

A method for the analysis of milk and egg allergens for the atopy patch test

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Abstract: The patch test with food antigens (atopy patch test, APT) has been reported as a more specific method than prick or RAST for the early detection of cow's milk and/or egg sensitizations in children. Standardization of APT extracts is a major issue on the road towards full clinical exploitation of this assay. Here, we used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to characterize sensitivity and specificity of commercial preparations of APT for milk and egg allergies, which are expected to improve the reliability of this test, when compared with fresh

food allergen sources. We found that: (i) SDS-PAGE is an appropriate technique for quality control of APT and (ii) commercial milk and egg APT are equivalent to fresh food preparations in terms of allergen content. Clinical trials aimed at characterizing sensitivity and specificity of APT in the diagnosis of food allergy in children will benefit from this technique.

Key words: allergy – atopy patch test – egg allergens – milk allergens – SDS-PAGE

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Background

The atopy patch test (APT) is from many aspects an innovative approach in the diagnosis of food allergies in children. In principle, APT allows the study of the presence of delayed, T-cell-dependent reactivity to allergens, with clinical expression in the skin (1–4). However, this technique, which has been in clinical practice for more than a decade (5) and represents in principle a useful *in vivo* diagnostic tool, has been criticized for its poor reproducibility (6, 7).

The working hypothesis underlying APT usage is that this test unveils delayed reactions to food allergens, similarly to specific IgE determination in the case of immediate type allergic reactions. However, there is controversy about this assumption, and a lack of association between delayed reactions to oral food challenge and positive APT was reported (1, 8). Another open issue is the lack of standardization of this technique, both in food preparation and in the concentration of the material used before application on the skin. In particular, the usage of fresh foods brings

about the issue of possible contamination by heterologous proteins. Recently, with the aim to improve standardization of this test and to reduce side effects observed with fresh food preparations, commercial kits for APT have been made available, which were prepared with freeze-dried proteins from the specific allergen sources (9). No data are available on the purity of these preparations, particularly on allergen content and on the presence of possible contaminants.

Questions addressed and experimental design

Here, we used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate whether commercial preparations of APT (Allergon AB, Angelholm, Sweden and ALK Abellò, Milan, Italy) for milk and egg allergens could be proficiently compared with fresh food allergen sources in terms of relative protein distribution and purity. Details of the allergen extracts we used for SDS-PAGE, of the electrophoretic analysis and of the statistical evaluation of results are reported in the supplementary file.

Abbreviation: APT, atopy patch test.

Results

We found that SDS-PAGE profiles of milk APT and fresh milk showed four main bands, which were identified, based on their electrophoretic run, as α -casein, β -casein, β -lactoglobulin and α -lactalbumin (Fig. 1). The detection of κ -casein was not possible, due to its relatively limited quantity (10). Equal electrophoretic mobility of corresponding bands was observed in compared samples. Moreover, analogous minor protein bands were also visible, such as bovine serum albumin (BSA).

The distributions of signals in the four main co-migrating bands from milk APT and from fresh milk were visually equivalent. This was confirmed using quantitative densitometric image analysis (supplementary Fig. S1). In the case of egg protein analysis, due to the more complex protein content, we made preliminary gels to co-identify bands corresponding to the most relevant major allergens. To this aim, commercially available purified ovalbumin, ovomucoid and lysozyme were run in parallel with fresh egg fractions, permitting a comparison between the electrophoretic runs and/or the molecular weight of any single band. Four major egg allergens (supplementary Table S1) were identified in fresh egg white and in whole egg preparation (supplementary Fig. S2). Subsequently, SDS-PAGE of egg APT, run in parallel with fresh egg white, allowed detecting four main bands, which were identified as ovotransferrin, ovomucoid, ovalbumin and lysozyme (Fig. 2). The electrophoretic runs of bands corresponding to these major allergens were then analysed using densitometry in

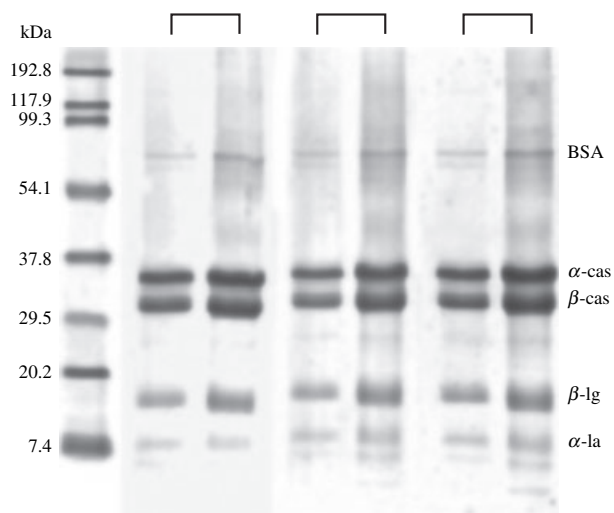


Figure 1. SDS-PAGE of milk APT products (left lane of each pair) compared with fresh cow's milk (right lane of each pair). Electrophoretic runs were from three independent analyses. α -Cas: alpha-Casein, β -Cas: beta-Casein, β -Lg: beta-Lactoglobulin, α -La: alpha-Lactalbumin, BSA: Bovine serum albumin. Pre-stained MW standards are displayed in the left most lane.

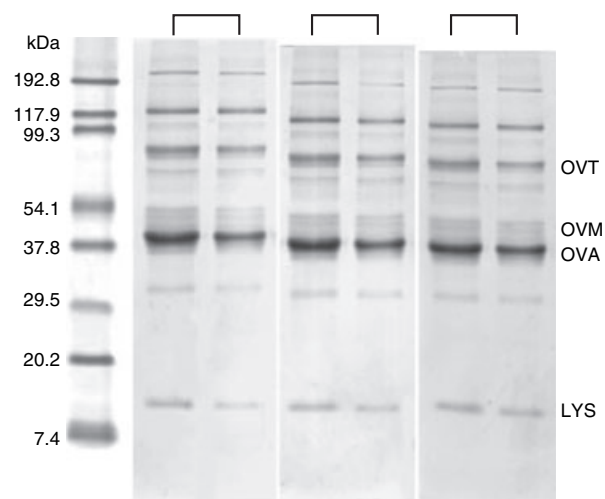


Figure 2. SDS-PAGE of the egg APT product (left lane of each pair) compared with fresh whole egg (right lane of each pair). Electrophoretic runs were from three independent analyses. OVA: ovalbumin, OVM: ovomucoid, OVT: ovotransferrin, LYS: lysozyme. Pre-stained MW standards are displayed in the left most lane.

the compared samples. In both samples, other minor protein bands were also visible, but they were equally represented in the compared samples. No extra bands were visible in the egg APT sample, when compared with the fresh egg preparation. The distributions of the bands corresponding to the main allergens from egg APT and from fresh egg white were visually equivalent. This was confirmed using quantitative densitometric image analysis (supplementary Fig. S3).

Conclusions

Thus, commercial, freeze-dried preparations of APT for milk and egg allergies can be critically analysed qualitatively and quantitatively using the SDS-PAGE method we describe. We suggest that manufacturers of APT use this approach as a standard quality control procedure. Our study also demonstrates that the freeze-dried preparations we considered were fully satisfactory in terms of their antigenic content.

Currently, EAACI/GA2LEN recommendations still suggest the usage of fresh food as preferred reagents (11), whereas recombinant allergens may have a role in the future. However, difficulties in standardization of preparatory procedures are well known (12). In this context, presently available commercial extracts may represent useful tools for the physician, as they warrant higher stability, better reproducibility of results and a batch-to-batch consistency (9). Moreover, as products of pharmaceutical grade, they are expected to reduce the risk of bacterial contamination, which is quite frequent in the preparation of fresh

food for APT. This is particularly relevant taking into consideration a recent report showing that colonization with exotoxin-producing bacteria may influence the outcome of patch tests in patients with atopic dermatitis (13). The procedure we used sets the stage for obtaining reproducible clinical results when using atopy patch test.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

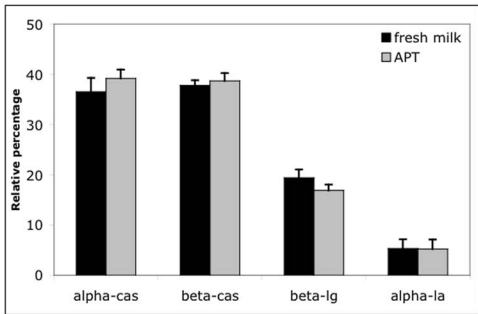
Figure S1. Relative distribution of four main milk allergens in APT product versus fresh milk.

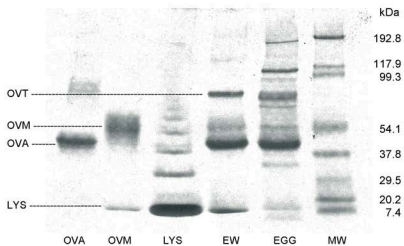
Figure S2. SDS-PAGE of fresh whole egg (WE) compared with fresh egg white (EW).

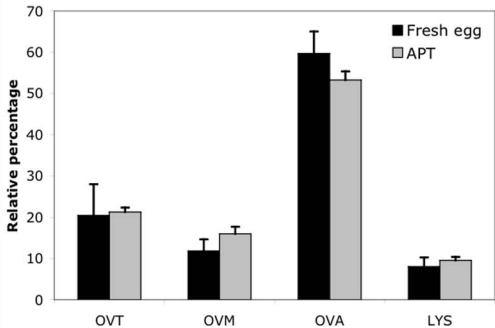
Figure S3. Relative distribution of four main egg allergens in APT product versus fresh whole egg.

Table S1. Major egg allergens (2).

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SUPPLEMENTARY MATERIAL

METHODS.

Allergens.

Purified egg proteins were from Sigma-Aldrich Fine Chemicals (Milan, Italy) at the highest available purification level (lyophilized powder, minimum 95% protein by electrophoresis). The 20% w/v freeze-dried APT protein preparations (in vaseline as carrier), which were used in this study are produced from Allergon AB (Ängelholm, Sweden) and are distributed as Rapid Patch Test by ALK Abellö (Milan, Italy)(egg: code 7053, batch 705305001; milk: code 7054, batch 705406001).

The APT products and the purified proteins were treated with sample buffer (containing 0.125M TRIS-HCl pH 6.8, 3,7% glycerol, 1% SDS, 2.5% β -mercaptoethanol) and heated in boiling water for 10 min at 100°C. Egg and milk APT reagents were diluted in sample buffer at the final concentration of 4 mg/mL and 1 mg/mL, respectively

Fresh products were treated as follows:

- a) fresh full cow's milk was diluted 1:10 (v/v) in sample buffer
- b) homogenized whole hen's egg was diluted to a final concentration of 4 mg/mL.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were loaded and separated on a polyacrylamide gel with the following characteristics:

Gradient running gel: 15% acrylamide; 0.4% bis-acrylamide; 0.36 M TRIS (Tris-hydroxymethyl amino methane)-HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylenediamine (TEMED)

Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS-HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% (TEMED).

Running buffer: 25mM TRIS, 0.19M glycine and 0.1% SDS (w/v), pH 8.8.

The electrophoretic runs were controlled by using a broad range molecular weight standard marker solution purchased from Bio-Rad (Richmond CA, USA). It contained: rabbit skeletal myosin muscle (192.8 kDa); *Escherichia coli* (beta

galactosidase (117.9 kDa); Bovine serum albumin (99.3 kDa); chicken egg ovalbumin (54.1 kDa); bovine carbonic anhydrase (37.8 kDa); soybean trypsin inhibitor (29.5 kDa); chicken egg lysozyme (20.2 kDa); bovine pancreas aprotinin (7.4 kDa).

After the electrophoretic run (90V at room temperature, for approximately 6h) gels were dyed with Coomassie Brilliant Blue G-250 according to the method of Neuhoff *et al.* (1). All materials and instruments were purchased from Bio-Rad (Richmond CA, USA).

Image analysis.

Quantitative analysis of protein bands was performed by a gel scanner (Sharp JX-330, Pharmacia Biothec) and the Image MasterTM 1D Software. It allows the quantification of proteins by calculating the average density of pixels across the band length and integrating over the band width. Each sample was analyzed three times.

With this approach, we first calculated the proportion of each band with respect to the total amount of signal of the four considered allergens in each sample, taken as unit (supplementary figures 1 and 2 for milk and egg allergens, respectively). Then, the relative abundance of each protein from APT was compared with the corresponding value from fresh allergen. No significant differences were observed between corresponding bands in compared samples, in a set of three experiments ($p > 0.10$) (supplementary figure 1)

Statistical analysis

The significance of the difference between the mean values was calculated by analysis of variance (ANOVA) and then by t-test (Graph Pad InStat for MacIntosh).

SUPPLEMENTARY TABLE 1.
Major egg allergens (2)

PROTEIN	CODE	MW	% of white egg proteins (3)
<i>Ovotransferrin</i>	Gal d 3	80 kDa	13.0
<i>Ovomucoid</i>	Gal d 1	28 kDa	11.0
<i>Ovalbumin</i>	Gal d 2	45 kDa	54.0
<i>Lysozyme</i>	Gal d 4	14 kDa	3.5

LEGENDS FOR ILLUSTRATIONS

SUPPLEMENTARY figure 1

Relative distribution of four main milk allergens in APT product versus fresh milk.

The quantification of the relative amount of milk allergens (see legend to figure 1) was performed by attributing a unit value to the sum of the four considered allergens from each of the two sources. Columns represent the mean + SD of three independent experiments.

SUPPLEMENTARY figure 2

SDS-PAGE of fresh whole egg (WE) compared with fresh egg white (EW).

For the identification of single molecular allergens, the following purified proteins were run in parallel.

OVA = ovalbumin	OVM = ovomucoid
OVT = ovotransferrin	LYS = lysozyme
MW = pre-stained MW standard	

SUPPLEMENTARY figure 3

Relative distribution of four main egg allergens in APT product versus fresh whole egg.

The quantification of the relative amount of egg allergens (see legend to supplementary figure 2) was performed by attributing a unit value to the sum of the four considered allergens from each of the two sources. Columns represent the mean + SD of three independent experiments.

REFERENCES IN SUPPLEMENTARY MATERIAL SECTION

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