

Allergens in celery and zucchini

The aim of this study was to confirm allergy to celery tuber and to zucchini, for the first time, by DBPCFC, and to identify the allergens recognized by IgE from DBPCFC-positive patients. Therefore, raw vegetables were hidden in a broccoli drink, and a DBPCFC-procedure was developed that consisted of a spit and swallow protocol, making sure that the procedure was safe for the patients and that reactions strictly localized to the oral cavity as well as systemic reactions could be reproduced by DBPCFC. The allergens in celery and zucchini extract were identified by immunoblot inhibition using allergen extracts, recombinant allergens and purified N-glycans as inhibitors. Celery allergy was confirmed in 69% (22/32) of subjects with a positive case history. Four subjects with a history of allergic reactions to zucchini had a positive DBPCFC to this vegetable. During DBPCFC, systemic reactions were provoked in 50% (11/22) of the patients to celery, and in 3/4 of the zucchini-allergic patients. The Bet v 1-related major celery allergen was detected by IgE of 59% (13/22) of the patients. Cross-reactive carbohydrate epitopes (CCD) bound IgE of 55% (12/22) of the celery-allergic patients and in 2/4 of the subjects with zucchini allergy. Profilin was a food allergen in celery in 23% (5/22) and in zucchini in 2/4 of the cases. A zucchini-specific allergen was detected by IgE from one patient. We conclude that ubiquitous cross-reactive structures are important in allergy to both, celery and zucchini, and that a specific association to birch pollen allergy exists in allergy to celery (mediated by Api g 1), but not in zucchini allergy.

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Introduction

According to published case histories, allergy to celeriac (celery tuber), a member of the *Apiaceae* family, belongs to the most frequent pollen-related food allergies in some European countries such as Switzerland, France, or Germany (1–6). By contrast, allergic reactions to ingestion of seeds and vegetables of the *Cucurbitaceae* family have rarely been reported (7–10). In particular, allergy to zucchini (*Cucurbita pepo*) has not been reported yet. In most of the studies in the area of pollen-related allergy to fruits and vegetables, food allergy diagnosis was based on the presence of specific IgE antibodies and on case history, or open challenge tests. Only very recently, double-blind placebo-controlled food challenges (DBPCFCs), the only conclusive test for food allergy (11), have been performed with hazelnut (12) and apple (13). Also, in three recent studies we were able to confirm celery and zucchini allergy by DBPCFC, and to identify the allergens reacting with IgE antibodies from subjects with confirmed food allergy (14–16).

Materials and methods

Details of the applied methodology can be read elsewhere (14–16).

Dbpcfc

DBPCFC with celery and zucchini was performed as described (14, 16). Briefly, raw celery or raw zucchini were hidden in a broccoli drink containing cooked broccoli, broccoli fond, cream, yoghurt, water, and salt. After excluding reactivity to the placebo by open challenges, a spit-and-swallow protocol was applied. Patients who did not react to increasing amounts in the spit-protocol were allowed to swallow the drinks. Subjects consistently complaining about symptoms to the active drink three times but never to the placebo drink were considered to be responders. One millilitre of the active drink contained 0.144 g of raw celeriac or zucchini.

N-glycans

To examine whether 'cross-reacting carbohydrate determinants' (CCD) play a role in celery and zucchini allergy we used a N-linked glycopeptide with the glycan structure Man α 1–6 (Xyl β 1–2)Man β 1–4GlcNAc β 1–4 (Fuc α 1–3)GlcNAc and just 2–4 amino acid residues (MUXF3) for inhibition experiments. We used this particular glycan because the main IgE-binding epitopes in cross-reactivity to glycoproteins are α 1,3-linked fucose and β 1,2-linked xylose (17). The preparation of the glycopeptide from pineapple stem bromelain was performed as described (18, 19). To check the purity of the sample, it was analyzed by mass spectrometry (MALDI-TOF MS). As negative control a glycopeptide with the common pentasaccharide core Man α 1–6 (Man α 1–3) Man β 1–4GlcNAc β 1–4GlcNAc (MM) was used. It was prepared from bovine fibrin as described (17). Both, MUXF3 and MM, were produced and supplied by Professor Friedrich Altmann (Vienna, Austria).

Total and specific IgE, EAST inhibition

Total serum IgE and specific IgE against standard inhalant allergens was measured with the CAP-FEIA system (Pharmacia & Upjohn, Uppsala, Sweden). Specific serum IgE against zucchini was determined by a commercially available Enzyme Allergosorbent Test (EAST) (Allergopharma, Reinbek, Germany). EAST classes correspond to RAST classes (Pharmacia, Freiburg, Germany). For EAST and EAST inhibition experiments, allergen extracts were coupled to cyanogen bromide activated paper disks (celery: 5 µg/disk, zucchini: 3 µg/disk). The EAST was performed according to the manufacturer's instructions. For EAST inhibitions, the sera were diluted 1:3, 1:4 or 1:5 with incubation buffer (0.05 M Tris-hydroxymethyl-aminomethane, 0.15 M NaCl, 1.0% Tween 20, 0.3% BSA, pH 7.4) and for each disk 50 µl serum dilution was incubated with 50 µl inhibitor solution (extracts: up to 500 µg/ml; recombinant allergens, N-glycans: up to 100 µg/ml diluted in incubation buffer) (15, 16).

Identification of allergens

Allergens were identified by IgE immunoblotting and IgE immunoblot inhibition as described (15, 16).

Results

Clinical study of allergy to celery root

DBPCFC was performed in 32 patients with a history of adverse reactions to celery. CAP test results with celery tuber were positive (>0.7 kU/l) in 16 of 22 patients with positive DBPCFC and in 5 of 8 patients with negative DBPCFC. Ninety-one percent of the DBPCFC-positive patients ($n=20$) had a positive CAP to birch pollen, 91% ($n=20$) for Bet v 1, 27% ($n=6$) for Bet v 2, 64% ($n=14$) for mugwort pollen, and 77% ($n=17$) for carrot (14). Seventy-three percent ($n=16$) of

the DBPCFC-positive patients had a history of food allergy to one or more other plant foods (15). The symptoms of the patients during DBPCFC and doses of raw celery that elicited the reactions are summarized in Table 1. In total, 22 patients were positive in the DBPCFC, with one subject exclusively showing respiratory symptoms after inhaling the vapour of the verum drink. Four patients were DBPCFC-negative, but reacted in the open challenge, and four were negative in both open and blinded challenge. Systemic symptoms occurred in 50% ($n=11$) of the DBPCFC-positive patients with a mean provocation dose of 19.3 ± 11.8 g.

Clinical study of zucchini allergy

Recently, we identified four patients with a history of systemic allergic reactions after ingestion of raw, or raw and cooked zucchini (16). Three of the four patients were sensitized to birch and grass pollen, and other kinds of pollen. One patient did not react with birch, but was positive in the skin test to grass and ragweed pollen. Also, three of the subjects had IgE to latex (CAP class 2). All patients were positive in the zucchini EAST with a low temperature extract from raw zucchini as solid phase antigen, that was prepared in our laboratory, with one class 3, two class 2, and one class 1 result. The results of total and specific IgE determinations in zucchini-allergic subjects are summarized in Table 2.

The DBPCFC provoked an oral allergy syndrome (OAS) with 0.7 g zucchini in the spit protocol in one patient, and systemic reactions in the other 3 patients (Table 3).

Table 1. Symptoms and dose of celery (g) producing symptoms in patients with positive DBPCFC to celery (14)

Patient	Symptoms	Dose of celery (g)
1	Itch of palate and lips (OAS)	0.7
2	Itch of palate and tongue (OAS)	0.7
3	Itch of lips (OAS)	0.7
4	Itch of lips and throat (OAS)	0.7
5	Rhinoconjunctivitis, cough, flush, generalized pruritus	28.5
6	Itch of lips (OAS)	0.7
7	Burning sensation of lips and tongue (OAS)	0.7
8	Flatulence, abdominal cramps	28.5
9	Conjunctivitis, flush, urticaria, cough, dyspnoea	28.5
10	Itch of lips and tongue (OAS)	5.6
11	Itch of lips (OAS)	0.7
12	Itch of throat and ears (OAS)	1.9
13	Itch of lips, palate and tongue (OAS)	0.7
14	Nausea and dyspnoea	1.9
15	Flush, angioedema of lips and eyelids	28.5
16	Itch of scalp and angioedema of lips	13.3
17	Itch of throat (OAS)	0.7
18	Urticaria	5.6
19	Angioedema of lips	28.5
20	Itch of palate (OAS), rhinoconjunctivitis, cough, dyspnoea	0.7
21	Nausea and emesis	28.5
22	Rhinoconjunctivitis by inhalation	—

Identification of celery allergens

Celery allergens were identified by immunoblotting and immunoblot inhibition with recombinant (r) celery allergens rApi g 1 (20), rApi g 4 (21), and the N-glycans MUXF3 and MM as inhibitors. In the DBPCFC-positive patient group, the major allergen Api g 1 (16 kDa) was recognized by IgE from 13/22 patients (59%). Another major allergen were CCD against which IgE was determined in 12/22 patients (55%). Celery profilin Api g 4, was recognized by IgE from 5/22 patients (23%). Figure 1 shows the identification of the three relevant celery allergens by immunoblot inhibition. EAST inhibition assays showed that Api g 1 and CCD were able to bind each more than 90% of celery-specific IgE in certain sera. IgE to the profilin Api g 4 always occurred as a coresponse in patients with IgE to other celery allergens, resulting in a maximal inhibition of celery EAST of 40% by Api g 4 (15).

Eight patients with suspected celery allergy were not responding to DBPCFC (14). Four of the patients had an OAS in an open challenge with raw celery. Since IgE antibodies against Api g 1, Api g 4, and CCD were

Table 2. Results of total and specific IgE determinations in zucchini-allergic patients (16)

Patient	Total IgE (kU/l)	EAST (kU/l) zucchini	CAP (kU/l)						
			melon	ragweed	celery	latex	birch	rBet v 1	rBet v 2
148	162	3.65 (3)	<0.35	<0.35	<0.35	n.d.	<0.35	<0.35	<0.35
225	125	1.16 (2)	2.25 (2)	2.51 (2)	2.25 (2)	2.02 (2)	2.46 (2)	<0.35	<0.35
291	207	2.08 (2)	1.03 (2)	n.d.	n.d.	0.85 (2)	32.2 (4)	31.9 (4)	8.1 (3)
652	156	0.38 (1)	<0.35	1.49 (2)	0.76 (2)	0.91 (2)	1.86 (2)	0.36 (1)	0.85 (2)

EAST-classes and CAP-classes are given in parentheses. n.d. = not determined.

detected in their sera, there were no obvious differences to patients with a positive DBPCFC (15). Only one of the four patients with negative open challenge to celery had a positive celery CAP and displayed an IgE response to Api g 4 and CCD.

Identification of zucchini allergens

Since no recombinant zucchini allergens have been produced so far, identification of pollen-related zucchini allergens was based on immunoblot inhibition with homologous birch pollen allergens, and the N-glycans MUXF3 and MM (Fig. 2A). Serum 148 strongly reacted with three proteins at about 16–17 kDa (lane 1). An almost complete inhibition was obtained only with zucchini extract for this serum (lane 2). Multiple bands above 29 kDa were recognized by IgE of the sera 225 and 291. IgE binding to almost all of these bands was inhibited by all plant extracts (lanes 2, 5, and 7) and the N-glycan MUXF3 (lane 3), but not with the control inhibitors skim milk extract (lane 8) and the control glycopeptide MM (lane 4). This points to a high cross-reactivity due to IgE against carbohydrate determinants. Serum 652 showed a totally different binding pattern. Only two bands at approximately 15 kDa were specifically detected. A complete inhibition of IgE reactivity to these bands was obtained with rBet v 2 (lane 6) and all plant extracts (lanes 2, 5, and 7). The same two bands were recognized by serum 291 too, but the staining intensity was weak when compared with high molecular weight bands, resulting in these bands being invisible in Fig. 2(A). Hence, the blot inhibition method was optimized to solve this problem: the low molecular ends of blotted NC strips were cut off and used for blot inhibition, so that the final staining could be carried out for a longer period. As seen in Fig. 2(B), under these conditions the bands at 15 kDa could be visualized with serum 291 (lane 1). They were successfully inhibited by zucchini and birch extract as well as by rBet v 2 (lanes

2–4). The sensitization of patients 291 and 652 to profilin was confirmed by a positive CAP to Bet v 2 (Table 2). From these experiments, we concluded that one patient (148) was sensitized to a non pollen-related allergen in zucchini, whereas the others had IgE to the cross-reactive allergens CCD (225), zucchini profilin (652), or both (291).

Discussion

According to reported case histories, celery allergy is very frequent and can be severe (2–6, 22, 23), whereas food allergy to zucchini is extremely rare. Since DBPCFC is the only scientifically accepted test for the confirmation of food allergy, other diagnostic methods can be validated only in patients in whom allergy was confirmed by DBPCFC (24). The spit- and swallow-protocol that was established in our study (14) ensured that OAS symptoms strictly localized to the oral cavity can be provoked by DBPCFC, and reduces the risk for severe reactions during challenge. By this procedure, we were able to confirm for the first time celery allergy in 69% (22/32) patients with a positive case history, and in four subjects who experienced allergic reactions after eating zucchini. The fact that 50% of the celery-allergic patients and 3/4 of the zucchini-allergic patients had systemic reactions during our very cautious challenge procedure demonstrates that food allergy to these two vegetables can be severe. The Bet v 1-related celery allergen, Api g 1, had an IgE prevalence of 59% (13/22) and was thus identified as a major allergen in patients with confirmed allergy to celery. By contrast, no member of the Bet v 1 allergen family was detected in zucchini. Cross-reactive carbohydrate epitopes (CCD) were identified as another major IgE-binding structure in celery-allergic patients, and in 2/4 of the zucchini-allergic patients. These structures are ubiquitous in plants (17). It has been

Table 3. Personal history: symptoms to ingestion of zucchini and to DBPCFC (16)

Patient	Zucchini	Symptoms to ingestion	Symptoms to DBPCFC	Provoking dose (g)	Protocol
148	Raw and cooked	OAS, urticaria, angioedema	OAS	0.7	Spit
225	Raw	Nausea, vomitus	Nausea	0.7	Spit
291	Raw and cooked	Palmoplantar pruritus, Urticaria	Persistent diarrhoea, nausea, pruritus	6.0	Swallow
652	Raw and cooked	OAS, stridor	Swelling of the throat, nausea	0.7	Spit

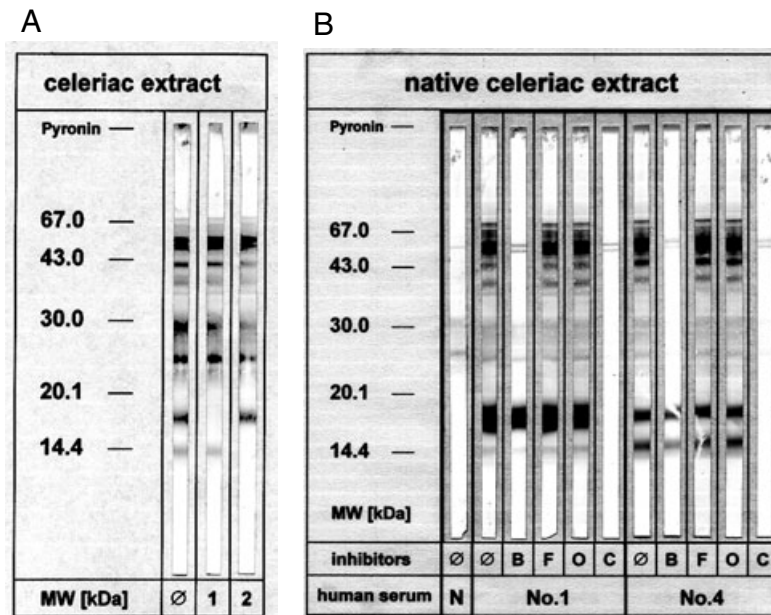


Figure 1. Immunoblot inhibition with extract from native celery tuber (celeriace) using sera from celery-allergic patients no. 4 (A) and nos. 1 and 4 (B) with specific IgE to Api g 1, Api g 4, and CCD, respectively. Lanes: no inhibitor (Ø); inhibitors: rApi g 1 (1), rApi g 4 (2), bromelain-glycopeptide MUXF3 (B), fibrin-glycopeptide MM (F), ovalbumin (O), native celeriac extract (C); nonallergic human control serum (N).

hypothesized that IgE antibodies to these structures have poor biological activity and thus are not able to elicit symptoms in sensitized patients (25–28). However, CCD were identified as the unique IgE binding structure in three of the celery-allergic patients

and one of the zucchini-allergic patients. If these IgE reactivities would be without clinical significance, the allergens responsible for the food allergy of these patients would remain unidentified. Currently, histamine release assays are underway in our laboratories to

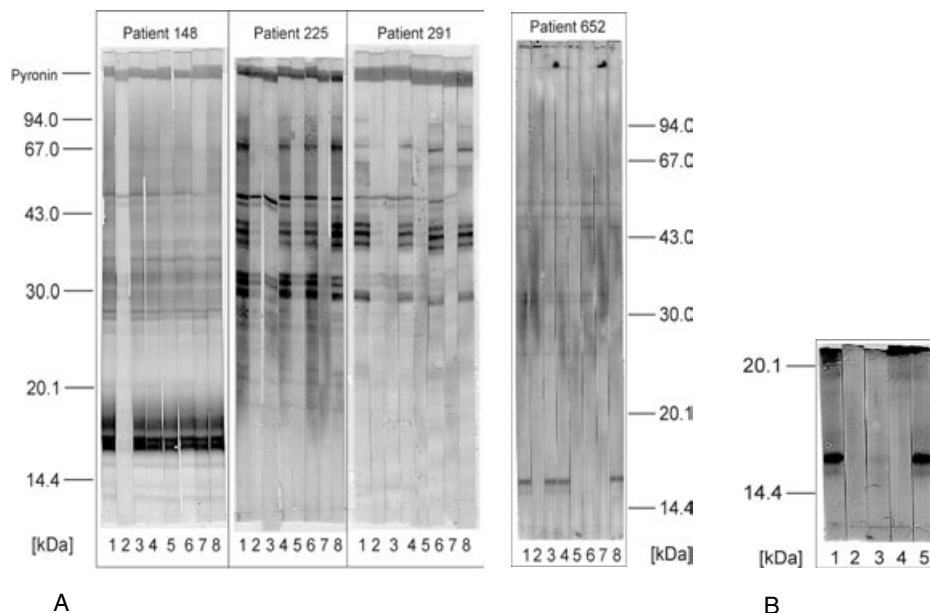


Figure 2. (A) Immunoblot inhibition experiments with sera from zucchini-allergic patients 148, 225, 291 and 652. Inhibitors: lane 1: without inhibitor, lane 2: 100 µg protein from zucchini extract, lane 3: 10 µg bromelain glycopeptide MUXF3, lane 4: 10 µg fibrin glycopeptide MM, lane 5: 100 µg protein from birch extract, lane 6: 10 µg rBet v 2, lane 7: 100 µg protein from melon extract, lane 8: 100 µg milk protein. (B) Optimized blot inhibition with serum from zucchini-allergic patient no. 291. Inhibitors: lane 1: without inhibitor, lane 2: 100 µg protein from zucchini extract, lane 3: 100 µg protein from birch pollen extract, lane 4: 10 µg rBet v 2, lane 5: 100 µg milk protein.

prove the biological activity of anti-CCD IgE. Another 'pan-allergen', profilin (29), was detected in celery extract by 23% (5/22) of the celery-allergic subjects and in zucchini extract by 2/4 of the subjects with confirmed allergy to zucchini. One patient appeared to be sensitized to a zucchini-specific allergen without cross-reactivity to pollen. The fact that in celery the same allergens, Api g 1, CCD and Api g 4, were detected by IgE from patients with positive and negative DBPCFC underlines that sensitization to all allergenic structures in celery can be clinically insignificant.

Recently, N-terminal sequences of a 55–58 kDa celery allergen, Api g 5, have been published that do not share homology with known protein sequences (30). So far, it is not clear whether Api g 5 is identical with one of the CCD-containing glycoproteins which were recognized by IgE from our celery-allergic patients. Also, a new isoform of the major celery allergen, Api g 1.0201 has recently been described (31). This isoform displayed only weak cross-reactivity to the isoform used in our investigation, Api g 1.0101, and to Bet v 1 from birch pollen. All our celery-allergic patients had IgE to rApi g 1.0101, and IgE binding to Api g 1 in celery extract was fully inhibited by birch pollen extract in all

Api g 1-sensitized patients we have investigated so far. Hence, we conclude that this new isoform, Api g 1.02.01 is of minor importance in patients with confirmed allergy to celery.

Taken together, our data demonstrate that pollen-related allergens are important in both allergy to celery and to zucchini, and that these allergens are able to cause systemic allergic reactions to food. The major allergen Api g 1 (20) in celery mediates a birch-specific cross-reaction, whereas profilin and CCD are not only present in tree pollen, but also in pollens of weeds and grasses. In our patient panel, we did not identify a celery allergen responsible for a mugwort-specific cross-reaction.

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