

Optimized allergen extracts and recombinant allergens in diagnostic applications

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Due to inherent enzyme activities and other factors, allergen extracts from fruits, vegetables and other plant foods often lack sufficient biological activity (1–4). This lability also leads to consistency problems with different lots of commercial extracts, or to large variations between extracts from different manufacturers. In certain cases, special extraction procedures are required to obtain extracts that are suitable for *in vitro* and *in vivo* diagnosis (4–6). Although this problem has been known for more than 20 years (7), this article will show that some problems still need to be solved. In general, allergen extracts from plant materials contain many glycoproteins with N-linked glycans that bind IgE in a subgroup of patients sensitized to pollen and food. Some authors think that these IgE responses are clinically irrelevant (8, 9), whereas others have shown that IgE responses specific for these so-called cross-reactive carbohydrate determinants (CCD) are frequently present in patients with adverse reactions to certain foods, and can be the unique detectable IgE response to these foods in such patients (10–12). Whether or not anti-glycan IgE is able to trigger allergic symptoms still needs to be answered. However, specific IgE responses in allergic patients are undoubtedly linked to the pathomechanism of the disease and should be taken into consideration. Diagnostic tools to pick up anti-CCD antibodies have been developed (10, 11) and are useful to highlight whether IgE reactivity to an allergen extract is caused by protein allergens or by

carbohydrate moieties. In this context, recombinant food allergens produced in bacteria that completely lack N-glycosylations may not only be useful to capture anti-protein IgE responses, but also to proceed from an extract-specific diagnosis to a component-resolved diagnosis (13). This has been achieved with inhalant allergens, including complex systems such as grass pollen (14), and initial studies have been published for foods such as peanut (15) and pear (13). Component-resolved diagnosis may also offer the opportunity to distinguish between IgE directed against “mild” and “severe” food allergens (9). In principle, the panel of recombinant or purified natural allergens used to replace the extract should be as complete as possible, and the recombinant compounds should closely resemble the IgE reactivity of their natural homologues. Here we report examples selected from the area of pollen-related food allergy that address the potential of optimized allergen extracts and recombinant allergens in the determination of food-specific IgE antibodies.

Cherry

Recently, we have studied 101 patients with a history of pollen-related allergy to cherry (16). All patients displayed IgE antibodies to birch pollen in skin test or serological assays. Eighty-three percent of the patients had oral allergy syndrome (OAS), with itching and swelling of the lips, tongue, and throat, when eating

fresh cherries. Seventeen percent of the patients had experienced systemic reactions. In the CAP assay, only 20% of the patients were IgE-positive to cherry, with nine class 1, and 11 class 2 results (Table 1). This result prompted us to retest the sera in an Enzyme Allergo Sorbent Test (EAST) with paper disks coated with a low temperature acetone powder extract prepared in our laboratory. In this assay, 97% of the sera were IgE-positive, including two class 4, 21 class 3, and 59 class 2 results. Extract quality was suspected as the reason of the observed discrepancy between the two assay systems. Since the extract used for preparation of the ImmunoCAPs was not available for further analysis, the low temperature extract prepared from whole cherries, a cherry peel extract prepared according to the method of Björkstén (7) and a commercial cherry prick test solution were separated in SDS-PAGE, and the gel was silver stained (Fig. 1). While the low temperature extracts contained many distinct protein bands in the mass range between 10 kDa and about 94 kDa, the proteins in both the peel extract and the prick test solution appeared to be strongly degraded.

Four cherry allergens have been characterized: The Bet v 1-related major allergen, Pru av 1 (17), a thaumatin-like protein, Pru av 2 (18), the profilin Pru av 4 (19), and a lipid transfer protein (LTP) (16). By IgE immunoblotting, we were able to show that extract from fresh cherries contained all relevant allergens, whereas neither Pru av 1 nor Pru av 4 were present in the prick test solution. The peel extract contained low amounts of Pru av 1 and Pru av 4, and a dominant LTP band. Due to its high stability (20), allergenic LTP was present in all three extracts (data not shown). Comparison of EAST data with the CAP results indicated that the major allergenic compound was not present on the ImmunoCAP. To identify the major cherry allergen in our patient group, an allergen profile was recorded by EAST using allergen disks coupled with recombinant (r) Pru av 1, Pru av 4, and cherry lipid transfer protein (Table 1). Ninety-four percent of the patients were sensitized to Pru av 1, 16% to cherry profilin Pru av 4, and only 3% to the cherry LTP. For three patients without IgE reactivity to the extract, EAST classes ranging from 1 to 2 were obtained with the recombinant allergens. In three subjects with IgE reactivity to the fruit extract, no specific IgE to the recombinant allergens was detectable. Since Pru av 1 was identified as the dominant major cherry allergen in our study population, we conclude that, similar to the prick test solution, a reduced Pru av 1 activity most likely caused the difference between CAP and EAST.

Exotic fruits

In addition to the latex-fruit syndrome, allergy to exotic fruits occurs in a small subgroup of pollen allergic

Table 1. Serological data of 101 patients with pollen-related allergy to cherry. Number of patients with a positive assay are given

Class	EAST cherry				CAP Cherry
	Extract	rPru av 1	rPru av 4	rLTP	
0	3	6	85	98	81
1	16	12	4	0	9
2	59	56	10	1	11
3	21	22	2	2	0
4	2	5	0	0	0

patients (21). Banana and pineapple were selected for optimization of extracts, as preliminary investigations indicated a major problem of extract quality with these fruits. Since the major protein components of pineapple are bromelain and other cysteine proteinases, commercial pineapple extract displayed a slightly degraded protein pattern when analyzed by SDS-PAGE (Fig. 2, lane 3). Even stronger degradation of high molecular weight proteins was observed with our low temperature acetone powder extract from pineapple (Fig. 2, lane 2). Significant improvement was achieved by the addition of the E64 cysteine protease inhibitor (Boehringer Mannheim, Mannheim, Germany) (Fig. 2, lane 1), but owing to the toxicity of this compound, such extracts are not suitable for *in vivo* applications. By immunoblotting, several of the high molecular weight bands in pineapple extract were identified as IgE-reactive, indicating the necessity to prevent degradation of these proteins (results not shown). In the case of banana, problems occurred both with simple buffer extraction and with the acetone powder method. Again, commercial prick test solutions presented a very poor protein pattern (Fig. 3, lanes 2–3). In the low temperature method, the main problem was gel formation of the solid material during extraction, most likely caused by polysaccharides, and leading to a very low yield of soluble protein (less than 200 µg/ml). Similar problems were observed with other fruits such as plum or strawberry. Addition of a food-grade pectinase (ROHAPECT® B5L, Röhm, Darmstadt, Germany, 1:10000) and treatment for 1 h during extraction resulted in a twofold increase of the protein yield from banana, and led to an extract that was well suitable for SDS-PAGE and contained more than 20 distinct protein bands (Fig. 3, lane 1). For the clinical part of the study, we selected 17 patients with a case history of banana allergy and 20 patients who complained about adverse reactions to pineapple. Fifteen of the 17 patients were positive in the banana CAP, and 12/20 had specific IgE to pineapple extract in this assay. Eight patients from each group had a positive CAP to Bet v 2, suggesting that profilin was an important cross-reactive allergen in these fruits. IgE immunoblotting was carried out with the optimized extract preparations, resulting in 12/17 and 13/20 of patients showing IgE-reactivity to bands in banana

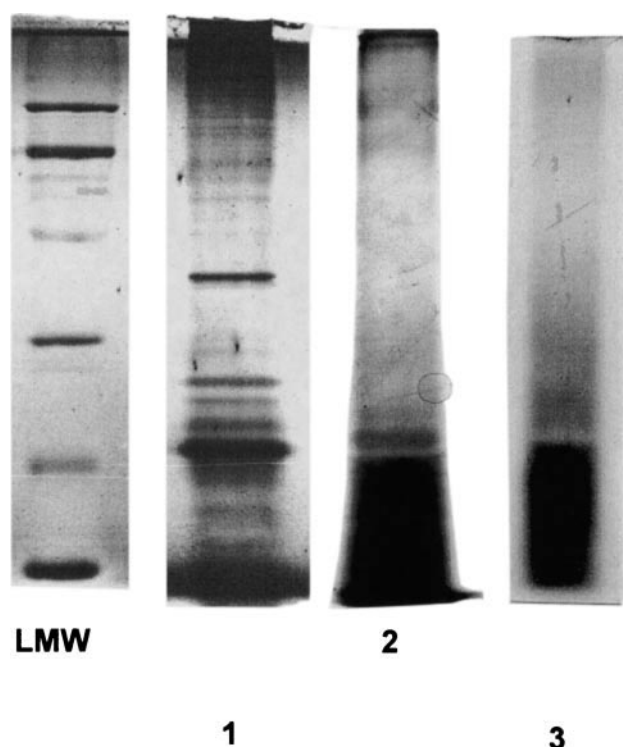


Figure 1. SDS-PAGE of allergen extracts from cherry. The gel was silver stained. Lane 1: extract prepared from whole fresh cherry by a low-temperature acetone powder method (5), lane 2: cherry peel extracted according to the method of Björkstén et al. (7), lane 3: commercial prick test solution. LMW: low molecular weight marker proteins (14.4, 20.1, 30.0, 43.0, 67.0 and 94 kDa).

extract and pineapple extract, respectively. Since the recognition of profilin on fruit extract immunoblots was weak (not shown) recombinant banana profilin and pineapple profilin were cloned by a PCR strategy, leading to significantly improved detection on immunoblots (not shown), and identifying 7/8 Bet v 2-sensitized banana-allergic and 8/8 Bet v 2-positive pineapple-allergic patients as being sensitized to the cross-reacting fruit profilins.

Hazelnut

In a previous communication, we have investigated the importance of various factors for the allergen extraction from hazelnut (HN) (22). However, when used in the EAST, even the optimized extract led to insufficient analytical sensitivity (Table 2). Sera from 43 patients whose hazelnut allergy had been confirmed by double-blind placebo-controlled food challenge (23), recruited at the University Hospitals in Copenhagen and Zürich, were analyzed in our laboratory. IgE from many of these sera did not show specific recognition of allergens when investigated by immunoblotting with HN extract. In the EAST, 11 of the 43 patients had a class 0 reaction to the optimized extract from raw hazelnut. Cloning of the major hazelnut allergen, Cor a 1.0401, solved this

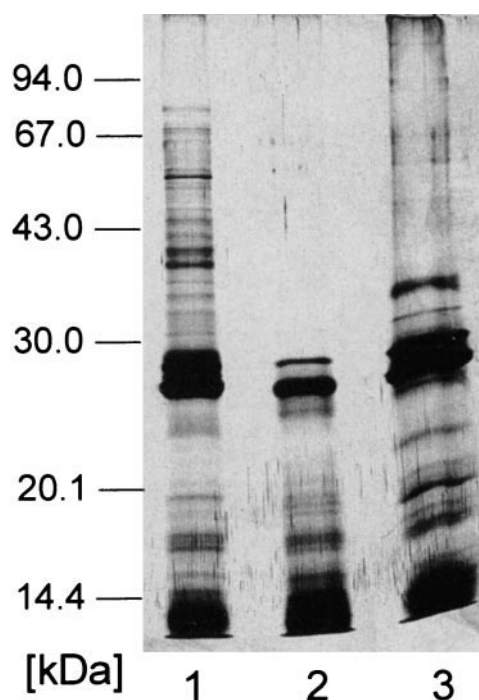


Figure 2. SDS-PAGE of pineapple extracts. The gel was silver stained. Lane 1: extract prepared from pineapple by a low-temperature acetone powder method (5) with addition of E64 protease inhibitor, lane 2: extract prepared from pineapple by a low-temperature acetone powder method (5) without protease inhibitor, lane 3: commercial prick test solution.

problem (13): the recombinant non fusion allergen was coupled to activated filter paper disks and tested in the EAST with sera from all 43 patients. With the exception of two sera, all were positive in the EAST, clearly demonstrating, firstly, that we had cloned the major HN allergen and, secondly, that the recombinant allergen displayed a very high biological activity. The tendency towards higher EAST classes to the recombinant major allergen is evident. We had only two class 4 results with extract compared to 13 class 4 measurements with the recombinant allergen. Inhibition assays demonstrated a complete cross-reactivity between natural and recombinant major HN allergen.

Discussion

The examples shown in this contribution clearly show that quality problems still exist with commercial

Table 2. Summary of EAST determinations with hazelnut allergens in 43 patients with positive DBPCFC to hazelnut

Allergen disk	No. of patients with EAST class				
	0	1	2	3	4
Raw HN	11	2	15	13	2
Roasted HN	38	5	0	0	0
rCor a 1.0401	2	3	9	16	13

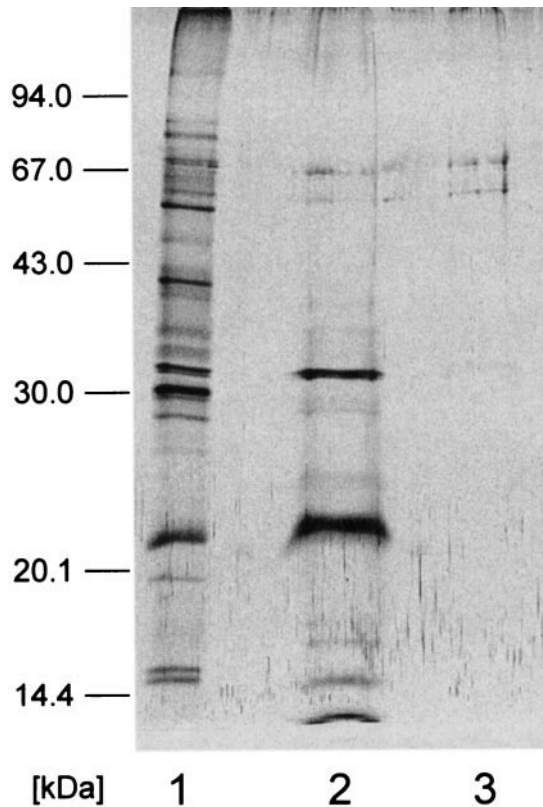


Figure 3. SDS-PAGE of banana extracts. The gel was silver stained. Lane 1: extract prepared from banana by a low-temperature acetone powder method (5), lane 2: commercial prick test solution A, lane 3: commercial prick test solution B.

allergen extracts from vegetable foods, and that several of these problems can be solved when extraction procedures are adapted to the specific source material. In addition, recombinant allergens can be beneficial in many ways: if the panel of cloned allergens is nearly complete, a component-resolved *in vitro* diagnosis can be achieved in a short time, allowing conclusions about

potential cross-reactivities, to distinguish between IgE binding to proteins or CCD, and possibly, to recognize sensitization to potentially severe food allergens. In the cherry study, three recombinant allergens largely resembled the IgE binding activity of cherry extract: only three patients were negative to all recombinants but positive to the extract, while three subjects who had a negative extract EAST were picked up as IgE-positive by the recombinant allergens. Interestingly, only three cherry allergic patients had IgE specific for the LTP, which is thought to be a severe food allergen (9) and which is a major fruit allergen in the Mediterranean area (20, 24).

In the case of hazelnut, the major allergen presented one of the minor components in the extract, being hardly recognizable among the very abundant storage proteins in a silver stained SDS-PAGE (13). This might also be the reason for the poor analytical sensitivity of the EAST, as the activated paper disks might have been blocked with the bulk of non allergenic storage proteins. Since a single allergen was dominant in this group of hazelnut-allergic patients, rCor a 1.0401 led to a dramatic improvement of the sensitivity of the *in vitro* assay. In conclusion, we think that both optimized allergen extracts and recombinant allergens should be applied in the *in vitro* diagnosis of food allergy, leading to more specific analytical data. As suggested by van Ree et al. (9) these data may result in increased clinical relevance of food allergy sero-diagnostics.

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