

Optimization of apple allergen preparation for *in vivo* and *in vitro* diagnostics

Rudeschko O, Fahlbusch B, Henzgen M, Schlenvoigt G, Herrmann D, Jäger L. Optimization of apple allergen preparation for *in vivo* and *in vitro* diagnostics.

Allergy 1995; 50: 262–268. © Munksgaard 1995.

The aim of our investigation was to obtain a well-characterized active apple extract suitable for both *in vivo* and *in vitro* diagnostics by a technically simple method. For this purpose, apple extracts were prepared by homogenization in potassium phosphate buffer or by precipitation in organic solvents and resolubilization in potassium phosphate buffer in the presence or in the absence of enzyme inhibitors. These extracts were comparatively investigated by means of SDS–PAGE, two-dimensional electrophoresis, immunoblotting, RAST inhibition, and prick test. The *in vitro* investigations indicated that extracts prepared by precipitation in organic solvents (diacetone alcohol) at -20°C have a higher allergen activity than those prepared by extraction in aqueous solutions. From the *in vivo* tests (prick test), it was concluded that application of inhibitors of cytoplasmic enzymes (phenol oxidases, peroxidases, proteases) already during extraction is an essential precondition for active prick test solutions. Correspondingly, the extract obtained by solvent precipitation in the presence of enzyme inhibitors appeared to be most suitable for clinical application.

O. Rudeschko, B. Fahlbusch,
M. Henzgen, G. Schlenvoigt,
D. Herrmann, L. Jäger

Institute of Clinical Immunology, Friedrich
Schiller University, Jena, Germany

Key words: allergen activity; apple; food
allergy; protein extract.

Olga Rudeschko
Institute of Clinical Immunology
Friedrich Schiller University
Bachstrasse 18
D-07740 Jena
Germany

Accepted for publication 4 October 1994

The phenomenon of pollen-associated food allergy represents an epidemiologically important, but widely unsolved recent problem. The sensitization to apples, nuts, and other fruits often observed in patients with birch-pollen allergy is due to common immunologic properties of pollen and food (3, 6–9). For investigation of such “cross-reactivities”, well-characterized allergen extracts are necessary.

As there are numerous problems concerning the availability of active apple extracts, while, on the other hand, sensitization to apple occurs relatively often, apple was chosen as a model to optimize preparation of allergen extracts from fruits.

In most cases, commercial apple extracts as a matter of experience do not fulfill the requirements of both *in vivo* and *in vitro* diagnostics because of their low protein and/or allergen content (18). A relatively high plant phenol contamination might be the cause of the deficient and variable extract quality (12). These phenols were oxidized during extraction by polyphenol oxidase to chinons, partly reacting with the extracted proteins. A further activity loss should be ascribed to endogenous and/or microbial proteases.

Björkstén et al. (1) demonstrated that it is possible to obtain apple extracts suitable for serologic

investigations by shortening the extraction period and by addition of enzyme inhibitors to the extraction medium. Recently, Vieths et al. (18) investigated apple extracts prepared by three different methods and analyzed them by electrophoresis (SDS–PAGE), isoelectric focusing, and immunologic procedures (ELISA, ELISA inhibition, immunoblotting), using pool and individual sera from patients with apple allergy. Preliminary skin tests on two probands indicated that apple extracts obtained by the low-temperature (-70°C) extraction method have especially high allergen activity (18).

The intention of our study was to prepare a well-characterized active apple extract, by a technically simple procedure, avoiding such a low temperature, that could be used for both *in vitro* and *in vivo* diagnostics. Therefore, we compared four different extraction procedures and characterized the obtained extract samples, using SDS–PAGE, immunoblot, RAST inhibition, and prick test.

Material and methods

Patients

The *in vivo* tests (prick test) were performed on 12 patients with clinically relevant allergy to birch pol-

len and apple, as verified by prick tests with fresh apple and birch-pollen extract (Allergopharma, Reinbeck, Germany) and by RAST, as well as on 10 nonallergic subjects.

For *in vitro* investigations, a serum pool from 32 patients (RAST class to apple >2 and to birch pollen >4) and individual sera (RAST classes to apple 3 to >4 and to birch pollen >4) taken from our serum bank were used. A serum pool from 50 non-allergic donors (RAST class 0 to birch pollen, apple, mugwort, wasp, honeybee, grasses) served as negative control.

Extract preparation

Apple extracts were prepared from small pieces of ripe "Golden Delicious" apples, with peel, but without core, by two different methodologic principles (see Table 1).

The first principle is based on extraction in aqueous media containing various enzyme inhibitors.

Extract A1 (extraction according to Björkstén et al. (1)). This sample was obtained by extraction in potassium phosphate buffer, containing 2% polyvinylpyrrolidone (PVP), 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium diethylthiocarbamate (DIECA), and 3 mM sodium azide (1).

Extract A2 (extraction in the presence of enzyme inhibitors). An amount of 100 g apple was mechanically homogenized at 4°C in 200 ml potassium phosphate buffer (pH 7.0), containing 2% PVP, 2 mM EDTA, 10 mM DIECA, 1 mM benzamidine hydrochloride (BAHC), 0.2 mM phenylmethanesulfonylflu-

oride (PMSF), and 10 mM ascorbic acid, at 4°C, with a high-speed rotating-blade homogenizer, followed by 5-min ultrasound homogenization. The homogenate was centrifuged for 45 min at 20 000 g, 4°C. The resulting supernatant was dialyzed at 4°C first against 2 mM EDTA, 10 mM DIECA, and 10 mM ascorbic acid.

The second principle is based on the fact that proteins precipitate in organic solvents.

Extract S1 (precipitation and resolubilization in aqueous medium). An amount of 100 g apple was mechanically homogenized at -20°C in 200 ml precooled diacetone alcohol (4-hydroxy-4-methyl-2-pentanone, Aldrich Chemie GmbH) for 10 min. The homogenate was mixed with 300 ml precooled acetone and equilibrated at -20°C overnight. After removal of the supernatant by water-jet vacuum, the precipitation products were twice washed with acetone and once with acetone/ether (1:1, v/v) and then dried carefully. The resulting pulverized remnant was resolubilized in 200 ml phosphate-buffered saline (PBS) by stirring for 1 h at 4°C and centrifuged for 45 min at 4°C. The sediment was discarded.

Extract S2 (precipitation and resolubilization in aqueous medium in the presence of enzyme inhibitors). This sample was prepared in the same way as sample S1 except that the precipitation products were dissolved and dialyzed in the presence of 2.0 mM EDTA, 5.0 mM DIECA, 0.5 mM BAHC, and 0.2 mM PMSF.

The enzyme inhibitors added prevent reactions between proteins and plant phenols (1) or decrease protease activity. Ascorbic acid is known to be an antioxidative agent. All extracts were lyophilized and stored at -20°C.

To avoid skin irritations in the *in vivo* tests, the remaining additives were removed from A2, S1, and S2 by extensive dialysis against distilled water. For the same reason, A1 was dialyzed against buffer.

The protein content of the extracts was determined by the method of Bradford (2).

Prick test

All patients were tested with both fresh apple material and the prepared apple extracts. The protein concentration of the extracts had been adjusted to 25 µg/ml with PBS containing 50% (v/v) glycerol. Histamine dihydrochloride (0.1%, w/v) served as positive control, and PBS/glycerol and physiologic sodium chloride solution as negative controls. Analogously, skin tests were performed on 10 nonallergic subjects to detect nonspecific reactions caused by histamine-releasing factors or pharmacologically

Table 1. Preparation procedures and characterization of apple extracts

Preparation steps	Extract A1	Extract A2	Extract S1	Extract S2
Mechanical homogenization by high-speed rotating blades	x	x*	x	x
Precipitation in diacetone alcohol at -20°C and precipitate resolubilization in aqueous medium			x	x
Extraction in aqueous medium	x	x		
Addition of enzyme inhibitors	x	x		x
Dialysis against buffer	x	x		x
Dialysis against distilled water		x	x	x
Characterization				
Protein content µg/ml	160	500	200	180
SDS-PAGE	x	x	x	x
Immunoblotting		x	x	x
RAST inhibition	x	x	x	x
Prick test	x	x	x	

active substances. The test result was read 15 min after contact of the skin with the corresponding substance. As a measure of activity, the wheal diameter (mean of the longest and the midpoint orthogonal diameters) was determined. The test was considered to be positive when the wheal diameter was greater than 3 mm.

For characterization of the individual skin sensitivity, the quotient of the diameters of the allergen-caused wheal and the histamine-caused wheal was calculated.

SDS-PAGE and immunoblotting

Electrophoretic analysis (as well as immunoblotting) of the extracts was performed with 12.5% polyacrylamide gels and sample buffer, as described by Laemmli (11). In the case of subsequent silver staining, 2 µg protein was applied per cm gel; in the case of immunoblotting, 5 µg.

The proteins were transferred to nitrocellulose membrane strips at 60 V and 200 mA/gel for 75 min. For prevention of unspecific binding, the membranes were treated with 0.5% Tween 20 in PBS.

For detection of specific IgE antibodies, nitrocellulose strips were incubated with patient pool sera diluted 1:6 with PBS/Tween 20 overnight at room temperature under shaking. The strips were subsequently incubated with rabbit antihuman IgE (DAKO GmbH, Hamburg, Germany; dilution 1:4000), biotinylated goat antirabbit IgG (DAKO GmbH, Hamburg, 1:6000), and streptavidin, peroxidase conjugate (Boehringer Mannheim GmbH, 1:20000) and stained with 3,3',5,5'-tetramethylbenzidine and diethylsodiumsulfosuccinate (17).

Two-dimensional (2-D) electrophoresis

Separation in the first dimension was carried out, according to the instructions of the manufacturer, with Pharmacia Immobiline dry strip kit, with pH gradient ranging from 3.0 to 10.5. An amount of 40 µg protein was applied per strip.

Separation in the second dimension was performed with ready-to-use Pharmacia Excel polyacrylamide gradient gels (8–18%).

RAST and RAST inhibition

For RAST inhibition studies, the different extracts (protein concentration 300 µg/ml) were coupled to bromocyan-activated cellulose paper disks (Allergopharma), modified according to Ceska et al. (4) and Yman et al. (20). This solid phase was used to measure IgE against apple allergens. A volume of 50 µl individual serum was added per test and incubated for 3 h. Immunodetection of IgE was performed with

¹²⁵I-labeled antihuman IgE. The results were expressed as percentage binding with related total counts added.

For RAST inhibition, the serum pool was preincubated for 2 h with serial dilutions of the extracts. The value for buffer with positive serum pool was taken as maximum (noninhibited sample), and buffer with negative serum pool as blank. The RAST determination was performed with 50 µl diluted sample and the apple allergen disk corresponding to the Phadebas RAST (Pharmacia GmbH).

Results

Extract preparation

The apple extracts prepared by the two basically different procedures, i.e., by extraction in aqueous medium and by precipitation in organic solvents (Table 1), revealed different properties. In addition, the quality of the extracts was found to depend remarkably on the presence of enzyme inhibitors present during preparation.

After lyophilization, the samples A1 and A2 obtained by extraction in aqueous media became more or less yellow. In contrast, the precipitation procedure yielded colorless samples. In this case, it has been observed that the supernatant discarded during preparation was deeply yellow and had a high content of phenolic compounds, flavoids, and tannin (13).

The protein content of the four extracts was nearly the same except for extract A2 (Table 1). The higher content of this extract could have been due to the additional ultrasound treatment of the apple tissue.

Our precipitation method using diacetone alcohol was found to be technically more simple than those procedures applying acetone (5, 18). The use of diacetone alcohol allows one to carry out homogenization without danger at a higher temperature (–20°C vs –70°C) because of its higher flash point (45°C vs –19°C).

Extract characterization – in vitro studies

Generally, the protein patterns of all extracts visualized by SDS-PAGE and immunoblot analysis were similar (Fig. 1; lanes b, d, e, f). However, the pattern of extract A1 was found to be more diffuse, although the same amount of protein applied to each lane. The molecular mass of the proteins varied over a broad range (from 12 to 90 kDa).

For immunoblot studies, the extracts had to be concentrated before SDS-PAGE. After that, A2 and especially A1 (Fig. 1, lane c) were separated poorly, whereas S1 and S2 demonstrated sharp protein patterns. Therefore, immunoblotting was performed only with extracts A2, S1, and S2.

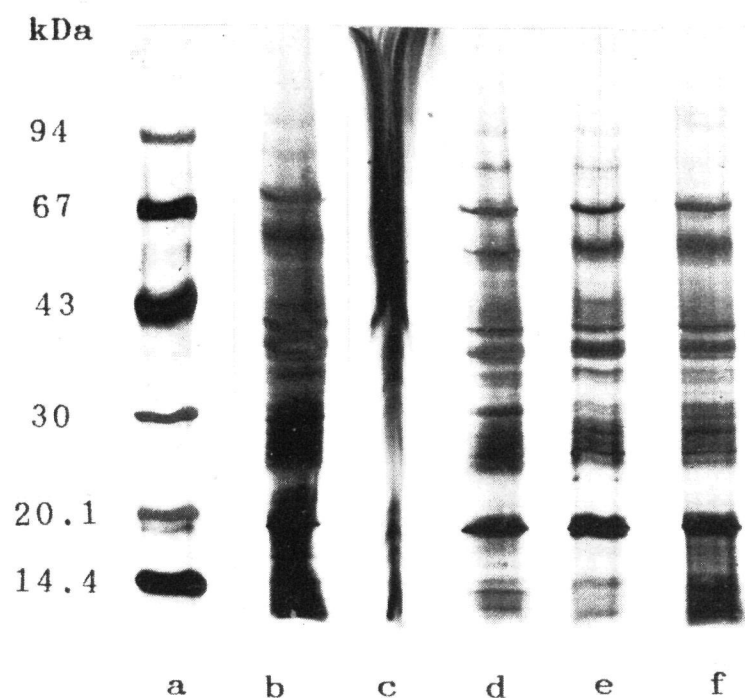


Fig. 1. SDS-PAGE of apple extracts: a) standard proteins, b) extract A1 (2 µg per lane), c) extract A1 (5 µg), d) extract A2 (2 µg), e) extract S1 (2 µg), f) extract S2 (2 µg); silver staining.

Immunoblot studies with serum pool (Fig. 2) revealed that specific IgE was bound mainly to proteins with molecular masses of 18 and 37 kDa. Additionally, IgE-binding was also detected in the range above 43 kDa in all extracts, but most obviously in S1 and S2. This binding was regarded as unspecific as it occurred also with negative serum pool (Fig. 2, lanes d–f).

Unlike the 37-kDa protein, the 18-kDa protein was also observed in all individual sera. But among the other IgE-binding components, there were remarkable differences in occurrence and intensity of single bands (Fig. 3). In general, extract S2 showed

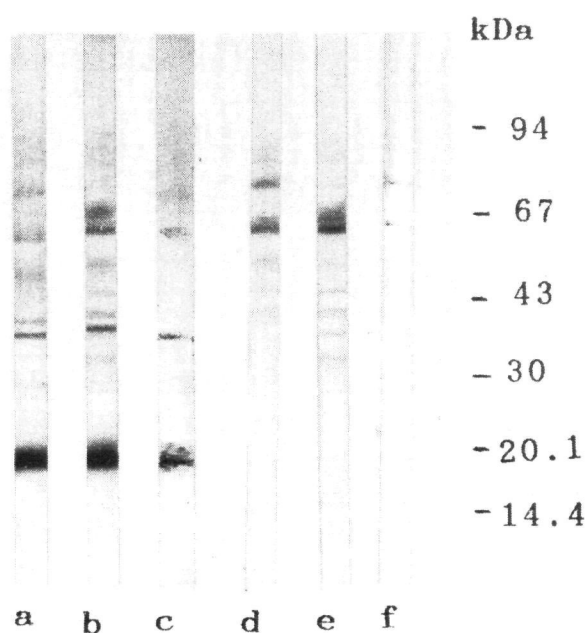


Fig. 2. Immunoblot of extracts performed with serum pool. Positive serum: a) extract S2, b) extract S1, c) extract A2; negative serum: d) extract S2, e) extract S1, f) extract A2.

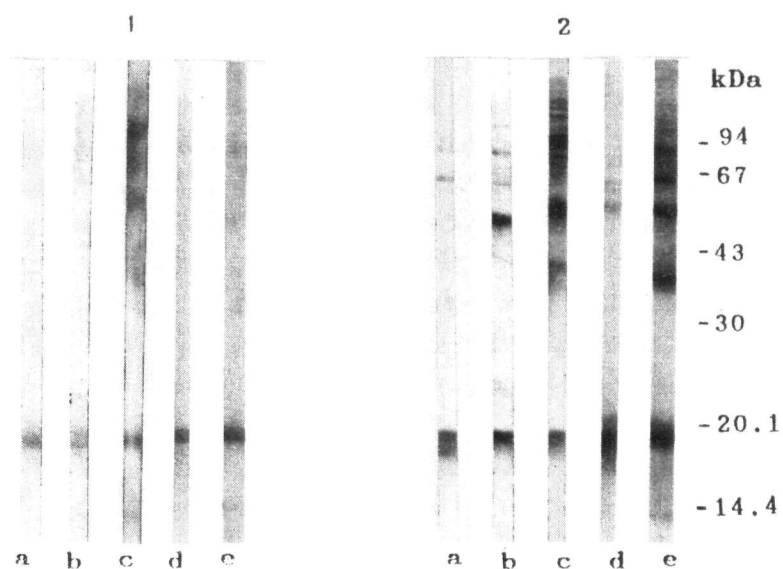


Fig. 3. Immunoblot of extracts performed with individual sera; 1) extract A2, 2) extract S2; a) serum 1, b) serum 2, c) serum 3, d) serum 4, e) serum 5.

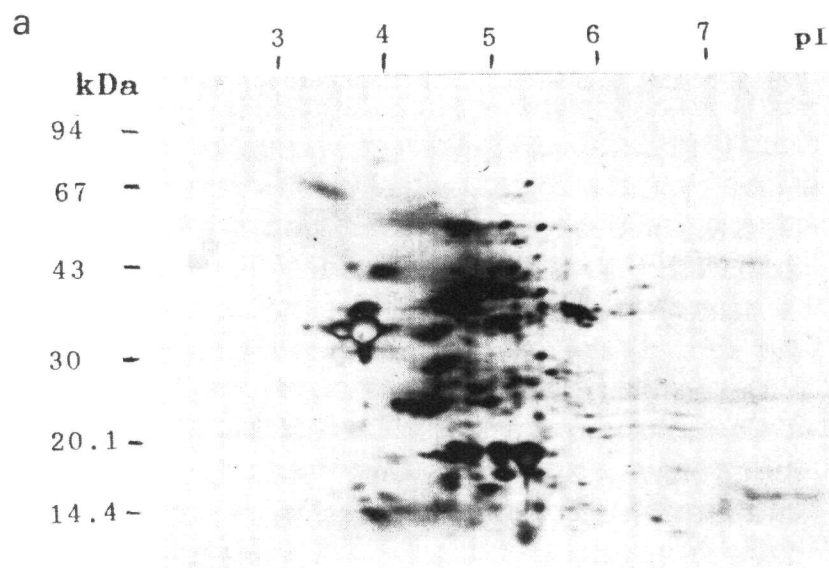


Fig. 4. 2-D electrophoresis of extract S2 (a) and corresponding immunoblot performed with serum pool (b).

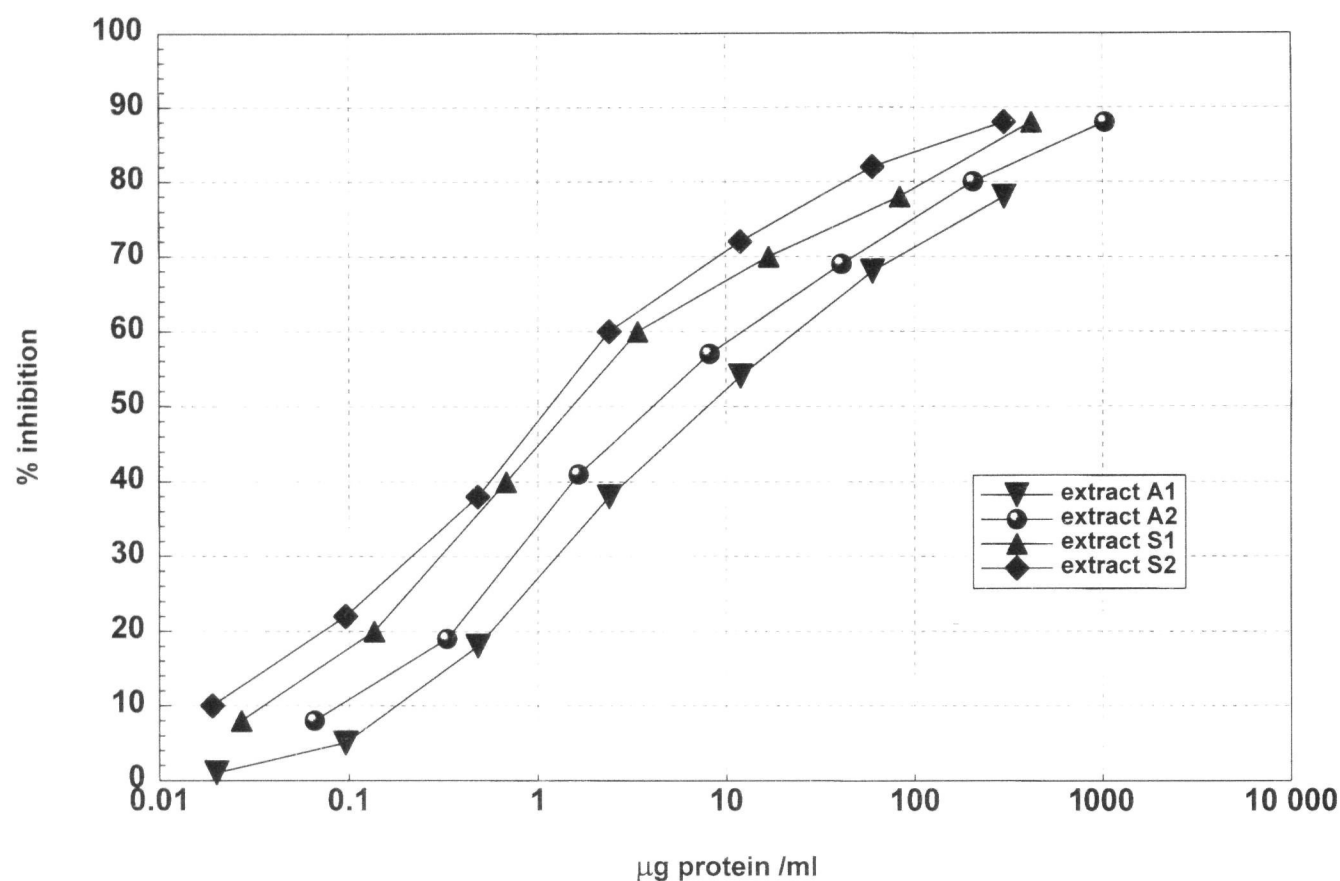


Fig. 5. RAST inhibition. Solid phase: extract A2. Inhibiting allergens: extracts A1, A2, S1, S2.

patterns with more numerous and intensive bands than A2.

After 2-D electrophoresis of extract S2 (and S1; not shown) and silver staining, numerous spots became visible (Fig. 4a), indicating a great heterogeneity in molecular mass and isoelectric point. Most of the proteins appeared within the range 12–90 kDa and at pI 4.0–6.0. The 18-kDa band revealed three main isoproteins with pI 4.5, 5.0 and 5.5, respectively. These isoproteins and two further proteins of 37 and 58 kDa, respectively, were found to bind IgE antibodies in immunoblot (Fig. 4b). The number of spots obtained from extract A2 by 2-D electrophoresis (result not shown) was not as high as in the case of extracts S1 and S2, and the separation pattern was diffuse.

For evaluation of the allergenic potency of the extracts, RAST inhibition studies were performed with serum pool and apple allergen disks of A2. The order of allergenic potency determined by RAST inhibition for each extract (Fig. 5) was $S2 > S1 > A2 > A1$. The same inhibition behavior was observed with each of the other extracts used as solid phase (not shown).

After covalent binding of the extracts to the disks, the percentage binding of IgE for the nine individual sera decreased in the following order (Fig. 6, sera 1–9): $S2 > A2 > A1$ (S1 was not examined because of its unsatisfactory results in the *in vivo* tests). The negative control showed very low values for all extracts (Fig. 6, negative serum pool).

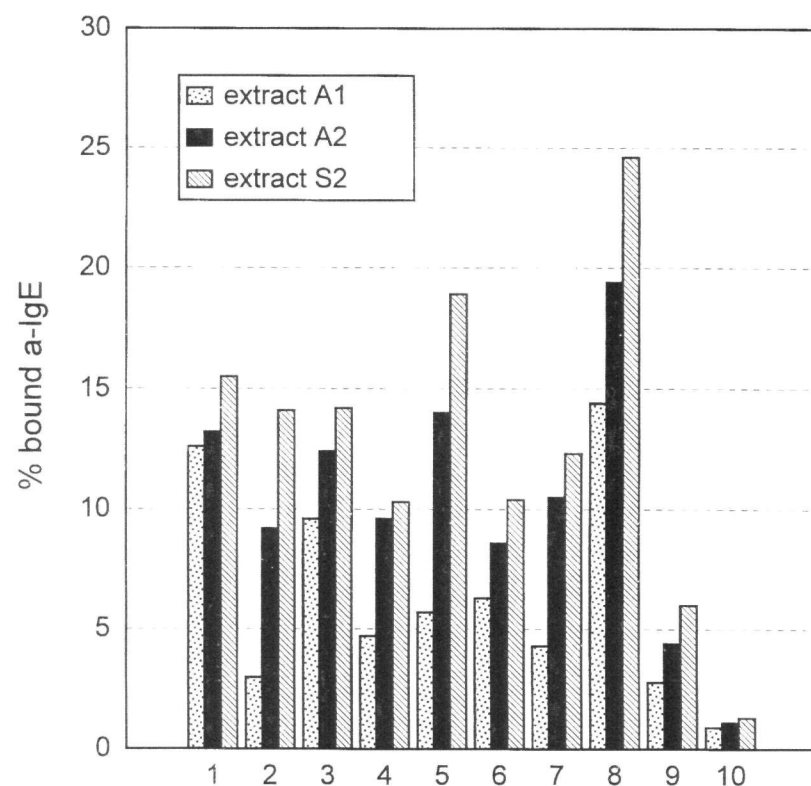


Fig. 6. Determination of IgE-binding to apple extracts coupled to bromocyan-activated disks by RAST. Disks were loaded with equal extract concentrations (300 µg/ml) and incubated with individual patient sera (1–9) and negative serum pool (11).

Extract characterization – *in vivo* studies

In the prick test, all extracts were found to have allergen activity, but to different extents (Table 2). The three extracts prepared in the presence of enzyme inhibitors had a higher allergen activity than extract S1. The order of allergen activity of the corresponding extracts was $S2 > A1 > A2 > S1$.

Table 2. Allergen activity of extracts and of fresh apple determined for 12 allergic patients by prick test. Activity is expressed as diameter quotient of allergen-caused and histamine-caused wheals

Patient no.	Extract A1	Extract A2	Extract S1	Extract S2	Fresh apple
1	0.75	1.00	0.75	0.60	2.00
2	0.67	0.83	0.33	0.75	0.60
3	0.67	0.67	0.50	0.80	0.75
4	1.25	1.00	0.75	1.25	1.00
5	1.00	0.67	0.33	2.40	0.50
6	1.00	0.80	0.80	1.66	1.40
7	0.67	0.83	0.33	1.00	1.25
8	1.00	1.00	0.75	1.33	1.20
9	1.25	1.00	0.75	1.66	1.50
10	1.20	0.80	0.80	2.00	1.25
11	0.80	0.80	0.80	1.00	0.75
12	1.20	0.80	0.40	1.25	1.00
Mean activity quotient	0.96	0.85	0.61	1.30	1.10

The nonallergic control subjects did not reveal any skin reaction to the apple extracts. All allergic patients showed negative reactions to extract-free PBS/glycerol and to physiologic sodium chloride solution.

Discussion

It is known that the allergen activity of commercially available apple extracts is frequently too low. Therefore, skin tests have to be performed additionally with fresh material to avoid false-negative results. On the other hand, the sensibility grade and, correspondingly, the results of prick tests may depend on the ripeness of the apple used (19).

To ensure reproducible results in diagnosis of apple allergy, a standardization of allergen extracts becomes necessary. In this paper, we studied extraction procedures for apple proteins as well as the composition, handling, and potency of the allergens obtained by *in vivo* and *in vitro* methods.

Independently of the preparation procedure, all extracts had a high protein content and were suitable for *in vivo* testing. All 12 birch-/apple-allergic patients had a positive skin prick test for the four apple extracts studied. In spite of remarkable individual features, extract S2 showed the highest activity (Table 2). Likewise, in the RAST studies with individual sera, the disks coupled with the different extracts revealed the highest IgE reactivity to S2 (Fig. 6). To exclude individual differences, we applied serum pool throughout for the *in vitro* studies to get a better comparability of the extracts prepared under different extraction conditions.

It has to be mentioned that there were some problems in *in vitro* characterization of the samples prepared by extraction in aqueous media. For example,

these samples were poorly separated by SDS-PAGE (especially after concentrating for immunoblotting and 2-D electrophoresis), a problem which might have been caused by nonprotein components such as pigments, lipids, or pectins. Usually, protein extraction is performed in phosphate buffer containing various additives, among them EDTA (1). EDTA should inhibit the reaction between proteins and phenol by decreasing phenol oxidase activity (12). But EDTA is also known to stimulate pectin extraction (14). The precipitation procedure described here has the advantage that it allows separation of the nonprotein components from the proteins already during treatment of apple tissue in organic solvents. Consequently, resolubilization of the precipitates results in relatively pure protein extracts quite suitable for SDS-PAGE, 2-D electrophoresis, and immunoblotting. As shown by immunoblot analysis, the extracts prepared in this way contained all apple allergens described up to now (19).

It is striking that there is a relatively great number of IgE-binding proteins above 43 kDa, mainly in the extracts S1 and S2. Other authors have also reported on IgE-binding in this range (10, 16). However, the association constant of IgE-binding to these proteins is remarkably lower than that to the 18-kDa protein (16). It has to be mentioned that our immunoblot investigations and those of Vieths et al. (16) showed that IgE-binding to proteins of the higher molecular mass range occurred also with negative serum. To a certain extent, RAST suggests low IgE-binding with negative serum, too. Vegetable extracts are known for their relatively high content of glycoproteins including lectins (15). These lectins can bind IgE through the carbohydrate side-chains on the F_c part of IgE. Therefore, we suppose that the extracts obtained by precipitation in organic solvents also contain some glycoproteins which might be responsible for the IgE-binding. Work is in progress with different individual sera in order to define single allergens in each extract better.

In spite of the relatively rough treatment of the proteins during precipitation in the organic solvent, the extracts S1 and S2 showed a higher allergen activity *in vitro* than those obtained in aqueous media (Figs. 3 and 5). Surprisingly, S1 revealed the lowest allergen activity in skin tests. This might be due to the absence of enzyme inhibitors during extraction. Further investigations have to be performed to give a satisfactory explanation.

In summary, our results indicate that extracts prepared by precipitation in organic solvents and resolubilized in aqueous media in the presence of enzyme inhibitors have high allergen activities and are suitable for *in vivo* and *in vitro* investigations. Therefore, this preparation procedure seems to offer basic preconditions for a standardization of apple extracts.

Acknowledgment

This study was supported by the Kernforschungszentrum Karlsruhe, Germany.

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