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Original article

Strategy for allergenicity assessment of 'natural novel foods': clinical and molecular investigation of exotic vegetables (water spinach, hyacinth bean and Ethiopian eggplant)

Background: Foods not commonly consumed in the European Union must be proven safe before being brought to market, including an assessment of allergenicity. We present a three-stepwise strategy for allergenicity assessment of natural novel foods using three novel vegetables, namely, water spinach, hyacinth bean, Ethiopian eggplant.
Methods: First, vegetable extracts were analyzed for the presence of pan-allergens [Bet v 1 homologous proteins, profilins, nonspecific lipid transfer proteins (LTP)] by immunoblot analysis with specific animal antibodies. Secondly, the IgE-binding of the food extracts was investigated by EAST (Enzyme-allergosorbent test) and immunoblot analysis using sera with IgE-reactivity to known pan-allergens or to phylogenetically related foods from subjects (i) allergic to birch, grass and mugwort pollen, (ii) with food allergy to soy, peanut, tomato, multiple pollen-related foods and (iii) sensitized to LTP. Thirdly, the clinical relevance of IgE-binding was assessed *in vivo* by skin prick testing (SPT) and open oral food challenges (OFC).
Results: Profilin and LTP were detected by animal antibodies in all vegetables, a Bet v 1 homologue selectively in hyacinth bean. IgE-binding to LTP, profilin and a Bet v 1 homologue was proven by immunoblot analysis and EAST. Positive SPT and OFC results were observed for all vegetables in pollen-allergic patients.
Conclusions: Our stepwise procedure confirmed the presence and IgE-binding capacity of novel vegetable proteins homologous to known allergens in endemic vegetable foods. *In vivo* testing proved the potential of the novel vegetables to elicit clinical allergy. Hence, our described algorithm seems to be applicable for allergenicity testing of natural novel foods.

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Novel foods, which have not been consumed to a relevant extent in Europe, may be introduced to the market for increasing the consumers' choice and for nutritional benefits. However, there is an unknown risk for atopic subjects to develop allergies against such novel foods. A relevant example is kiwi fruit, which became popular in Europe in the 1970s. Kiwi is nowadays one of the most frequent causes of fruit allergies in Central Europe affecting subjects allergic to birch pollen and latex as a consequence of IgE cross-reactivity, but also eliciting primary food allergy by *de novo* sensitization in children (1, 2).

As novel foods are not commonly consumed in the European Union (EU), they must undergo a safety assessment according to the Regulation (EC) No. 258/97 of the European Parliament and of the Council concerning novel foods and novel food ingredients (3) for obtaining a marketing authorization. The assessment of potential allergenicity is an integral part of the safety assessment process. The main aim is to minimize the risk of health and environmental concerns caused by novel foods such as genetically modified foods (GMO foods), foods with an altered physico-chemical structure and natural novel foods not commonly consumed in the EU such as the exotic vegetables investigated in this study.

Detailed guidance on how to perform such a risk assessment is mainly available for GMO foods (4–6), but not for natural novel foods that are not yet commonly consumed in the EU. Therefore, we adopted the strategy of targeted serum screening for the assessment of allergenicity of natural novel foods. As examples, we studied the following three exotic vegetables: Water spinach (*Ipomea aquatica*; *Convolvulaceae*), hyacinth bean (*Lablab purpureus, syn.Dolichos lablab*; *Fabaceae*), and Ethiopian eggplant (*solanum gilo*; *Solanaceae*), which mainly originate from Africa, Asia and South America.

Our assessment strategy was based on scientific publications (7–9) and the Commission Recommendation 97/618/EC (10). One approach that has been suggested for allergy assessment of GMO foods is the so-called targeted serum screen (4). Such attempt would include the investigation of the presence of known pan-allergenic structures such as Bet v 1 homologous proteins (11), profilins and nonspecific lipid transfer proteins (LTP). Moreover, targeted screening with sera from patients sensitized to botanically related foods should be performed to investigate for potential cross-reactivity. In a case of a positive result, skin prick testing (SPT) and food challenge are applicable methods to investigate the clinical relevance of IgE-binding properties of the novel foods (7).

In this study, we adopted the targeted serum screening to the testing of novel exotic vegetables. By using *in vitro* and *in vivo* methods, we wanted to assess the allergenic potential of the three exotic vegetables for patients allergic to plant derived food or pollen. *In vitro* and *in vivo* testing indicated that the new vegetables may have the potential to elicit clinical food allergy in subjects showing cross-reactive IgE-binding.

Material and methods

Study design

Our strategy of allergenicity assessment of natural novel foods was performed in three steps: First, we investigated the presence of proteins related to pan-allergens (Bet v 1 homologous proteins, profilins and LTPs) and of important allergenic storage proteins of the fabaceae family using allergen-specific polyclonal antisera and monoclonal antibodies (see Table 1A).

Secondly, we determined the IgE-binding of extracts by immunoblot and EAST (Enzyme-allergosorbent test) analysis using selected sera of four different patient groups (see Table 1B). Thirdly, SPT and open oral food challenges (OFC) with the natural novel vegetable foods were performed to investigate the *in vivo* relevance of the *in vitro* findings.

Study groups

The selection of appropriate sera for the allergenicity assessment of natural novel foods is essential. For the screening, we considered sera from allergic patients with IgE-reactivity to known pan-allergens on one hand and confirmed IgE-reactivity to phylogenetically related foods, on the other hand. To meet these requirements, we included four different patient groups.

Group 1: We prospectively included 60 patients from the Allergy Unit of the University Hospital Zürich with pollen allergy, who had not been exposed in the past to the three vegetables under investigation. These patients were allocated according to the reported main season of respiratory symptoms to group 1a: birch pollen-allergics ($n = 20$), group 1b: grass pollen-allergics ($n = 20$) and group 1c, mugwort pollen-allergics ($n = 20$). The majority of the included patients were sensitized to several pollen species. They underwent SPT with commercial pollen extracts (birch, grass, mugwort, ALK) and extracts of the three vegetables. In case of a positive SPT test result to one or more of the vegetables, the patients underwent OFC. Additionally, blood samples were taken for further *in vitro* investigations.

Moreover, sera from three further study groups were investigated *in vitro*. Group 2 consisted of patients with different food allergies. This group comprised 11 patients with a soy and 11 patients with a peanut allergy because of a possible cross-reaction to Hyacinth bean (Fabacae), 10 patients with a tomato allergy, respectively, because of a possible cross-reaction to Ethiopian eggplant (Solanaceae) and finally 10 patients with multiple pollen-associated food allergy because of the fact that those patients are frequently sensitized to pan-allergens such as profilin and Bet v 1 homologous proteins.

Nonpollen-related allergies to fruits, nuts and vegetables are mainly observed in the Mediterranean area and are usually accompanied by more severe symptoms than allergies to the same foods in Central and Northern Europe. These allergies are mainly caused by nonspecific LTP (12, 13). Therefore, six sera from donors with food allergies caused by LTPs were included in group 3. Group 4, i.e. the control group, consisted of 18 nonatopic subjects.

Animal antisera and monoclonal antibodies

The animal antisera and monoclonal antibodies used to investigate the presence of putative homologous plant food allergens in the selected vegetables are listed in Table 1A.

Extracts

The three exotic vegetables were grown at the research orchards of the University Wädenswil and extracted from prepared acetone powder (14) at a ratio of 1 : 10 (w/v) in phosphate-buffered saline (PBS, 8 mM phosphate, 150 mM NaCl, pH 7.1) at 4°C within 1 h. Extracts were sterile filtered (0.2 μm cellulose acetate filters; Sartorius, Göttingen, Germany), protein quantified (Roti®-Nanoquant; Carl Roth GmbH, Karlsruhe, Germany) and the freeze-dried extracts were stored at −80°C until use. For SPT, PBS extracts with an adjusted protein concentration of 1 mg/ml were prepared accordingly by the clinical investigators.

Sodium dodecylsulphate polyacrylamide electrophoresis (SDS-PAGE)

For the immunodetection with various allergen-specific antisera and monoclonal antibodies (Table 1A), the vegetable extracts were separated by SDS-PAGE according to Lämmli (15) under reducing conditions in 15% gels at 20 μg/cm protein load. For immunodetection with patients' sera, the samples were separated in a 12.5% Lämmli gel. For the immunodetection with sera from LTP-sensitized patients, the extracts were separated under nonreducing conditions in a 15% Lämmli gel.

Immunoblotting

The separated proteins were transferred onto a nitrocellulose membrane by semi-dry blotting according to Kyhse-Andersen (16). For the immunodetection with animal antibodies and patients' sera, the membranes were blocked with 5% nonfat dry milk and 0.3% Tween 20, respectively, diluted in tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4). The nitrocellulose strips were incubated over night with 600 μl of the diluted antisera and antibodies respectively.

Rabbit and mouse antibodies were incubated at appropriate dilution (rabbit-antisera 1 : 20 000; mouse monoclonal supernatant

Table 1. Summary of allergen specific polyclonal antisera and monoclonal antibodies used for the detection of homologous putative allergens (A) and sera from patients with pollen and/or food allergy applied in targeted serum screening (B)

A

	Specificity		
Antibody source	Allergen	Plant	Protein family
Rabbit, polyclonal	Gly m 4*	Soybean	PR-10
Rabbit, polyclonal	Api g 1*	Celery, celeriac	PR-10
Rabbit, polyclonal	Amb a 8	Ragweed pollen	Profilin
Rabbit, polyclonal	Pyr c 4	Pear	Profilin
Rabbit, polyclonal	Pru p 3	Peach	LTP
Rabbit, polyclonal	Cor a 8	Hazelnut	LTP
Rabbit, polyclonal	Ara h 2	Peanut	2 S albumin
Mouse, monoclonal mP16	Bet v 1	Birch pollen	PR-10
Mouse, monoclonal mP20	Api g 1.01*	Celery, Celeriac	PR-10
Mouse, monoclonal 2B2	Mal d 1*	Apple	PR-10
Mouse, monoclonal 7B2	Ara h 3/4	Peanut	7 S glycinin
Mouse, monoclonal PN-t	Ara h 1	Peanut	11 S vicilin

B

Study subjects

Group	Allergy status	Sensitization to	Number of sera	Selection criteria for serum testing
1	Pollen-allergic	(a) Birch	20	Sensitization to pan-allergens such as profilins and PR-10 proteins
		(b) Grass	20	
		(c) Mugwort	20	
2	Food-allergic	Soybean	11	Sensitization to Fabacae
		Peanut	11	Sensitization to Fabacae
		Tomato	10	Sensitization to Solanaceae
		Multiple, pollen-associated	10	Sensitization to pan-allergens such as profilins and PR-10 proteins
3	Food-allergic	LTP	6	Non-pollen related sensitization, frequently with severe symptoms
4	Non-atopic	None	18	Control panel

*Bet v 1-homologous protein

1 : 10; mouse monoclonal 1 : 5000 and 1 : 10 000 respectively) and detected with biotinylated goat-anti-rabbit IgG (DAKO, Glostrup, Denmark, 1 : 10 000) and biotinylated rabbit-anti-mouse IgG (Jackson, West Grove, PA, USA, 1 : 10 000), respectively, following incubation with NeutrAvidin-horseradish peroxidase conjugate (Pierce, Rockford, IL, USA, 1 : 50 000). The specific immunodetection was visualized by chemiluminescence (LumiGLO Reserve™ Chemiluminescent Substrate Kit, KPL via Medac, Hamburg, Germany) and recorded electronically with a Lumi Imager F1 (Roche Diagnostics, Mannheim, Germany) CCD camera.

For IgE immunoblot analysis, nitrocellulose strips were incubated over night with patients' sera diluted 1 : 10 in 600 µl of TBS containing 0.5% bovine serum albumin and 0.05% Tween 20. Bound IgE was detected with alkaline phosphatase-conjugated mouse monoclonal anti-human IgE (Pharmingen, San Diego, CA, USA, 1 : 750) following a colorimetric staining (AP Conjugate Substrate Kit; BioRad Laboratories, Hercules, CA, USA).

EAST

Specific IgE in human sera was quantified by EAST according to the manufacturer's instructions (Spez IgE ELISA; Allergopharma, Reinbek Germany), except for an overnight incubation with human sera at room temperature for increased assay sensitivity. The vegetable extracts were coupled to cyanogen bromide-activated paper disks at a protein concentration of 10 µg per disk. All 126 patients' sera including the nonatopic controls were tested for their IgE-binding properties to the exotic vegetable proteins.

Skin prick testing

All pollen-allergic patients ($n = 60$) underwent SPT that was performed on the flexor aspect of the forearm with a standardized prick needle (Stallerpoint, Stallergènes). Histamine dihydrochloride (10 mg/ml) was used as positive control, the glycerol-containing diluent of the prick solution (Soluprick, ALK) as negative control. SPT was performed with commercial pollen extracts of birch, grass and mugwort (ALK), as well as with the PBS extracts of Ethiopian eggplant, hyacinth bean and water spinach. The reactions were recorded after 15 min. A wheal diameter of >3 mm was considered as a positive result (17).

Open food challenge

Patients with a positive SPT result to one or more of the vegetables underwent an OFC. The hyacinth bean as well as the Ethiopian eggplant were shortly steamed without addition of spices. The water spinach was applied raw as it is used in vegetable salads. Four servings with an increasing dosage (Ethiopian eggplant: 10, 20, 40 and 80 g; hyacinth bean: 5, 10, 20 and 40 g; water spinach: 2, 4, 8 and 16 g) at intervals of 15 min were ingested by the patients.

Results

Allergen-specific antibodies bind to pan allergen structures in vegetable extracts

Polyclonal antisera and monoclonal antibodies raised against members of important plant pan-allergen families (Table 1A) were used to test for cross-reactive binding to members of these allergen families in extracts of the three exotic vegetables.

Proteins could be detected in the molecular weight range of LTP, profilin and Bet v 1 homologues with the anti-Pru p 3 (peach LTP), anti-Cor a 8 (hazelnut LTP), anti-Amb a 8 (ragweed profilin) and the anti-Gly m 4 (Bet v 1 homologous protein in soybean) in the hyacinth bean extract (Fig. 1). Binding to putative LTPs in the range of 8–10 kDa could be observed in the extract of eggplant with anti-Pru p 3 and anti-Cor a 8 respectively. Furthermore, strong binding at 12 kDa was obtained with the anti-Amb a 8 while the reaction was weaker with the anti-Pyr c 4 (pear profilin, data not shown). In the extract of water spinach, a positive protein double band could be detected in the range of 14–16 kDa with the anti-Amb a 8. Moreover, an 8 kDa band was observed with the anti-Cor a 8. In all vegetables no antibody-binding was observed with the rabbit-anti Ara h 2 and the monoclonal antibodies directed against Ara h 1 and Ara h 3/4 (data not shown).

Immunoblot analysis with sera from allergic subjects confirms the presence of common plant food allergens

IgE immunoblot analyses of the vegetable extracts were performed with sera from all allergic patients (Fig. 2). The specificity of the immunoblotting was in general proven with the nonatopic controls. Unfortunately, in some experiments we observed unspecific binding, such as in the extract of the Ethiopian eggplant (Fig. 2B) which, however, could be clearly distinguished from the positive IgE reactivities. Sera from the tomato-allergic subjects were only tested on the extract of the hyacinth bean because only low amounts of sera were available. For the same reason, serum from one LTP-sensitized patient (LTP1) could only be tested by EAST.

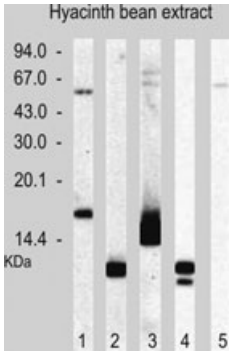


Figure 1. Immunoblot analysis of hyacinth bean extract with sera from rabbits (lane 1: anti-Gly m 4; lane 2: anti-Pru p 3; lane 3: anti-Amb a 8; lane 4: anti-Cor a 8; lane 5: buffer control).

Examples of typical immunodetection results are shown in Figure 2A–D. IgE-binding in the range of profilin (14–16 kDa), Bet v 1 homologues as well as in the higher molecular weight range were observed with sera from various patients in the extract of hyacinth bean (Fig. 2A). A similar pattern was observed in the extracts of Ethiopian eggplant (Fig. 2B) and water spinach (Fig. 2C). IgE-binding to a presumed LTP (8–10 kDa) was also observed for all three vegetable extracts with some sera of LTP sensitized patients (Fig. 2D).

EAST reveals strong IgE-binding capacity of all three vegetables

Specific IgE to the three vegetables was measured by EAST. The results are summarized in Table 2. Furthermore, the percental distribution of the reactivities is illustrated in Fig. 3. Because of a limited amount of serum, tomato-allergic patients were only tested on the eggplant extract. The test results of the nonatopic control panel (n = 18) were negative for all vegetables. The EAST data corresponded to the immunoblot results for most of the allergic patients.

Allergic reactivity to exotic vegetables confirmed by clinical investigations

Results of skin testing and food challenges are summarized in Table 3. Nine of 13 pollen-allergic patients with a positive skin prick result with extract from hyacinth bean agreed to undergo OFC. Six patients developed an oral allergy syndrome (OAS) under challenge. Positive skin tests to Ethiopian eggplant were observed in one grass and seven mugwort allergic patients. OFC with Ethiopian eggplant were positive in five of six challenged patients. All responded with OAS. Skin testing with water spinach was positive in seven patients. However, just one of five challenged patients responded under OFC with water spinach with local oral symptoms.

Discussion

Novel non-GMO foods may offer to the consumer an increased choice with the possibility of nutritional benefits (7). However, bearing in mind a considerable percentage of the population suffering from food allergies, care needs to be taken to avoid an increased burden with novel allergenic foods.

The authorization of novel foods is harmonized in the EU as outlined in the introduction. For the first overview concerning the presence and the molecular weight of potential allergens in these novel vegetables, extracts were screened with polyclonal antisera and monoclonal antibodies raised against known pan-allergens from pollen

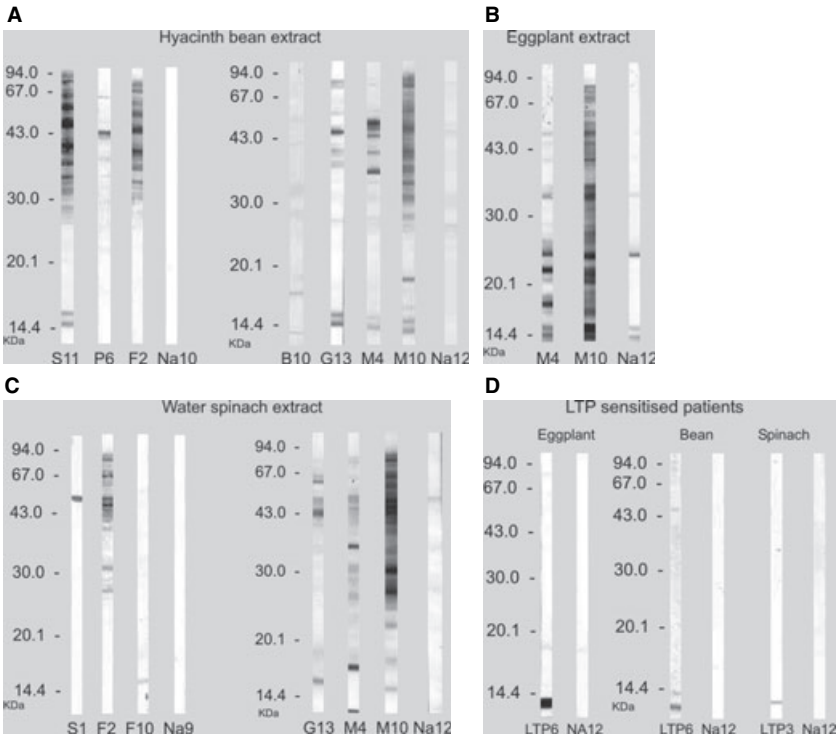


Figure 2. IgE-immunoblot analysis of (A) hyacinth bean, (B) Ethiopian eggplant, (C) water spinach and (D) hyacinth bean and water spinach with selected sera from patients having either birch, grass or mugwort pollinosis, and/or allergies to various foods (sera from subjects with allergy to: B10: birch pollen; G13: grass pollen; M4, M10 mugwort pollen; S1, S11: soybean; P6: peanut; F2, F10: multiple food; LTP3, LTP6, LTP12: sensitization to LTP; Na9, Na10, Na12: non-allergic control).

and foods (PR-10 proteins, profilins, LTPs) using immunoblot analysis.

Bet v 1 is the predominant allergen in birch pollen allergy. It is related to the so-called Pathogenesis-related protein family 10 (PR-10) and it mediates the majority of pollen-related food allergies. Proteins homologous to Bet v 1 have been identified in several fruits, nuts, vegetables and legumes such as in apple (Mal d 1), cherry (Pru av 1), carrot (Dau c 1), celery (Api g 1), hazelnut (Cor a 1.04), soybean (Gly m 4), peanut (Ara h 8) and mungbean (Vig r 1) (11, 18, 19). Profilin, another pan-allergen, was first identified as Bet v 2 in birch pollen. Many pollen allergic patients show IgE-binding to Bet v 2 homologous proteins from e.g. apple, pear, carrot, celery and hazelnut (11, 19, 20). However, a weaker correlation with clinical food allergy has been reported for the sensitization to profilin in comparison with the sensitization to Bet v 1 homologous proteins (21, 22). LTPs are 8–10 kDa molecules with eight cysteines forming disulfide bonds stabilizing the tertiary structure, resistant to higher temperatures, pH changes and gastro-intestinal digestion (12, 13, 23, 24). In the Mediterranean area, LTPs seem to

Table 2. IgE levels in positive sera determined by EAST

	Specific IgE (kU/l) measured by EAST to			Median IgE levels (kU/l) in positive sera (numbers of positive sera x/y)		
Study subjects	Hyacinth bean	Ethiopian eggplant	Water spinach	Hyacinth bean	Ethiopian eggplant	Water spinach
Birch pollen-allergic	0.50–2.09	1.4–3.1	0.76–1.7	1.64 (5/20)	1.45 (3/20)	1.40 (3/20)
Grass pollen-allergic	0.64–2.7	No reactions	No reactions	0.85 (6/20)	–	–
Mugwort pollen-allergic	0.47–9.5	0.55–2.7	0.71–2.0	1.25 (8/20)	1.32 (3/20)	1.56 (3/20)
Soybean allergic	0.46–6.3	0.99–3.5	1.2–2.6	3.40 (8/10)	3.13 (5/11)	2.15 (4/11)
Peanut allergic	0.40–2.0	No reactions	No reactions	0.57 (3/11)	–	–
Tomato allergic	nd	0.56–5.6	nd	–	1.95 (8/10)	–
Multiple, pollen-associated food allergic	0.61–6.7	2.6–3.2	1.1–2.9	1.34 (5/10)	2.87 (2/10)	2.02 (2/10)
Sensitized to LTP	1.6–9.3	1.3–5.6	1.1–2.3	5.91 (6/6)	2.88 (5/6)	1.57 (5/6)

nd = not determined, because of limited amount of serum.

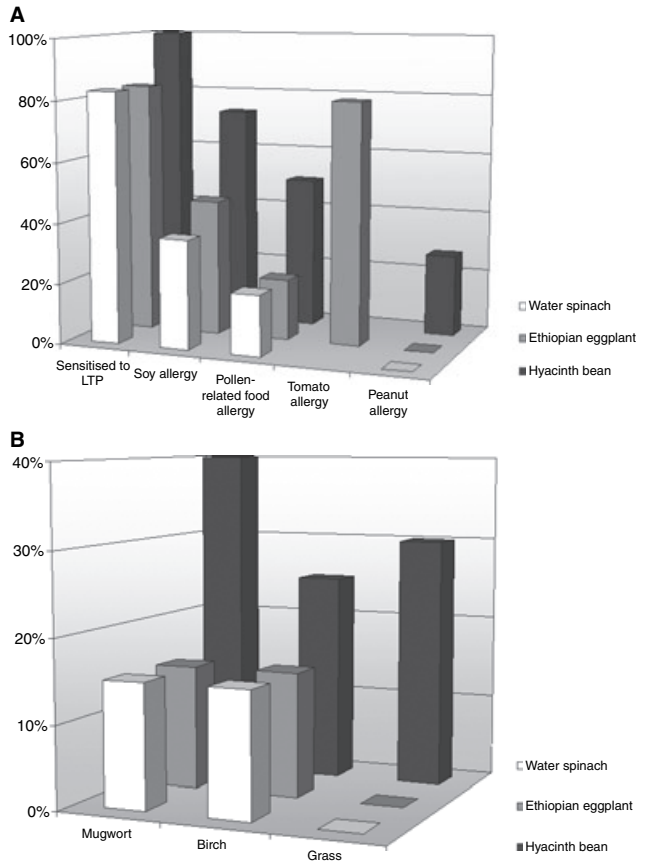


Figure 3. Percental distribution of the reactivities resulted from the enzyme-allergosorbent test analysis of the food allergic patients (A) and of the pollen allergic patients (B) to water spinach, Ethiopian eggplant and hyacinth bean on the solid surface respectively.

Table 3. Results of the clinical investigations with the pollen allergic patients

	SPT			OFC		
Patients	Hyacinth bean	Ethiopian eggplant	Water spinach	Hyacinth bean	Ethiopian eggplant	Water spinach
B6	+++	–	–	+	nd	nd
G5	+	–	–	nd*	nd	nd
G6	+	–	–	+	nd	nd
G12	+	–	–	nd	nd	nd
G13	+	–	–	+	nd	nd
G18	–	+	–	nd	+	nd
G20	+	–	+	+	nd	+
M4	–	++	–	nd	+	nd
M7	–	–	+	nd	nd	–
M9	–	+	–	nd	+	nd
M10	+	+	+	nd	nd	nd
M11	+	–	+	–	nd	–
M12	+	+	+	+	+	–
M13	+	–	–	–	nd	nd
M15	++	++	+	+	+	–
M16	+	+	+	nd	nd	nd
M17	+	+	–	–	–	nd

B, birch pollen allergic patients; G, grass pollen allergic patients; M, mugwort pollen allergic patients; nd, not determined; SPT, skin prick testing; OFC, open oral food challenges.
nd* not determined because ACE-inhibitor could not be discontinued.

be responsible for allergies to vegetables and fruits independent from a co-existent sensitization to pollen. Allergens belonging to this family were identified in different plant derived foods such as peach, cherry, apricot, apple, tomato, hazelnut, corn, rice and lettuce (13, 25).

Our approach was to screen both for the presence of the pan-allergens of the PR-10 protein family, profilin, and LTP, and for cross-reactive structures according to phylogenetic relationship. The results of our study demonstrate that the choice of immunoreagents was appropriate: Proteins belonging to two or more of these families were detected in all of the three investigated vegetables using immunoblot analysis. Because all vegetables contain LTPs and profilins, and Bet v 1 homologous proteins were particularly detected in hyacinth bean, it was assumed that patients allergic to plant proteins probably show cross-reactive IgE-binding to these vegetables.

A remarkable IgE-binding capacity was found when sera of patients from the different study groups were investigated by EAST. The highest and most frequent IgE-binding was directed to hyacinth bean extract in all patient groups except for the tomato sensitized patients who were only investigated for IgE-binding to eggplant. As expected, most of the tomato allergic patients were IgE-reactive to the Ethiopian eggplant. This finding is attributed to the botanical relationship between tomato and eggplant (Solanaceae). IgE-binding of sera from soybean-allergic patients was surprisingly frequent and strong and similar to the IgE-binding pattern of the group with pollen-related food allergy but not to the peanut allergic patients. One explanation may be the fact that the soybean allergic subjects were European adults who are predominantly pollen-allergic and sensitized to Gly m 4, the Bet v 1 homologous allergen of soybean (26). In contrast, in peanut allergic patients, pollen-related allergy to peanut is rare and most of our patients were sensitized to Ara h 2, the major peanut allergen in Europe (data not shown).

Most of the patients with a positive IgE reaction to proteins in the vegetable extracts showed also positive reactions in the immunoblot, and clear IgE-binding to Bet v 1 homologues, profilins and LTPs. Slightly divergent results between different assay systems were obtained and are likely to be explained by methodical differences. Although the most likely reason for the observed IgE-reactivity is cross-reactivity, the final proof for this hypothesis is lacking as we did not perform inhibition experiments with purified allergens. In addition to profilins, LTPs and the Bet v 1 homologues, IgE-reactivities were detected in the higher molecular weight range. We

assume that these are caused by cross-reacting carbohydrate determinants (11, 27, 28).

To investigate the biological activity and thus potential allergenicity of the exotic vegetables *in vivo*, pollen allergic patients were tested by SPT. The SPT results clearly showed that the IgE-binding components are biologically active and have the potential to activate mast cells specifically. Further evidence for this conclusion resulted from positive test results in the subsequently performed OFCs. Unfortunately, we could not perform DBPCFCs to prove the outcome of the open challenges for reasons of limited availability of fresh exotic vegetables.

Sten et al. investigated the allergenic components of Nangai nut (9). In accordance with our results they reported an *in vitro* and *in vivo* IgE-cross-reactivity in pollen-allergic subjects. In addition, they assumed that the IgE-cross-reactivity was, at least in part, related to carbohydrate epitopes, when investigated by RAST-inhibition. Unfortunately, apart from the pollen allergic groups, the groups of food allergic patients including LTP-sensitized patients could, for practical reasons such as mentioned above and time course of the investigation, not be included in further clinical investigations. Such investigations or at least basophil activation tests would be meaningful to substantiate the biological activity and assess the allergenic risk by these exotic vegetables for these groups of patients.

Nonetheless, the applied three-stepwise procedure is applicable to assess the allergenicity of natural novel foods before introducing them to the market. We recommend as a first screening step to confirm the presence of proteins homologous to known allergens by specific animal antibodies or antisera. Thereafter, the IgE-binding potential should be investigated by using targeted serum screening. It is important to select sera from allergic patients for the screening with IgE-reactivity to known pan-allergens on one hand and confirmed IgE-reactivity to phylogenetically related foods (i.e. to peanuts if screening for a novel legume). Finally, the clinical relevance of *in vitro* IgE-binding has to be verified by provocation tests. Our data on *in vitro* and *in vivo* allergenic potential of the investigated natural novel vegetables water spinach, hyacinth bean and Ethiopian eggplant underlined the usefulness of such an allergenicity assessment strategy: All three novel vegetables may have the potential to cause allergies in previously unexposed pollen and plant food allergic patients. In patients sensitized to LTPs, an allergen family associated with severe allergic reactions to several plant derived foods (13), similar reactions to the exotic vegetables investigated in our study cannot be excluded.

The fact that an allergenic potential of novel vegetables is indicated by cross-reactive IgE-binding in a targeted serum screen involving sera from subjects allergic to pollen and plant derived food is not surprising and may even be viewed as trivial. It seems very likely that all kinds of newly introduced plant derived foods would give a similar result, when investigated according to the current recommendations for GMO foods.

The meaningfulness of such studies with special regard to market authorization of novel nonGMO foods may be questionable when considering that *in vitro* IgE-binding properties in targeted serum screening and even clinical reactivates in preselected allergic patient groups may be observed with any novel vegetable or fruit. Nonetheless, the continuing performance of comparable studies with novel foods can improve our knowledge about the allergenic potential of novel foods. Having sets of data on different novel foods, those foods with an extraordinary allergenic potential may be easier to identify. However, as long as no validated methods for assessing a *de novo* sensitization capacity are available, the overall allergenic potential of novel foods is impossible to predict.

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