

Investigation of the stability of apple allergen extracts

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To determine optimal conditions for allergen preservation, we investigated the influence of different stabilizing additives and of storage temperature on the allergen activity of apple protein preparations, obtained by extraction in phosphate buffer or by precipitation in diacetone alcohol and resolubilization in phosphate buffer in the presence or absence of enzyme inhibitors. For this purpose, the extracts were stored for 6 months either in frozen state at -20°C or in lyophilized state at -20°C , 4°C , or room temperature and were characterized by SDS–PAGE, immunoblot, ELISA inhibition, and prick test. The highest stability revealed the extracts that were prepared by precipitation in the organic solvent in the presence of enzyme inhibitors, lyophilized, and stored at -20°C . For storage of extract solutions at 4°C , PBS/glycerol and cysteine/sodium citrate/glycerol were found to be the most effective stabilizing additives.

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In spite of great advances in allergology, there are still problems concerning *in vivo* and *in vitro* diagnostics of fruit and vegetable allergies, especially apple allergy. These problems are mainly due to deficient and nonpermanent apple extract quality caused by the action of endogenous enzymes, leading to remarkable reactivity of the apple allergens with non-protein constituents. Frequently, fresh material is required for skin tests since commercial apple extracts usually have low allergen activity (18, 20), often leading to false negative results. For the same reason, the *in vitro* tests can also give unreliable results.

Allergen activity might decrease already during extraction of proteins from apple tissue as a result of reactions between the proteins and endogenous phenols involving polyphenol oxidase and proteinase activity (1, 7, 11–13). Further allergen activity is lost during storage (1).

For minimization of allergen degradation, the presence of enzyme inhibitors is necessary already in extraction media (1, 16). Recently, it has been demonstrated that it is possible to obtain apple extracts with especially high activities, suitable for both *in vivo* and *in vitro* diagnostics, by protein precipitation in an organic solvent and resolubilization in aqueous media containing enzyme inhibitors (16).

To our knowledge, there have been no systematic investigations of allergen stability and storage con-

dition of apple allergen extracts and consequently of their usability for *in vivo* tests over long periods. To find out how storage conditions affect allergen activity, we compared lyophilized and aqueous apple extracts stored at different temperatures and in the presence of different stabilizers by means of SDS–PAGE, immunoblot, ELISA inhibition, and skin prick tests. In this way, we tried to get basic information on the preparation of stable apple allergens that could be reproducibly applied for diagnostic purposes over longer periods.

Material and methods

Patients

The *in vivo* tests (prick tests) were performed on 12 patients with clinically relevant allergy to birch pollen and apple whose diagnosis was proved by prick tests with fresh apple and birch pollen extract and RAST, as well as on 10 nonallergic persons.

For *in vitro* investigations, a serum pool obtained from 12 allergic patients (RAST class to apple >2) and individual sera from patients with birch-pollen and apple allergy (RAST classes 3 to >4), taken from our serum bank, were used. A serum pool from 50 nonallergic donors (RAST class 0 to birch pollen, apple, mugwort, wasp, honeybee, grasses) served as negative control.

Extract preparation

The allergen extracts were prepared from one lot of Golden Delicious apples, purchased at the local market, as recently described (16):

Extract A2: prepared by extraction in potassium phosphate buffer in the presence of enzyme inhibitors (EDTA, disodium diethyldithiocarbamate, phenylmethanesulfonylfluoride, benzamidinchloride)

Extract S1: prepared by precipitation in diacetone alcohol (4-hydroxy-4-methyl-2-pentan-2-one) and resolubilization in potassium phosphate buffer without enzyme inhibitors

Extract S2: prepared by precipitation in diacetone alcohol and resolubilization in potassium phosphate containing enzyme inhibitors (see extract A2).

The extracts contained 200–500 µg/ml protein, determined by the method of Bradford (2), with bovine serum albumin as standard.

For lyophilization, the extracts were frozen at –80°C immediately after preparation and dried at 0.01 mbar.

Storage and extract stabilization

One part of the prepared extracts was stored frozen at –20°C; another part was stored in lyophilized state at –20°C, 4°C, or room temperature for up to 6 months.

For *in vivo* diagnostics, the activity of allergen solutions should be preserved over longer periods. To determine the optimal conditions for preservation, the lyophilized extracts were resolubilized in media containing the following different stabilizing agents and stored at 4°C for up to 6 months: 1) PBS/50% glycerol; 2) 0.4% phenol/0.15 M sodium chloride/50% glycerol (commercial prick test solution); 3) PBS/ascorbic acid/0.7% sodium citrate/50% glycerol; 4) PBS/0.12% cysteine/0.7% sodium citrate/50% glycerol; 5) PBS/0.1% sodium benzoate/50% glycerol. For the *in vivo* and *in vitro* tests, the protein concentration was adjusted to 25 and 500 µg/ml, respectively.

Skin prick tests

The lyophilized extracts were resolubilized in the presence of different stabilizing agents, sterilized by filtration, and tested in fresh state in June and after 6-month storage in December, i.e., outside the birch pollen season. The corresponding media without apple extract and physiologic sodium chloride solu-

tion served as negative controls. Fresh apple (Golden Delicious) and histamine were used as positive controls.

As measures of extract activity, the wheal diameter (mean of the longest and the midpoint orthogonal diameter) was determined. The obtained results were statistically processed by the Mann-Whitney test.

SDS-PAGE

For electrophoretic characterization, ready-to-use polyacrylamide ExcelGel SDS gradient 8–18% gels (Pharmacia) were applied. For separation, the extracts were dissolved in sample buffer according to Laemmli (10). Amounts of 2 and 5 µg of total protein were applied per lane for silver staining and immunoblotting, respectively.

Immunoblotting

The proteins were transferred to nitrocellulose (NC) membranes (Pharmacia LKB GmbH, Freiburg, Germany) as we have described (16). Specific IgE antibodies were detected after incubation of the cut NC strips (3 mm) in serum pool, followed by incubations in antihuman IgE from rabbit (dilution 1:4000, DAKO GmbH, Hamburg), biotinylated goat antirabbit IgG (1:6000, DAKO GmbH, Hamburg), streptavidin peroxidase conjugate (1:20000, Boehringer Mannheim GmbH), and 3,3',5,5'-tetramethylbenzidine/dioctyl sodium sulfosuccinate (16, 19).

ELISA inhibition

Microtiter plates (MAXISORB, NUNC) were coated with extract S2 (10 µg/ml protein) overnight at room temperature (9).

The extracts (500 µg protein/ml) were diluted with PBS/Tween 20 in 1:5 steps down to 0.03 µg/ml. For inhibition, two volumes of diluted extract were mixed with one volume of positive serum and preincubated for 2 h. The value for buffer with positive serum pool was taken as maximum (noninhibited sample) and buffer with negative serum pool as blank. A volume of 50 µl of the diluted preincubated sample was applied per well.

Specific IgE was detected in the patient serum pool by sequential incubation in the following:

- rabbit antihuman IgE (diluted 1:4000, 2 h)
- biotinylated goat anti-rabbit IgG (1:6000, 1.5 h)
- streptavidin peroxidase conjugate (1:10000, 1.5 h)
- *o*-phenyldiamine/H₂O₂/citric acid-phosphate buffer, pH 5.0 (4 mg/5 µl/10 ml, 0.5 h).

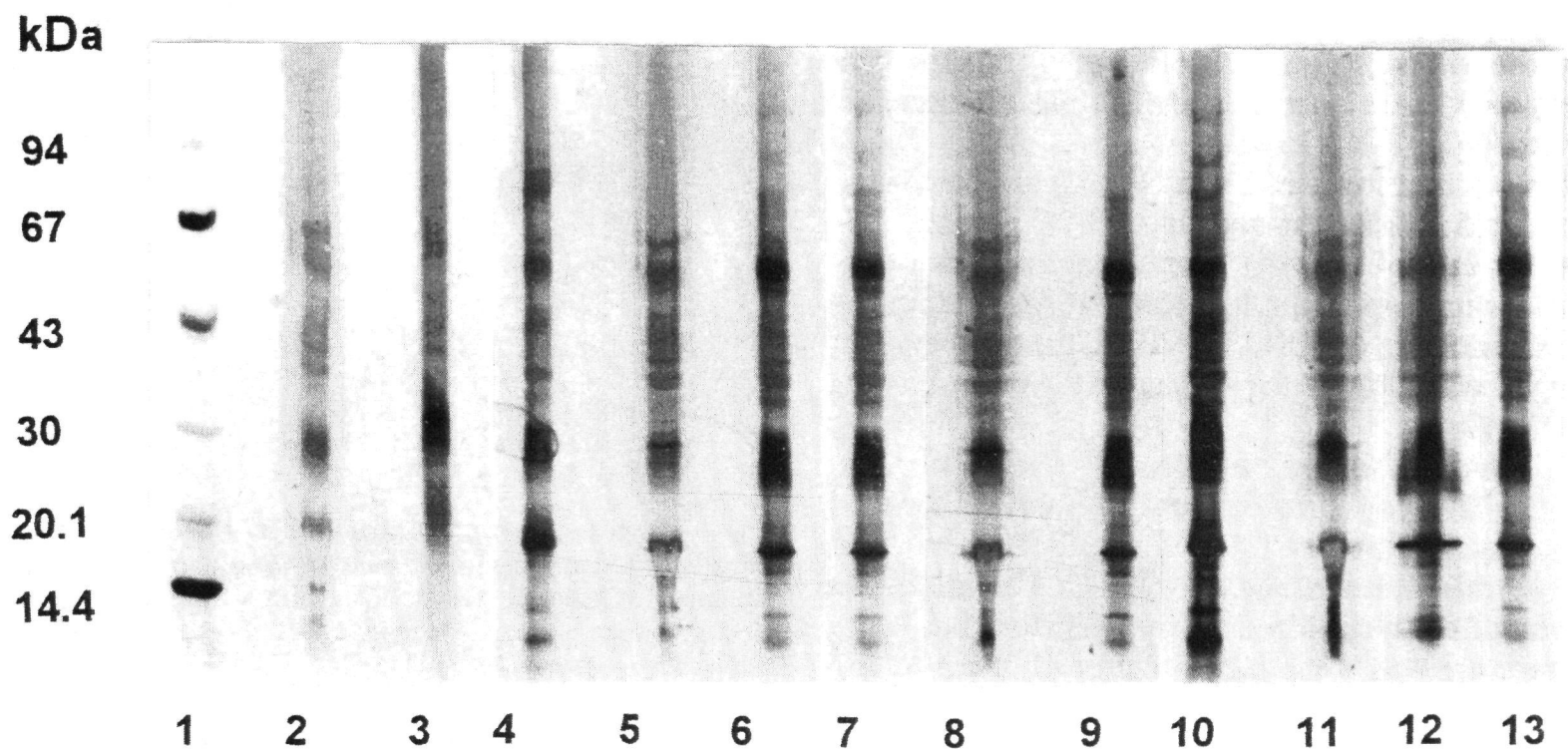


Fig. 1. SDS-PAGE of apple extracts after 6-month storage. Lane 1) protein standards; 2-4) lyophilized extracts A2, S1, and S2 after storage at room temperature; 5-7) lyophilized extracts A2, S1, and S2 after storage at 4°C; 8-10) lyophilized extracts A2, S1, and S2 after storage at -20°C; 11-13) resolubilized extracts A2, S1, and S2 after storage at -20°C.

Between the incubation steps, the samples were washed three times with PBS/Tween 20. Photometric measurement was performed at 492 nm with a microplate reader (SLT). All determinations were run in duplicate.

Results

Storage

Independently of the preparation procedure, the fresh extracts revealed a similar protein pattern in SDS-PAGE (16). However, depending on the stor-

age conditions, significant differences between the corresponding protein patterns became visible after 6 months. Storage at room temperature led to partial protein degradation that resulted in unsharp bands (Fig. 1, lanes 2 and 3). This concerned especially extract S1, which was prepared without enzyme inhibitors. In this case, all proteins above 30 kDa were degraded almost completely. The same was found for the 18-kDa protein, which is considered to be the major apple allergen (6, 17, 18). Unlike S1, in extract S2, all proteins remained preserved.

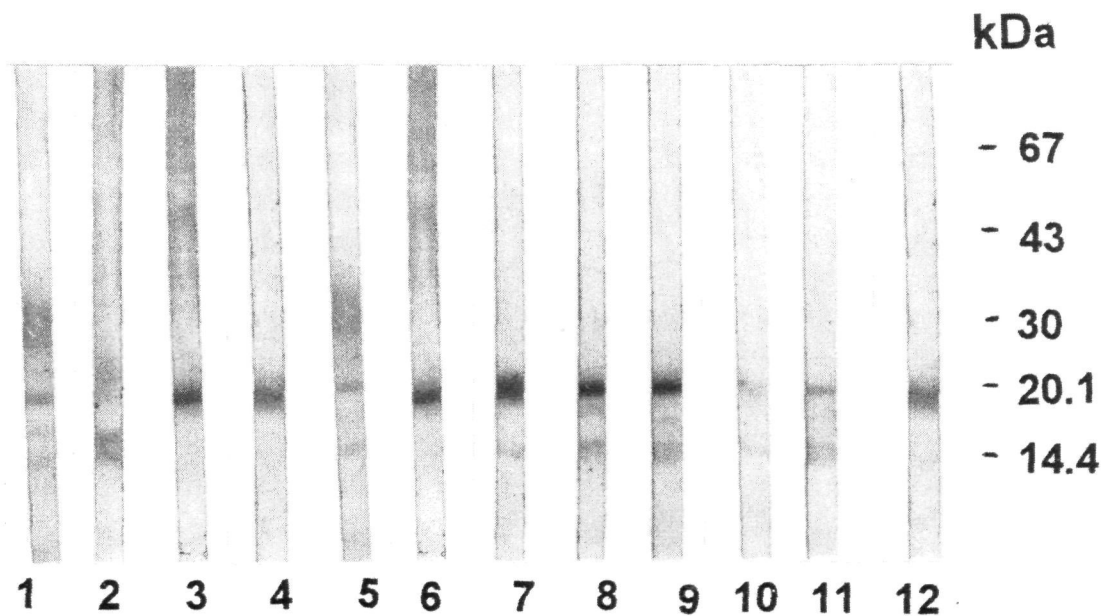


Fig. 2. Immunoblot of apple extracts after 6-month storage. Lanes 1-3) lyophilized extracts A2, S1, and S2 after storage at room temperature; 4-6) lyophilized extracts A2, S1, and S2 after storage at 4°C; 7-9) lyophilized extracts A2, S1, and S2 after storage at -20°C; 10-12) resolubilized extracts A2, S1, and S2 after storage at -20°C.

After storage of the extracts at 4°C and at -20°C, all protein bands could be recovered, but most of them were more intensive in S2 than in the other extracts (Fig. 1).

Immunoblot investigations (Fig. 2) indicated that lyophilized extracts stored at -20°C revealed the lowest losses of activity. The strongest activity decrease occurred in samples stored at room temperature. Coinciding with SDS-PAGE, the most evident changes were found for extract S1, which lost its 18-kDa band.

ELISA inhibition confirmed these findings (Fig. 3). The lyophilized extracts S1 and S2 showed the strongest inhibition (50% values for both at about 0.05 µg/ml) after storage at -20°C. The inhibition activity of the lyophilized extract S2 stored at room temperature was weaker by a factor of about 4. Extract S1 stored under the same conditions but prepared without enzyme inhibitors had the lowest inhibition potency. Its activity was approximately 40 times lower than after storage at -20°C.

Stabilization of resolubilized samples

Immediately after resolubilization, all extracts were colorless. After storage, several samples revealed color changes (Table 1). In contrast to A2 and especially S2, extract S1 changed its color at both concentrations (25 and 500 µg/ml) in all stabilizing media used.

In the *in vivo* investigations, all nonallergic persons did not react to both the apple extracts prepared and the extract-free control solutions (stabilizing media). Commonly, the allergic patients also did not reveal reactions to the control solutions, with the exception of two (out of 12) patients who reacted to sodium benzoate. All patients were positive to all fresh apple extracts (Fig. 4). The activity of extract S1 was lower than those of A2 and S2, in every case. Extract S2 in PBS/glycerol and cysteine/sodium citrate was the most active one after storage.

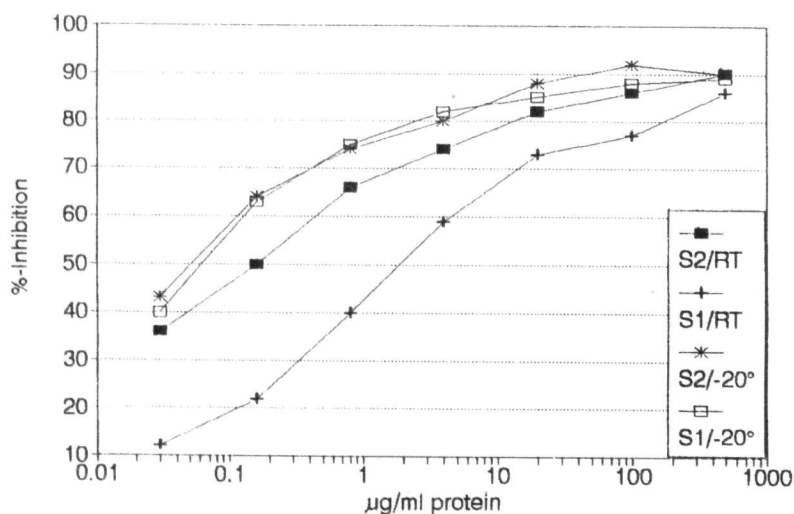


Fig. 3. Effect of storage on activity of apple extracts determined by ELISA inhibition. RT: room temperature.

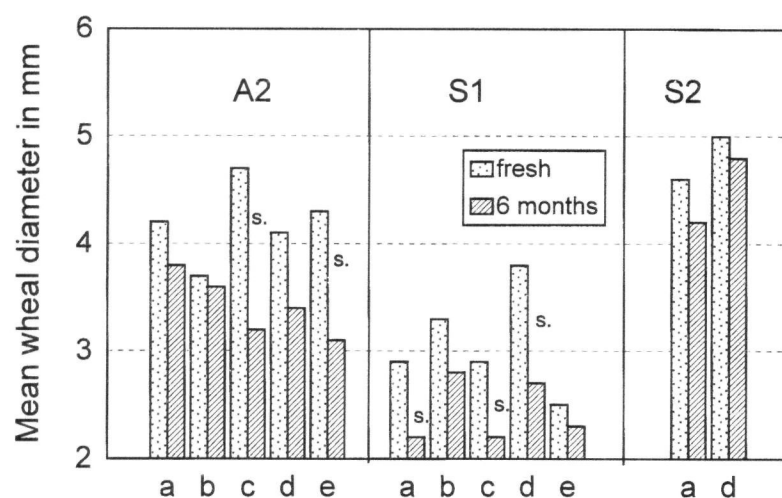


Fig. 4. Prick test results of apple extracts after 6-month storage in different stabilizing solutions. a) PBS/glycerol; b) prick test solution; c) ascorbic acid/sodium citrate/glycerol; d) cysteine/sodium citrate/glycerol; e) sodium benzoate/glycerol. Significant differences indicated by "s".

In vitro investigations by ELISA inhibition showed that the extracts resolubilized in prick test solution and in ascorbic acid/sodium citrate had the highest 50% inhibition values; i.e., lost the most activity (Table 2). Mixtures of PBS/glycerol, cysteine/sodium citrate/glycerol, and sodium benzoate/glycerol were found to be the most favorable stabilizing media. Under these conditions, extract S2, both fresh as well as after storage, had the highest activity.

These results were confirmed by immunoblotting experiments (Fig. 5). After resolubilization in prick test solution or ascorbic acid/sodium citrate, all three extracts lost a great part of their allergen activity, whereas PBS/glycerol, cysteine/sodium citrate/glycerol, and sodium benzoate/glycerol preserved allergen activity widely.

Discussion

Active and stable allergen extracts are an essential precondition for a precise and reliable diagnosis of allergy to fruit and vegetables. Problems arise not only from allergen preparation but also from allergen storage. It has been shown that the loss of allergen activity during extraction can often be reduced by enzyme inhibitors (1, 16).

Moreover, it has been demonstrated that the stability of allergen extracts may depend on the conditions of storage (1, 3). Our results indicate that, as expected, the apple allergen activity depends on storage temperature and physical state (solid or fluid). Storage in lyophilized state and at -20°C was found to be the most favorable condition to preserve allergen activity of apple extracts, independently of the preparation procedure used (Figs. 2 and 3). This might be explained by the decreased reactivity of enzymatic components in the dried state.

The highest activity loss was observed in extract S1 at room temperature. As extract S1 was pre-

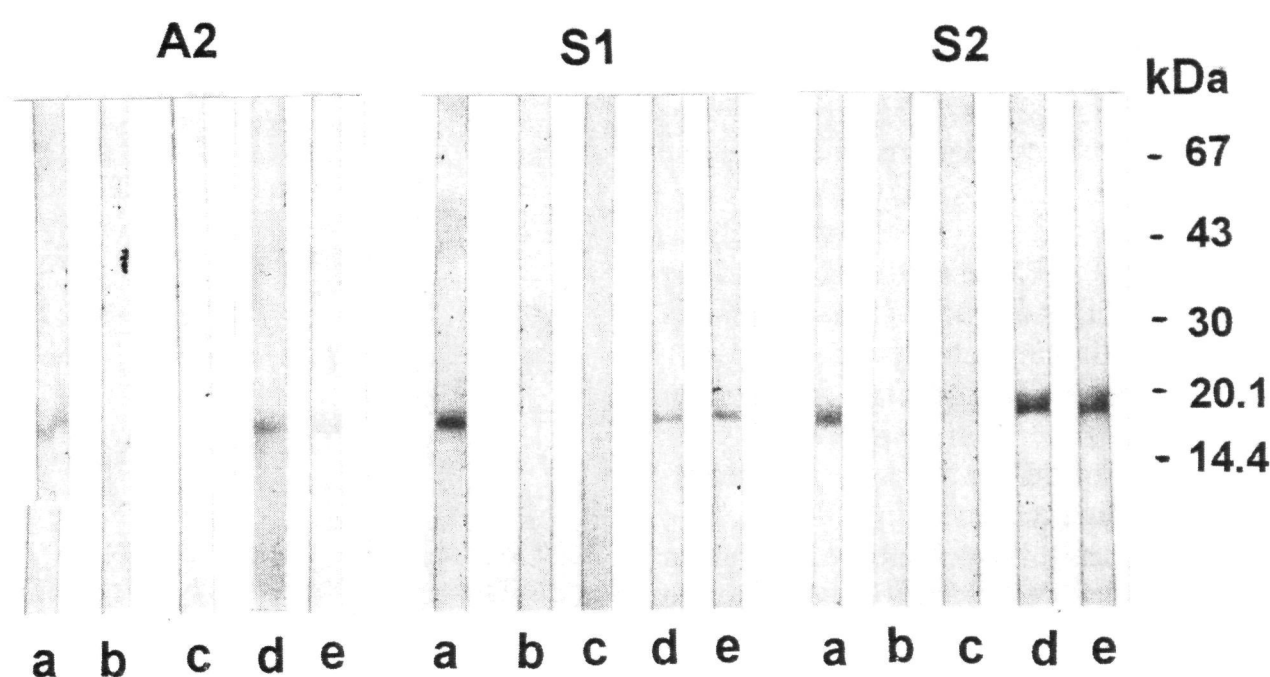


Fig. 5. Immunoblot of the apple extracts after 6-month storage in different stabilizing solutions. Lane a) PBS/glycerol; b) prick test solution; c) ascorbic acid/sodium citrate/ glycerol; d) cysteine/sodium citrate/glycerol; e) sodium benzoate/glycerol.

Table 1. Color of apple extracts after 6-month storage in different stabilizing solutions

Stabilizer	Extract A2		Extract S1		Extract S2	
	25 µg/ml	500 µg/ml	25 µg/ml	500 µg/ml	25 µg/ml	500 µg/ml
PBS/glycerol	Colorless	Yellowish	Yellowish	Yellowish	Colorless	Colorless
Prick test solution	Colorless	Brownish	Brownish	Dark brown	Colorless	Brownish
Ascorbic acid/sodium citrate/glycerol	Yellow	Dark yellow	Dark yellow	Dark yellow	Colorless	Yellowish
Cysteine/sodium citrate/glycerol	Colorless	Yellowish	Yellowish	Yellowish	Colorless	Colorless
Sodium benzoate/glycerol	Colorless	Yellowish	Yellowish	Yellowish	Colorless	Colorless

pared without enzyme inhibitors, polyphenol oxidase and/or proteinases obviously could inactivate the allergen extract during storage. In contrast, the high activity of extract S2 obtained in the presence of enzyme inhibitors remained preserved. Therefore, we conclude that beside storage temperature and the physical state of extracts the history of extract preparation might affect the stability of apple allergen remarkably.

For *in vivo* diagnostics, it is necessary to have allergen solutions whose activity is preserved over long

periods. In clinical practice, extracts in prick test solution are usually used. However, the prick test solution itself contains 0.4% phenol that apparently, like the endogenous phenols of apple, might interact with apple proteins involving polyphenol oxidase, possibly causing enzymatic browning (1, 7, 11–14). This seems to be the reason that the prick test solution was not suitable for stabilizing apple extracts (Table 2 and Fig. 5).

Sodium citrate and cysteine are widely used in the food industry as inhibitors of enzymatic browning (14, 15) and sodium benzoate as an antimicrobial agent. Correspondingly, the allergen activity of the extracts A2 and S2, prepared in the presence of enzyme inhibitors, was not remarkably lowered during storage in cysteine/sodium citrate/glycerol or sodium benzoate/glycerol (Table 2). However, sodium benzoate/glycerol caused nonspecific reactions in skin tests in some cases. Moreover, PBS/glycerol stabilized the allergen activity to a satisfactory extent, possibly due to the protein-conserving properties of glycerol (4, 5). Although ascorbic acid is also known to inhibit enzymatic browning (14), it was found not to be suitable to preserve the activity of apple allergen extracts during storage.

Table 2. ELISA inhibition of apple extracts in different stabilizing solutions. Values correspond to protein content for 50% inhibition in µg/ml

Stabilizer	Extract A2		Extract S1		Extract S2	
	Fresh	6 months	Fresh	6 months	Fresh	6 months
PBS/glycerol	1.0	1.0	0.5	0.8	0.2	0.4
Prick test solution	3.0	> 10	1.4	> 10	0.4	> 10
Ascorbic acid/sodium citrate/glycerol	> 10	> 10	> 10	> 10	0.3	1.0
Cysteine/sodium citrate/glycerol	1.3	1.8	0.8	1.2	0.3	0.4
Sodium benzoate/glycerol	1.0	1.0	1.2	2.0	0.4	0.4

It has to be mentioned that the enzyme inhibitors obviously present during extract preparation were sufficiently removed by dialysis. This could be inferred from the lack of any skin reactions in the *in vivo* skin tests with nonallergic persons.

When we compared the individual extract solutions with each other, it became evident that S1 (as in the case of storage of this extract in the lyophilized state) had the lowest allergen activity independently of stabilizing medium used (Fig. 4). Only in cysteine/sodium citrate could a relatively high activity be measured for this extract before storage, confirming the polyoxidase-inhibiting action of cysteine (8, 15).

Furthermore, our results show a close correlation between extract activity and extract color. Active extracts were colorless or yellowish, whereas the extracts that lost much activity changed their color more strongly. Thus, color changes might be regarded as a visual indicator of extract quality.

To summarize the results, we conclude that lyophilization and storage at -20°C are suitable conditions to preserve allergen activity in apple extracts. For storage of apple allergen solutions over longer periods, it seems to be necessary to add stabilizing agents. Mixtures of PBS/glycerol and cysteine/sodium citrate/glycerol were found to be the most effective stabilizing media.

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