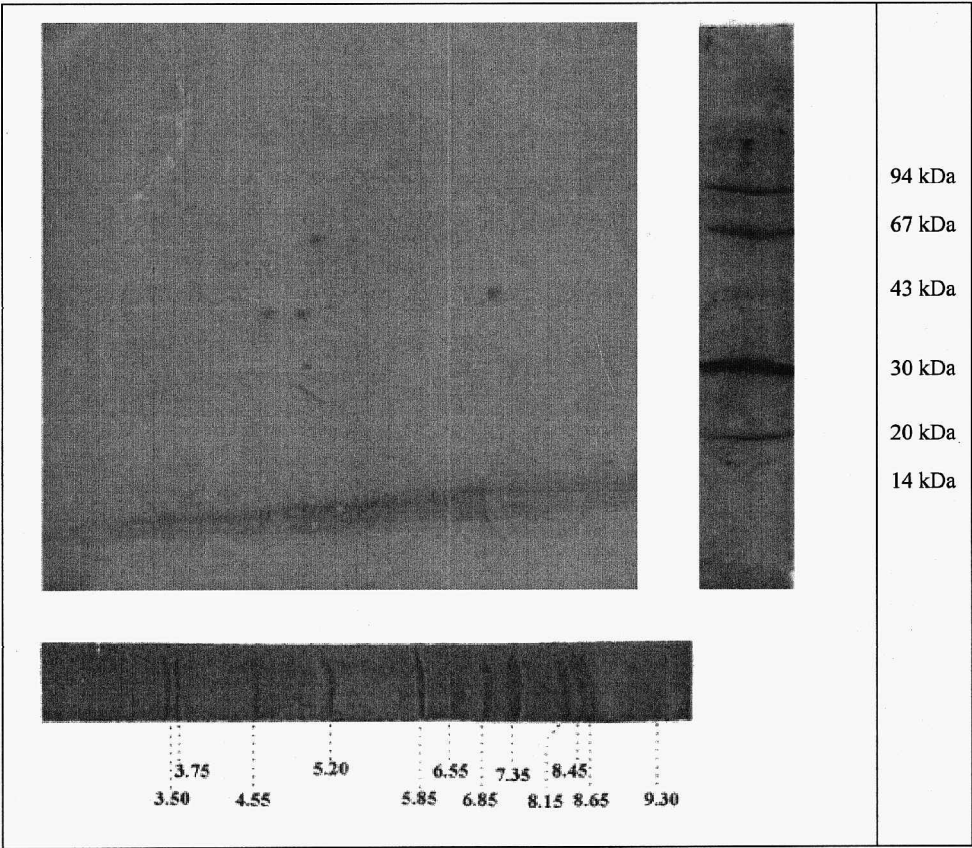


FIG. 3. Immunoblotting of mango fruit extract (variety Tommy Atkins) separated by 2D-electrophoresis and transferred to a NC-membrane; detection with pooled serum.

SDS-PAGE/immunoblot inhibition. Immunoblot inhibition was carried out with the extract from the fruits obtained 40 days after harvesting using the pooled serum described before. IgE binding to all allergens in this sample was inhibited by pre-incubation with the other samples described above and vice versa. No significant inhibition or non-specific binding was observed with ovalbumin and the control serum, respectively (data not shown).

EAST inhibition. In order to estimate the extent of IgE-binding patterns during ripening EAST inhibition experiments were performed using the pooled serum described above. The protein concentrations leading to an inhibition of IgE binding to the mango allergen disks (5 days after harvest) at the 50% level and maximum inhibitions in parentheses were determined as follows: 5 days after harvest: 1.3 mg l⁻¹ (99%), 12 days after harvest: 0.6 mg l⁻¹ (99%), 21 days after harvest: 0.8 mg l⁻¹ (99%), 35 days after harvest: 1.1 mg l⁻¹ (99%), 40 days after harvest: 1.1 mg l⁻¹ (98%). The inhibition of IgE binding to the sample protein extraction was performed 40 days after harvesting the fruit by the other samples and conversely showed that all samples led to an identical inhibition of IgE-binding. All four inhibition graphs were nearly identical (not shown). Using the statistical post-hoc comparison technique there was no difference between the mean values of an inhibition of IgE binding to the allergen disks at the 50% level (*P* < 0.05). With ovalbumin there was no significant inhibition observed in all cases.

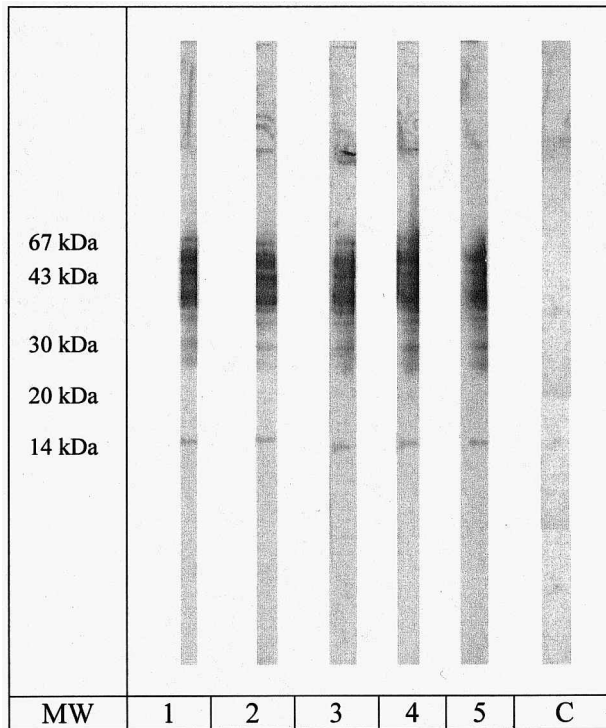


FIG. 4. Immunoblotting of mango fruit extract (variety Tommy Atkins) separated by SDS-PAGE and transferred to a NC-membrane; 1: 5 days after harvest (a.h.); 2: 12 days a. h.; 3: 21 days a. h.; 4: 35 days a. h.; 5: 40 days a. h.; C: control serum of a non-allergic individual.

DISCUSSION

The topic of the paper was the detection of IgE-related proteins in mango fruit. Furthermore the occurrence of IgE-binding patterns of mango fruit allergens during ripening was examined.

In order to identify the molecular weights of IgE-binding proteins SDS-PAGE/immunoblot was performed, 52 sera from patients with sensitization to mango fruit were used. Forty-six of the 52 patient sera detected two IgE-binding proteins with molecular weights of approximately 40 and 30 kDa, therefore these can be described as the so-called major allergens. IgE-binding proteins with molecular weights of approximately 67 (43) kDa were detected by 21 (17) patient sera. Twelve sera showed an additional IgE-binding protein with a molecular weight of approximately 50 kDa and eight sera detected an IgE-binding protein with a molecular weight of approximately 14 kDa. Two IgE-binding proteins with molecular weights of approximately 25 and 16 kDa were detected by six sera. Wellhausen *et al.* (1996) described a minor allergen of birch pollen with a molecular weight of 35 kDa cross-reacting with a mango fruit allergen of similar molecular weight. This allergen was not detected in this study, but it might be possible that one of the examined IgE-binding proteins with molecular weights of approximately 40 and 30 kDa was detected by these authors. Two mango fruit allergens with molecular weights of approximately 30 and 33 kDa were described by Möller *et al.* (1996) using 10 patient sera.

Using IEF two IgE-binding proteins with isoelectric points of approximately 4.55 and 5.20 were detectable. The final identification of the allergens in mango fruit was accomplished by 2D-electrophoresis followed by immunoblotting. It could be proved that the main IgE-

binding protein with a molecular weight of approximately 40 kDa exists as two isoallergens with isoelectric points of 4.6 and 4.8. Both important IgE-binding proteins with molecular weights of approximately 30 and 67 kDa had an isoelectric point of 4.9. An isoelectric point of 6.7 could be assigned to the IgE-binding protein with a molecular weight of approximately 43 kDa. The IgE-binding protein with an apparent molecular weight of 14 kDa was not detectable. This is the first comprehensive report on these food allergens. According to the international rules, the 40 kDa allergen was named *Mangifera indica* 1 (Man i 1) and the 30 kDa allergen *Mangifera indica* 2 (Man i 2) (Marsh *et al.*, 1987).

Investigations of different apple strains showed major differences in the allergenic potency of these strains. In addition the allergenic potency increased during maturation (Vieths *et al.*, 1993, 1994; Li-Shan *et al.*, 1995). As apple and mango fruits are both climacteric, the assumption that there is an alteration of the occurrences of different IgE-binding proteins during ripening seemed to be justified. The SDS-PAGE/immunoblotting showed no significant differences between the occurrence of IgE-binding proteins during ripening. These results could be confirmed by means of immunoblot-inhibition. This indicates an identical occurrence of allergens in all fruit maturities. A final confirmation was achieved by an investigation by means of EAST-inhibition. All inhibition curves of different fruit maturities are almost comparable and significant differences were not observed. Using statistical examination by means of LSD (lowest significant difference) test it could be observed that the average values of 50% inhibition do not differ significantly from each other. As described before there is also no difference in the allergenic potency between different varieties (Kinder *et al.* 1999).

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