

Extraction and Properties of Apple Allergens

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It is shown that apple allergens are probably proteins and that they can be extracted in an active form only if reactions with phenolic compounds present in apple are inhibited. This is accomplished by incorporating chelators and solid polyvinylpolypyrrolidone in the extraction medium. Phenol, commonly used as a preservative, should not be added. With the RAST, serum-IgE antibodies capable of reacting with apple allergens were detected in 90% of patients with clinical apple allergy, in 44% of patients with clinical birch-pollen allergy and in 5–10% of patients with other atopic allergies. RAST inhibition indicated that apple and birch-pollen allergens cross-react.

Key words: allergen; allergy; apple; birch pollen; extract; RAST.

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Immediate skin reactions to fresh apple can be demonstrated for instance by a scratch-chamber test (5, 8) or a prick test (1). Allergens from apple seed can readily be extracted, but the preparation of stable allergen extracts from apple fruit tissue has been found more difficult (7).

Allergens causing immediate hypersensitivity are generally proteins and usually more than one allergenic protein is found in an allergen source such as birch pollen (6). It is known that the presence of phenolic compounds makes it impossible to isolate proteins in the native state by conventional techniques from many plant tissues. Phenols combine with proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensation (10). Apple is known to contain phenolic compounds, which belong to the

hydrocinnamic acids, flavoids and tannins (12, 13).

In the present study we extracted proteins from apple under conditions in which reactions with phenols were inhibited (9) and studied the properties of the allergens in the resultant solutions.

METHODS

Allergen extracts were prepared from locally purchased apples from which peel and core were removed. When not otherwise mentioned, the Granny-Smith strain was used and extraction was performed with a 10 mmol/l potassium phosphate buffer (pH 7.0) containing 2% suspended solid polyvinylpolypyrrolidone (=Plasdone XL = PVPP), 2 mmol/l ethylenediaminetetraacetic acid disodium salt

(EDTA), 10 mmol/l sodium diethyldithiocarbamate (DIECA) and 3 mmol/l sodium azide (NaN_3). PVPP, EDTA and DIECA were added to inhibit reactions between proteins and phenolic compounds (9). NaN_3 is an antimicrobial agent.

For extraction, small pieces of apple were suspended in the buffer in a polypropylene centrifuge tube and homogenized with a high-speed, spinning-blade homogenizer for 1 min. The homogenate was centrifuged for 30 min at 40,000 *g*. These steps were performed at room temperature. The supernatant was dialyzed for 20 h at 4° against 10 mmol/l potassium phosphate buffer (pH 7.0) containing 3 mmol/l NaN_3 . If any precipitate was apparent, a second centrifugation was performed at 3,000 *g* for 10 min. When necessary, the resultant solution was stored at -20°.

A 'control extract' was prepared in identical manner except that apple was omitted and 1 g/l human serum albumin was added to the extraction medium.

The proteins in apple and control extracts were coupled to cyanogen-bromide activated Munktell 00H (Grycksbro Pappersbruk, Sweden) filter paper discs using 200 μl extract per disc, except when otherwise mentioned (3). The discs and Pharmacia RAST (radioallergosorbent test) reagents (Pharmacia Diagnostics Sweden) were used in RASTs and RAST-inhibition experiments. The quantity of specific IgE bound to a disc is given in Phadebas RAST units per ml (PRU/ml) (11). For the present purposes we have arbitrarily defined results equal to or above 0.35 PRU/ml as 'positive'. The traditional RAST score 1 equals 0.35–0.70 PRU/ml.

An 'apple-positive' serum pool was collected from equal volumes of sera from 20 patients allergic to apple as judged by history, skin test and apple-seed RAST (7). In some RAST-inhibition experiments also a 'birch-positive' pool collected from sera known to contain birch-specific IgE (RAST score 3–4) was used.

For RAST inhibition we employed a birch-pollen allergen preparation (*Betula verrucosa*, Pharmacia Allergen Extract 1:10, freeze-

dried). The preparation had been freeze-dried with mannitol and was reconstituted using solvent provided by the manufacturer. The solvent contained 0.5% sodium chloride, 0.4% phenol, disodium hydrogen phosphate and EDTA.

RAST inhibition was carried out as described by Yman et al. (14). In short, a dilution series was prepared from the stock allergen solution through further dilution with 100 mmol/l potassium phosphate buffer, pH 7.4, containing 3 mmol/l NaN_3 . Twenty μl of pooled serum and 30 μl of each allergen dilution were added to a polystyrene tube. The tubes were incubated for 3 h at room temperature. A birch-pollen (Pharmacia Diagnostics) or apple-allergen RAST disc was added, and the assay was continued using standard RAST technique.

To study the occurrence of positive apple-RAST results, sera from four groups of patients were analyzed. Groups A, B and C had been studied in detail previously (7).

In *group A* there were 20 patients allergic to both birch and apple; history, and skin tests with birch pollen and apple allergens were positive.

In *group B* there were 16 patients allergic to birch but not to apple by the same criteria.

Group C contained 21 patients with no evidence of birch or apple allergy. Approximately 70% of these patients were atopics with other allergies.

In *group D* there were 10 patients with high total IgE (range 500–3400 U/ml) and a negative birch-RAST result. These patients had allergies other than birch and apple allergy.

RESULTS

Useful quantities of apple antigens capable of reacting with serum IgE antibodies could not be extracted with phosphate buffer alone. Better results were obtained when compounds known to inhibit reactions between proteins and phenolic compounds (9) were added to the extraction medium. The most effective

Table 1
 Apple allergen extraction in the presence of various inhibitors, which were added to prevent reaction between allergenic proteins and phenolic compounds

Inhibitor	Apple: extraction medium ratio (w:v)	IgE bound (PRU/ml)
<i>No inhibitor</i>		
—	1:5	0.4
—	1:1	0.3
<i>Adsorbents</i>		
4% Servachrome XAD-2 polystyrene resin (Serva)	1:1	0.4
8% Servachrome XAD-2 polystyrene resin (Serva)	1:1	0.9
4% Servachrome XAD-4 polystyrene resin (Serva)	1:5	1.0
10% Servachrome XAD-4 polystyrene resin (Serva)	1:5	0.5
4% Servachrome XAD-4 polystyrene resin (Serva)	1:1	3.0
8% Servachrome XAD-4 polystyrene resin (Serva)	1:1	3.6
4% Servachrome XAD-7 polystyrene resin (Serva)	1:1	2.6
8% Servachrome XAD-7 polystyrene resin (Serva)	1:1	1.6
<i>Chelating agents</i>		
2 mmol/l Ethylenediaminetetraacetic acid·2Na (EDTA)	1:1	11.0
10 mmol/l Ethylenediaminetetraacetic acid·2Na (EDTA)	1:1	7.8
2 mmol/l Sodium diethyldithiocarbamate (DIECA)	1:1	6.6
10 mmol/l Sodium diethyldithiocarbamate (DIECA)	1:1	10.0
<i>Detergents</i>		
0.5% Tween 80 (polyethylene sorbitanmonooleate)	1:5	1.4
1.0% Tween 80 (polyethylene sorbitanmonooleate)	1:5	2.5
<i>Insoluble hydrogen-bonding polymers</i>		
2% Polyvinylpyrrolidone (PVPP) (Fluka)	1:1	19.0
10% Polyvinylpyrrolidone (PVPP) (Fluka)	1:1	12.8
<i>Ion exchange resins</i>		
5% Dowex 1 × 8, 200/400 mesh, anion-exchange resin	1:5	0.3
10% Dowex 1 × 8, 200/400 mesh, anion-exchange resin	1:5	0.3
20% Dowex 1 × 8, 200/400 mesh, anion-exchange resin	1:5	0.3
<i>Reducing agents</i>		
2 mmol/l 1,4-Dithiothreitol (Cleland's reagent)	1:5	3.8
10 mmol/l 1,4-Dithiothreitol (Cleland's reagent)	1:5	0.4
20 mmol/l 2-Mercaptoethanol	1:1	3.9
100 mmol/l 2-Mercaptoethanol	1:1	4.9
1 mol/l 2-Mercaptoethanol	1:1	4.7
2 mmol/l Sodium disulfite (sodium pyrosulfite)	1:5	0.4
10 mmol/l Sodium disulfite (sodium pyrosulfite)	1:5	0.4
<i>Soluble hydrogen-bonding polymers</i>		
2% Polyethylene glycol, MW 20000 (Fluka)	1:5	2.8
10% Polyethylene glycol, MW 20000 (Fluka)	1:5	1.4
2% Polyvinylpyrrolidone K 15, MW 10000 (Fluka)	1:1	21.0
10% Polyvinylpyrrolidone K 15, MW 10000 (Fluka)	1:1	11.2
2% Polyvinylpyrrolidone K 30, MW 40000 (Fluka)	1:5	0.9
10% Polyvinylpyrrolidone K 30, MW 40000 (Fluka)	1:5	0.9

The extraction medium contained 10 mmol/l potassium phosphate (pH 7.0), 3 mmol/l NaN₃ and one of the inhibitors listed. The proteins in 200 µl extract were coupled on a cyanogen-bromide activated paper disc. The quantity of apple-specific IgE bound to the allergen disc from 50 µl of pooled serum was measured with the RAST and is given in PRU/ml units.

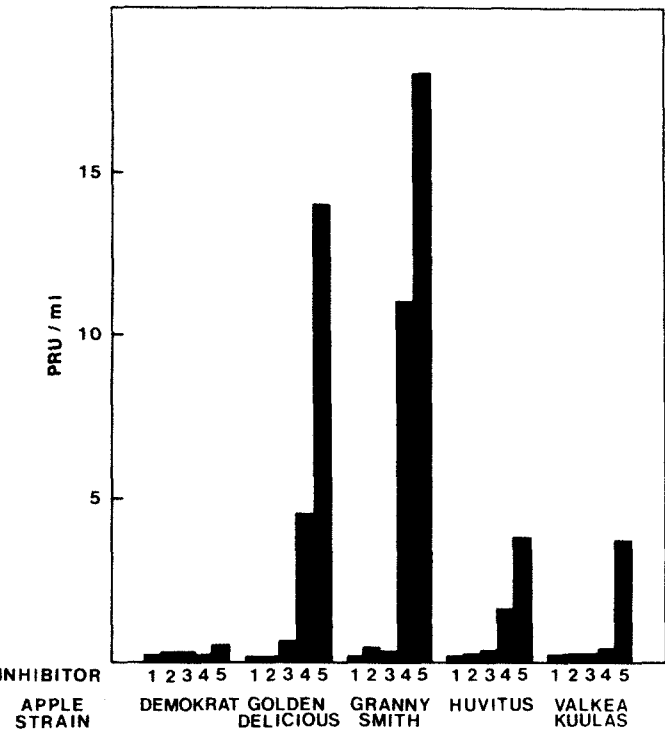


Fig. 1. Apple allergen extraction in the presence of various inhibitors added to prevent reaction between allergenic proteins and phenolic compounds. The inhibitors were (1) none, (2) 2 mmol/l EDTA, (3) 10 mmol/l DIECA, (4) 2% PVPP and (5) all three inhibitors. The apple: extraction medium ratio was 1:1 (w/v). For further experimental details see Table 1.

inhibitors were PVPP and polyvinylpyrrolidone (Table 1). Of these PVPP was chosen for routine use, since it is insoluble and can easily be removed through centrifugation. Other inhibitors have poorer and less consistent results (Table 1). EDTA and DIECA were included in the routine extraction medium, since they had an effect additive to that of PVPP (Fig. 1).

The quantity of allergens extractable from apples of various strains varied greatly (Fig. 1). The largest quantities were obtained from the Granny-Smith strain, and it was used for most experiments.

Allergens could always be extracted from Granny-Smith apples, but we observed considerable differences in the quantity or quality obtained in different experiments. The differences could have been due to variability between individual apples or apple lots. When the proteins in 50 µl of a good Granny-Smith extract were coupled on a cyanogen-bromide

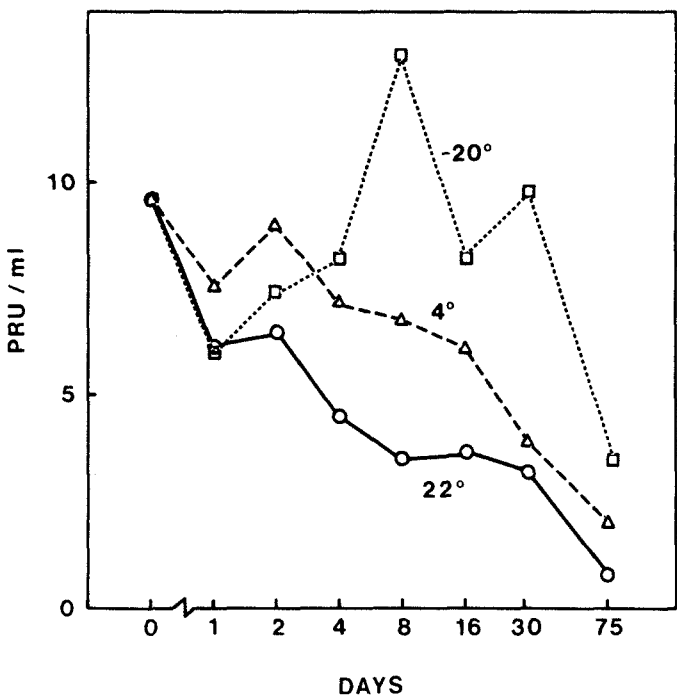


Fig. 2. Effect of storage time and temperature on the allergen content of an apple extract. The extract contained 10 mmol/l potassium phosphate (pH 7.0) and 3 mmol/l NaN₃. The allergen content was examined as described in Table 1.

Table 2
Effect of volume of apple extract used per RAST disc on quantity of specific IgE bound

Volume (µl)	IgE bound (PRU/ml)
10	7.6
20	13.0
50	> 30.0
100	> 30.0
200	> 30.0

activated paper disc, all apple-specific IgE in 50 µl of our serum pool was bound to it in the RAST (Table 2). With a poorer extract, 200 µl did not yield an equally good disc.

Extracts which had been frozen tended to give somewhat variable results after thawing. If extracts were stored non-frozen, the apparent allergen concentration fell steadily (Fig. 2).

IgE antibodies (0.35 PRU/ml or more) specific to the extracted apple allergens were found with the RAST in the sera of 90% of patients considered allergic to apple by other

Table 3
Occurrence of apple-specific IgE antibodies in the sera of various patient groups

Patient group	Number of patients	Apple-specific IgE (PRU/ml)			Prevalence of results ≥ 0.35 is significantly different from that in groups*
		< 0.35	0.35 – 0.69	≥ 0.70	
		Prevalence (%)			
A. Allergic to apple and birch	20	10	5	85	B, C and D
B. Allergic to birch but not to apple	16	56	13	31	A and C
C. Non-allergic to apple and birch	21	95	5	0	A and B
D. High total IgE, birch-RAST negative	10	90	0	10	A

*Fisher's exact test, two-sided, $P < 0.01$

criteria (patient group A). In addition, such positive results were obtained in 44% of patients allergic to birch pollen but not to apple (patient group B). In patient group A there were significantly more positive results than in any one of the groups (B–D) containing patients with no known apple allergy (Fisher's exact test, one-sided, $P < 5 \times 10^{-3}$) (Table 3).

Ten sera from patient group A were also tested using human albumin control-RAST discs prepared in a manner identical with that for the apple-RAST discs except that apple allergen was replaced by 1 g/l human serum albumin. All results were under 0.35 PRU/ml. With the apple-RAST discs the same sera gave results in the range 2.7–29 PRU/ml.

RAST-inhibition experiments were carried out to study the cross-reactivity of apple- and birch-pollen allergens. It was found that birch-pollen allergens could bind all or nearly all IgE specific to apple in an 'apple-positive' serum pool. On the other hand, when apple allergen was used as inhibitor for the 'birch-pollen-positive' serum pool, no inhibition could be demonstrated (Fig. 3).

DISCUSSION

The extraction properties of apple and related apple-seed (7) allergens and their binding to cyanogen-bromide activated paper discs suggest that these allergens, like most

allergens causing immediate hypersensitivity (6), are proteins. Difficulties, which have previously been encountered in the study of the properties of apple allergens (1), have

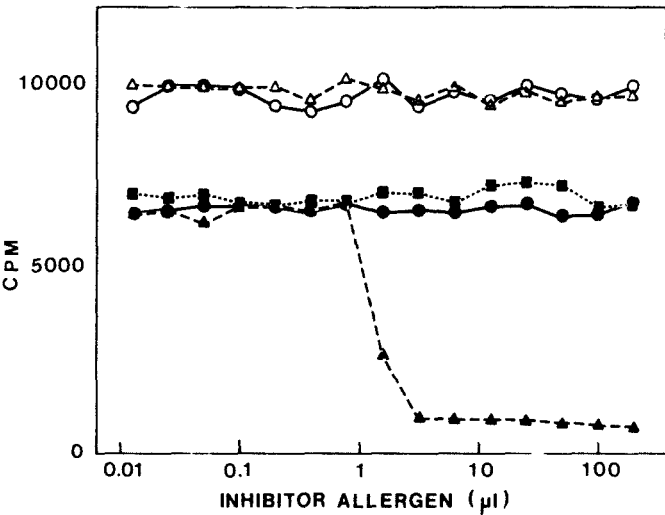


Fig. 3. RAST-inhibition experiments performed to detect cross-reactions between apple and birch-pollen allergens. In each experiment a dilution series was prepared from the inhibitor-allergen stock extract (on the x-axis). An equal amount of pooled serum was added to each dilution. After 3 hours, a RAST was carried out on each allergen-serum sample to determine the quantity of specific IgE still capable of binding to the disc (given as counts per minute on the y-axis). Altogether five experiments were performed:

Symbol	Inhibitor allergen	Serum pool	Allergen coupled to RAST disc
○	None	birch positive	birch pollen
△	Apple	birch positive	birch pollen
●	None	apple positive	apple
▲	Birch pollen	apple positive	apple
■	Apple	apple positive	birch pollen

probably been due to chemical reactions between the phenolic compounds present in apples (12, 13) and the allergenic proteins (9, 10).

We found that apple allergens can readily be extracted with media containing additives, which inhibit the protein-phenol reactions (Table 1 and Fig. 1). In previous studies on the solubilization of various plant proteins (9) and also in the present experiments, polyvinyl-polypyrrolidone (PVPP) has been found to be a particularly useful inhibitor. It is added as an insoluble powder, to which phenolic compounds are bound through hydrogen bonding.

An even better extraction result was obtained when—in addition to PVPP—a chelating agent was added. Chelators bind metal ions and thus inhibit both the enzymatic and non-enzymatic oxidation of phenols to reactive quinones, which bind covalently to proteins. We found EDTA and DIECA suitable for routine purposes (Fig. 1). DIECA also has reducing capacity, which may be useful. After extraction, EDTA and DIECA were removed through dialysis and PVPP with bound phenols through centrifugation.

The antigenic activity in apple-allergen extracts remained fairly stable for at least 1 month, when extracts were stored frozen. Non-frozen extracts suffered considerable loss of antigenic properties (Fig. 2). We used NaN_3 as an antimicrobial agent. When phenol was tried for the purpose, the apple-allergen content fell rapidly, and very little remained after a few days. This could be due to the presence of a phenol oxidase.

The apple allergens can readily be coupled to RAST discs and these can be used to assay specific IgE. The clinical usefulness of the assay is, however, limited by the observation that IgE antibodies are found with this assay not only in the sera of patients allergic to apple, but also in 44% of sera from patients allergic to birch pollen with no clinical apple allergy (Table 3).

IgE binding by apple-RAST discs is not

unspecific in an immunological sense. Sera from patients with apple allergy yield a positive result significantly more often than sera from patients with no apple or birch allergy even when the total IgE contents of the latter sera are high (Table 3). There was no binding of IgE to reagent-control discs prepared from an extract with no apple in it.

Our RAST results appear to illuminate the causes of the frequent co-occurrence of immediate hypersensitivity to birch pollen and apple, which can be detected both clinically and with skin tests. In addition, the same patients are often hypersensitive also to hazelnut, carrot, parsnip and potato, and sometimes to other vegetables (1, 2, 4, 5, 7, 8).

Belin (2) has shown that birch-pollen and hazelnut allergens cross-react, and Lahti and coworkers (7) found evidence for the cross-reactivity of birch-pollen and apple-seed allergens. The present RAST-inhibition experiments suggest cross-reactivity between birch-pollen and apple allergens. However, binding of IgE antibodies to birch-pollen RAST discs could not be inhibited with our apple-allergen stock solution (Fig. 3).

Patients clinically allergic to apple but not to birch pollen are rare, and we have not been able to include such a group in our study. This and the RAST-inhibition results could be explained by assuming that birch-pollen allergens are usually the primary immunogens and that apple allergens are bound to birch-specific IgE in a casual cross-reactions with a relatively low association constant.

Two patients (in groups C and D) included in Table 3 had a positive apple and a negative birch-pollen RAST result. These patients had serum-IgE antibodies against other pollens. There appears to be some degree of cross-reactivity also between apple and pollens other than birch (Björkstén et al., unpublished).

Allergy to apple and hazelnut and to a lesser extent to carrot, parsnip, etc. is of clinical importance in countries where the birch is a prevalent species. This is probably due to cross-reactions between the allergens.

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