

Improvement of fruit allergenic extracts for immunoblotting experiments

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A method based on ion-exchange column chromatography to enhance the protein content of fruit allergenic extracts was found to help make the subsequent SDS–PAGE immunoblotting assays possible; otherwise, they were difficult to achieve due to the high carbohydrate content. Fractionated extracts of apple, pear, and peach (peel and pulp) were obtained by anion-exchange chromatography (Q-SepharoseTM column), providing clear electrophoretic patterns which allowed IgE detection by enzymatic assays of the transferred membranes. This chromatographic method produced in one single step an enriched extract directly from the standard crude aqueous one, with an increment in the protein content of more than sixfold, on average; thus, it proved to be more suitable than the usual chemical fractionation procedures.

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The SDS–PAGE immunoblotting technique is a very useful tool for diagnosis and research in allergy. Since food and vegetable allergenic extracts contain large amounts of carbohydrate molecules and very little protein, these extracts have to be greatly concentrated in order to obtain detectable levels of protein in electrophoresis.

Concentration of protein by ultrafiltration, inverse dialysis, and the usual chemical precipitation procedures also causes an increase in sugar concentration which prevents electrical current flow inside the gel, giving rise to distorted protein patterns as a final result of electrophoresis techniques. As a consequence, IgE detection by immunoblotting is very poor or even impossible.

Eating certain fruits and vegetables causes allergic disorders, especially oral allergy syndrome (OAS). In these disorders, the involvement of IgE has been clearly demonstrated (1, 2). However, in studies performed to detect the allergens involved by means of IgE binding in SDS–PAGE immunoblotting, especially for the most frequently sensitizing Rosaceae fruits (apple, pear, and peach), the protein bands (and consequently IgE bands) are either undetectable (3, 4) or difficult to detect (4), or the SDS–PAGE pattern is simply not shown (5, 6).

Recently, Vieths et al. (7) have described a low-temperature, “acetone powder” method to obtain apple extracts which yields convenient extracts for electrophoretic assays, but involves complex homogenization of apples in acetone/dry ice at –60 to –65°C with further steps at this temperature.

The present work aimed to establish an easy method for the enrichment of fruit allergenic extracts in order to facilitate the evaluation of IgE-binding proteins (allergens) by immunoblotting.

Material and methods

Production of allergenic extracts

Apples, peaches, and pears were purchased at a local market; 1 kg of each was used for pulp extraction and 3 kg for peel extraction. First, the fruits were peeled, and the resulting pulp and peel were processed separately but by the same procedure.

Extraction was started by homogenization in a blade homogenizer (Waring type, Braun, Kronberg, Germany) (5 min, 20 000 rpm) and was followed by magnetic stirring (2000 rpm) at 4–8°C for 1 h, the Björkstén solution (Björkstén et al. [3]) being used as a solubilizing agent. This solution

consists of 10 mmol/l potassium phosphate buffer, pH 7.5, containing 2% suspended solid polyvinylpyrrolidone (PVPR), 2 mmol/l ethylenediaminetetraacetic acid disodium salt (EDTA), and 10 mmol/l sodium diethyldithiocarbamate (DIECA). All these components prevent the loss of allergenicity of the extracts, since, according to Björkstén et al. (3), they avoid the reversible combination of the cosolubilized phenolic compounds with proteins by hydrogen bonding, or the irreversible binding after first being oxidized to quinones. The extract was then clarified by centrifugation at 43 000 *g* for 30 min. The pellet was discarded, and the supernatant containing the solubilized proteins was dialyzed by diffusion in Visking tubing (Medicell, London, UK). The dialyzed extract was sterilized by filtration through a 0.22- μ m pore diameter membrane and freeze-dried. Average yields after this process were 1.4% for pulp and 4.1% for peel.

Ion-exchange chromatography

Anion-exchange chromatography was achieved by passing the fruit extracts (1.0 g) dissolved in 25 ml of 0.05 M HCl-Tris buffer, pH 8.8, through a 2.2 $\times$ 10 cm column of fast-flow Q-SepharoseTM (Pharmacia LKB, Uppsala, Sweden) equilibrated in the same buffer.

Stepwise elution was performed with a constant flow rate of 26 ml $\cdot$ cm⁻² $\cdot$ h⁻¹. Molecules which did not adsorb to the resin (mainly sugars) were firstly recovered by passing 100 ml of the starting buffer. Then, adsorbed proteins were eluted with 100 ml of 2 M NaCl, 0.05 M HCl-Tris buffer, pH 8.8. Collected fractions were evaluated for their protein and carbohydrate contents by the anthrone and dye-binding-assay (Bradford) methods, respectively (8, 9). The last eluted 2 M NaCl fraction was dialyzed, filtered, and freeze-dried until its further use in electrophoretic techniques.

Chemical fractionation methods

Ammonium sulfate precipitation was carried out by adding the salt to the extract solution up to 95% saturation. The precipitation process was left for 30 min at 4°C. The precipitated fraction was separated by centrifugation at 11 600 *g* for 10 min, and then redissolved in distilled water, dialyzed, and freeze-dried.

Acetone precipitation was achieved by adding five volumes of the organic solvent to the extract solution. The precipitation process was left for 1 h at 4°C. Recovering of the precipitated fraction was done as described for salt precipitation.

Attempts to remove the carbohydrate portion from the extracts were also carried out by metaperiodate treatment (10) with subsequent dialysis.

SDS-PAGE immunoblotting

SDS-PAGE was carried out by the method of Laemmli (11). Polyacrylamide concentrations of 12.5% and 3% were used for separating and stacking gels, respectively. Proteins dissolved in 0.125 M HCl-Tris, pH 6.8, were dissociated with 0.1% SDS and 5% β -mercaptoethanol by treatment at 100°C for 5 min. An amount of 20 μ g protein estimated by Bradford's method (9) was applied per lane. Nine different samples were separated by SDS-PAGE: six fruit extracts (apple, pear, and peach; pulp and peel) and three pollen extracts (*Artemisia vulgaris*, *Lolium perenne*, and *Betula verrucosa*).

After electrophoresis, gels were stained by diffusion in 0.1% Coomassie brilliant blue R-250 dissolved in methanol/acetic acid and distilled water (4 : 1 : 5). Destaining was also performed by diffusion in the same mixture without dye. Separated protein bands were electrophoretically transferred to Immobilon-P membranes, essentially by the method of Towbin et al. (12). Then, IgE-binding proteins were immunochemically revealed by first blocking the Immobilon membrane with 9% defatted dry milk in Tris-borate saline (TBS) (1 h at 37°C). After three washes with TBS, the blots were incubated with 0.5 ml of undiluted human serum (16 h at 4°C). The blots were washed three times with TBS and incubated with 0.5 ml for 1 : 25 peroxidase-conjugated rabbit immunoglobulins to human ϵ -chains (DAKO, Glostrup, Denmark). Bound peroxidase was detected by the addition of 3 ml of freshly prepared 0.06% w/v of 4-chloro-1-naphthol solution (Bio-Rad, Richmond, CA, USA) and 0.01% H₂O₂ in TBS. After 30 min, the blots were rinsed in distilled water and air-dried. Antigen-antibody binding was identified by purple stain deposition (13).

A serum pool was used to reveal the IgE-binding components of fruit extracts. It was formed by combining equal aliquots of sera from 10 patients allergic to Rosaceae fruits and pollens, showing positive skin tests to the nine extracts used in the SDS-PAGE immunoblotting. The resulting serum pool presented the following RAST values: 4.44, 8.02, 15.76, >17.5, 4.54, 5.04, 16.50, 10.90, and >100 PRU/ml to apple peel, apple pulp, pear peel, pear pulp, peach peel, peach pulp, *B. verrucosa* pollen, *A. vulgaris* pollen, and *L. perenne* pollen, respectively.

SDS-PAGE and SDS-PAGE immunoblotting patterns were evaluated by image analysis in the Bio-Image System (Millipore, Bedford, MA, USA).

The system allows a high performance digital scanning of gels and membranes at a resolution of 600 dots per inch, which can be processed by the Whole Band Analyzer program. The relative mobility of a set of protein standards (LMW Calibration Kit, Pharmacia LKB, Uppsala, Sweden) was plotted logarithmically against their mol. wt., and the resulting curve was used for estimating the mol. wt. of the resolved bands in the different samples analyzed. Quantification of bands was expressed in terms of integrated intensity, defined as the volume of a band calculated by summing the volume of each pixel within the boundaries of a band (area $\times$ height) and subtracting the background for each pixel.

Results

The effectiveness of the enrichment of protein content in fruit extracts after anion-exchange chromatography is shown in Tables 1 and 2, which present the protein and carbohydrate contents of conventional and purified extract (after ion-exchange chromatography) for the six extracts. The increase in protein percentage (with respect to the total lyophilized extract) ranged from 3.6-fold for peach peel to 10.0 for apple pulp (average: 6.2-fold). This process caused the loss of a small amount of basic proteins ($pI > 8.8$) which were eluted together with the sugars in the unretained fraction. The loss was lower than 3% of the total protein content in all the cases.

Classic chemical precipitation methods were tested on the peach extracts; the improvement obtained was clearly lower than with the chromatographic procedure (Table 3).

The ion-exchange fractionated extracts were subjected to SDS-PAGE (Fig. 1), giving rise to clearly detectable bands after use of Coomassie brilliant blue R-250, bands that could not be detected in the nonfractionated extracts (not shown). The protein pattern was evaluated with the Bio-Image System (Millipore), yielding the results that are expressed in Table 4. From 10 to 20 different bands could be resolved for the extracts.

The protein pattern obtained in SDS-PAGE proved to be clear enough to obtain an IgE-binding scheme after SDS-PAGE immunoblotting of a serum pool from patients allergic to Rosaceae fruits and pollen (the same serum pool for the incubation of the nine extracts), as shown in Fig. 2B. No IgE-binding bands could be detected in the SDS-PAGE immunoblotting of the conventional (nonchromatographically fractionated) fruit extracts after incubation with the same serum pool (Fig. 2A). In Fig. 2B, the intensity of IgE binding can be compared with that of some pollen extracts

Table 1. Improvement of protein content in fruit extracts, estimated by Bradford's method

Source (allergen)	Percentage of protein in freeze-dried material		Improvement (-fold)
	Conventional extract	Chromatographed extract	
Apple peel	1.52	11.30	7.4
Apple pulp	0.25	2.54	10.0
Pear peel	1.47	6.05	4.1
Pear pulp	0.58	2.80	4.8
Peach peel	0.72	2.62	3.6
Peach pulp	0.37	2.60	7.0
Average improvement: 6.2-fold.			

Table 2. Carbohydrate content in freeze-dried extract, estimated by anthrone method

Source (allergen)	Percentage of carbohydrates in freeze-dried material		Decrease (-fold)
	Conventional extract	Chromatographed extract	
Apple peel	32.0	0.4	80.0
Apple pulp	79.8	5.5	14.5
Pear peel	28.7	3.8	7.5
Pear pulp	48.2	4.2	11.4
Peach peel	17.4	2.7	6.4
Peach pulp	58.1	7.5	4.5

Table 3. Protein content* of peach extracts fractionated by various methods

	Source	
	Peach peel %	Peach pulp %
Conventional extract	0.72	0.37
Chromatographic extract	2.62	2.60
Ammonium sulfate fractionated	0.41	0.25
Acetone fractionated	0.54	0.35
Metaperiodate (treatment)	1.47	0.78

* Protein estimated by Bradford's method.

such as *B. verrucosa*, *A. vulgaris*, and *L. perenne*, all of them showing high protein content in the conventional aqueous form with values of 20–40% (weight of protein with respect to weight of freeze-dried material) by Bradford's method. No bands were detected with a pool of negative control sera, thus indicating the specificity of the IgE-binding bands.

Discussion

The results presented in this paper demonstrate how a conventional allergenic extract with high carbohydrate content (which causes difficulties

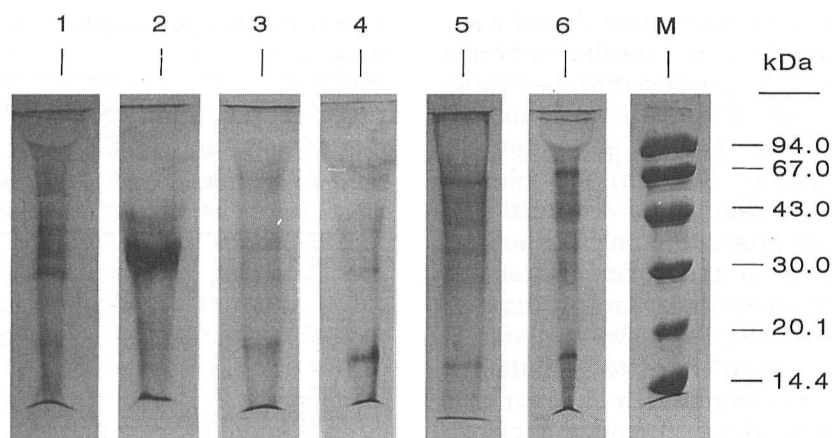


Fig. 1. SDS-PAGE of fruit allergenic extracts after Coomassie brilliant blue R-250 staining. Lane 1) apple peel; 2) apple pulp; 3) peach peel; 4) peach pulp; 5) pear peel; 6) pear pulp; M) mol. wt. marker.

Table 4. SDS-PAGE protein bands of fruit allergenic extracts fractionated by ion-exchange chromatography

Pear peel		Pear pulp		Apple peel		Apple pulp		Peach peel		Peach pulp	
kDa	%I*	kDa	%I*	kDa	%I*	kDa	%I*	kDa	%I*	kDa	%I*
124.4**	44.4	128.1**	16.9	146.4**	15.1	152.9**	41.6	131.1**	26.0	139.8**	15.6
115.3**	5.4	116.0**	5.6	134.3**	2.1	42.9	2.8	123.7**	5.2	67.4	8.1
67.4	1.3	83.6	5.7	94.0	8.6	41.6	2.4	62.1	2.4	40.7	2.2
57.3	16.4	63.4	7.0	69.5	4.9	39.0	0.7	59.1	5.6	36.6	6.3
49.0	4.5	60.6	5.7	62.3	3.5	35.2	1.5	40.7	0.6	33.6	5.1
46.8	5.0	51.8	4.9	55.5	1.6	32.2	10.4	36.2	1.6	17.2	43.3
42.7	1.2	47.6	2.8	46.3	1.4	30.0	3.6	34.2	3.2	15.2	3.0
40.0	4.0	45.4	2.0	44.4	1.3	21.0	2.2	27.2	2.4	13.5**	16.4
33.1	4.9	42.6	4.7	40.6	4.7	15.9	2.3	18.8	2.0		
29.5	0.8	40.4	1.5	36.8	5.7	13.5**	32.4	18.3	11.2		
26.0	0.9	37.1	3.4	35.1	1.5			17.6	1.8		
23.8	0.6	33.7	2.3	33.4	3.6			17.0	2.8		
17.2	2.2	30.9	3.6	31.0	7.9			13.1**	11.4		
16.5	7.1	29.2	4.0	30.0	8.1			13.0**	23.7		
13.8**	1.0	27.2	0.7	24.9	3.0						
12.5**	0.4	17.5	10.9	18.5	5.6						
		16.2	1.7	15.4	3.6						
		15.4	1.2	13.2**	17.6						
		14.6	1.3								
		12.9**	14.1								

* Percentage of integrated intensity of each band with respect to whole lane optical density.

** Extrapolated values.

during the electrophoretic analysis) can be improved in a single step of column chromatography, thus allowing the detection of IgE-binding bands, as is indeed necessary for diagnosis and research purposes. This is the case for fruit extracts, especially those of the Rosaceae family, such as apple, peach, and pear, which are important food allergens, causing mainly oropharyngeal symptoms, and being frequently associated with pollinosis (6, 14).

Anion-exchange chromatography was carried out in a column of medium semipreparative scale with a soft resin as support (Sephacrose), to which a strong exchanger group, positively charged, was

covalently bound (Q-Sepharose, Pharmacia AB, Sweden). In this way, the negatively charged molecules of the extract, at the pH of starting buffer, are retained inside the column, while the positive or neutral molecules pass straight through the column. Most of the interfering carbohydrate molecules of the extracts are neutral sugars; therefore, the retained fraction, once eluted with sodium chloride, contained a higher ratio of protein than the total freeze-dried mass (Table 1). The presence of carbohydrate molecules remaining in the 2 M NaCl eluted fraction (Table 2) can be explained by the occurrence of charged sugars (e.g., aminated) and the association with protein molecules as pros-

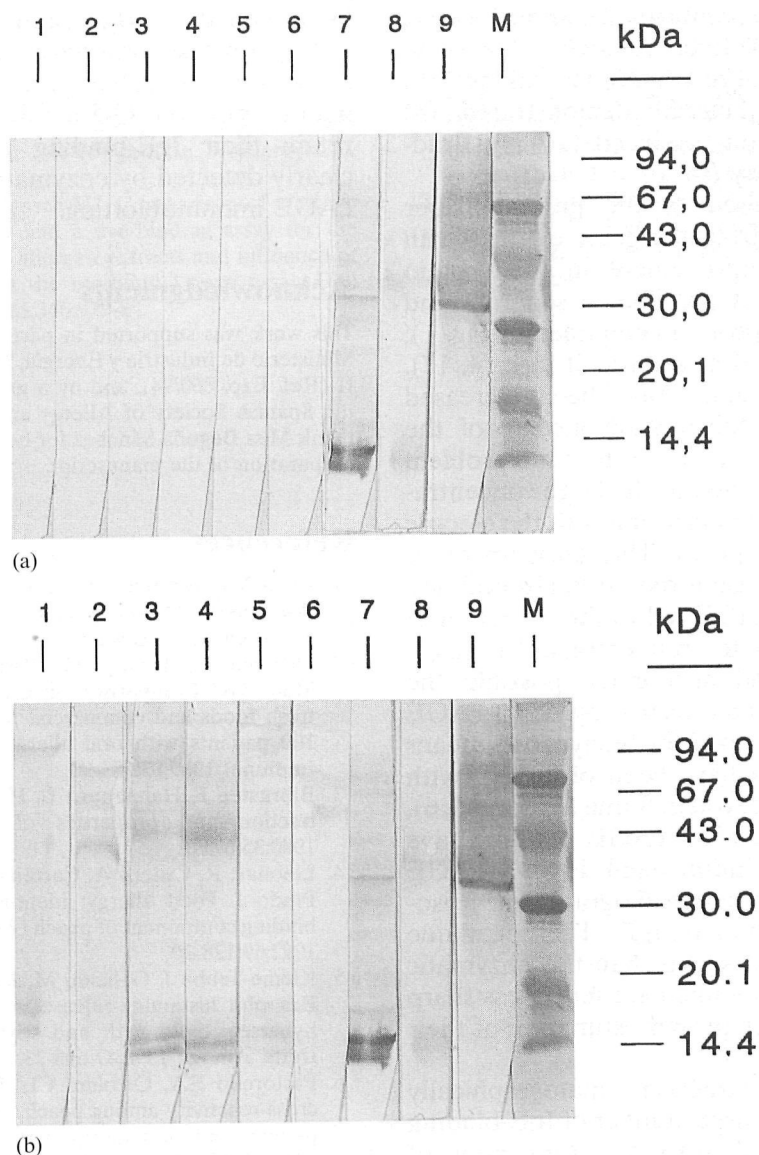


Fig. 2. SDS-PAGE immunoblotting of fruit extracts with serum pool from patients allergic to Rosaceae fruits and pollen. Lane 1) apple peel; 2) apple pulp; 3) pear peel; 4) pear pulp; 5) peach peel; 6) peach pulp; 7) *Betula verrucosa* pollen; 8) *Artemisia vulgaris* pollen; 9) *Lolium perenne* pollen; M) mol. wt. marker stained with amido black 10B. Panel A corresponds to conventional (nonfractionated) fruit extracts, and panel B to chromatographically enriched ones.

thetic groups. When the ion-exchange process was attempted in the batch mode instead of the column one, the degree of protein enrichment reached was lower (data not shown). The Q-Sepharose chromatographic step caused only minimal loss of the protein content of the extracts, representing less than 3% of the total protein composition, on average. This loss due to basic proteins of pI higher than 8.8 was not relevant if we take into consideration the fact that allergens are most frequently acidic proteins and also that no basic allergens have been reported for these fruit extracts in the other studies when protein fractionation was achieved by other means (7). Moreover, in the present work,

negative values were found by RAST and SDS-PAGE immunoblotting with the pooled serum from fruit-sensitized patients, while the Q-Sepharose column retained basic proteins from fruit extracts as solid phases.

Chromatography was clearly superior to chemical fractionation with both peach extracts, according to the protein contents estimated by Bradford's method (Table 3). The ion-exchange-processed extracts reached 2.6% of protein level followed by the ones subjected to metaperiodate treatment, which showed approximately twofold improvement in protein content. Salt and organic solvent precipitation did not enrich the protein content of

the extracts. Protein percentages shown in Tables 1 and 3 should be considered relative values with which to compare different methods, taking into account the known and already demonstrated (15) protein underestimation produced (16) by Bradford's dye-binding assay (9).

The method proposed in the present paper allowed us to obtain fruit allergenic extracts with an average sixfold improvement in the protein content, thus making it possible to separate and detect their different protein components (Fig. 1); this had been described as a difficult task (4, 17). Obviously, detection could have been increased about 50-fold with a Silver stain instead of the Coomassie brilliant blue one, but the main problem was distortion of bands due to the large concentration of sugar molecules interfering with the electric flow through the gel pores. This enrichment of protein content which gave rise to horizontal and sharp bands in SDS-PAGE led to the detection of up to 20 bands in some fruit extracts by image analysis (Table 4) and also made possible the detection of IgE-binding bands by SDS-PAGE immunoblotting developed by enzymatic means (Fig. 2) that could not have been observed with the conventional fruit extracts. Some authors (5, 6), who did not show the SDS-PAGE pattern, have obtained intense IgE-binding bands in SDS-PAGE blottings developed by autoradiography with isotopically labeled antihuman IgE. This technique gives a more amplified signal than the enzymatic one, albeit the bands obtained are much less sharp and definite, preventing proper estimation of their molecular weights.

The fruit allergenic extracts chromatographically enriched gave rise to a large number of IgE-binding bands when incubated with a serum pool of patients allergic to fruit and pollen (Fig. 2).

The clinical significance of such bands does not lie within the scope of the present methodological paper, and discussion of that aspect will be published elsewhere. In Fig. 2, three immunoblotting lanes corresponding to pollen extracts can be seen to illustrate the comparison of IgE-binding intensity with allergenic extracts of high protein content.

Recently, a method has been described to overcome the problem of electrophoretic analysis of fruit extracts: the procedure of Vieths et al. (7). This method consists of the preparation of low-temperature acetone powder extracts, requiring sophisticated laboratory equipment for homogenization of raw materials (fruits).

Other authors have postulated the requirement of adding enzyme inhibitors to obtain fruit allergenic extracts with preserved allergen activity (4, 18). However, the results shown in the present paper demonstrate that fruit allergenic extracts can

be obtained by Björkstén's method (3) without adding protease inhibitors, and that the resulting extracts can be enhanced by anion-exchange chromatography on Q-Sepharose columns and still retain their IgE-binding properties, as can be clearly detected by enzymatically developed SDS-PAGE immunoblotting.

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