

New Perspectives for the Diagnosis of Food Allergy

by Ronald van Ree, Jaap H. Akkerdaas, W. Astrid van Leeuwen, Montserrat Fernández-Rivas, Riccardo Asero, Vlasta Knul-Brettlova, André Knulst, & Rob C. Aalberse

Skin tests and *in vitro* diagnostic tests for food allergy have poor predictive value. This can be improved by taking advantage of the progress that has been made in the identification of major food allergens, and in their subsequent immuno- and physico-chemical characterization. Potential improvements to the current diagnostic tests have one thing in common: they aim at the detection of immunoglobulin E (IgE) antibodies against selected allergenic structures instead of measurement of specific IgE against the whole food-allergen extract. One way to achieve this is by application of purified (natural or recombinant) food allergens. In addition, binding to whole extracts can be limited to those structures that are (potentially) of clinical relevance by inhibiting binding to irrelevant epitopes. Finally, distinction can be made between “severe” and “mild” food allergens by pepsin-digestion of allergen extracts. Together, these modifications will result in increased clinical relevance of food allergy sero-diagnostics.

“The golden standard for the diagnosis of food allergy still is the double-blind placebo-controlled food challenge.” This statement always turns up in reviews and presentations on food allergy diagnostics. Opinion leaders in the field of food allergy all agree with it. “However, nobody puts it into daily practice,” the same opinion leaders would have to admit. Double-blind placebo-controlled food challenges (DBPCFC) are almost exclusively used in a research setting. For routinely performed diagnosis, the technique is simply too laborious. A thorough history, combined with skin testing and/or *in vitro* serologic testing (e.g., CAP or radioallergosorbent test, RAST) is common practice. In difficult cases, open food challenges or elimination diets are sometimes used. Both skin tests and *in vitro* tests for food allergy have poor predictive value, when compared to those used for diagnosing inhalant allergies. Skin tests are affected by both false-negative and false-positive results. False-negative results are mainly due to instability of allergens in food extracts used for skin testing [1]. Fruit extracts are notorious in this respect. For *in vitro* testing, false-negative results are less of a problem. A positive CAP or RAST, however, is frequently not accompanied by clinical food allergy [2].

How are we to improve food allergy diagnosis? By trying to convince practitioners to start using DBPCFC? Probably not, because of practical issues. A significant increase in the predictive value of skin tests and *in vitro* assays would be a major step forward. Over the past decade, our knowledge of food allergens has increased enormously. The cause of crossreactivity between pollen and vegetable foods has been largely elucidated [3, 4]. More recently, progress has been made in the identification of crossreactive allergens in latex and foods [5]. In addition, many food-specific allergens were characterized and, in some cases, cloned and expressed as recombinant proteins. How can this knowledge be exploited to improve *in vitro* diagnostic tests for food allergy?

False-Negative Tests

The oral allergy syndrome (OAS) caused by fruits such as apple and peach is closely linked to birch pollinosis [6]. The main responsible crossreactive structure is the major birch pollen allergen Bet v 1 [3, 7, 8]. The homologous structure in fruits, such as Mal d 1 in the apple, is degraded rapidly upon disruption of the fruit. This was already known before Mal d 1 was identified as the major apple allergen [1]. The lability of Bet v 1-related structures is perhaps best illustrated by the daily experience of practitioners with commercial skin test products for fruit allergy: They frequently result in false-negative diagnoses [9]. More recently, the lability of Bet v 1 homologues in foods was also confirmed by laboratory experiments [10–12]. The second pollen-related food allergen, profilin, has been reported to be more stable than the Bet v 1-related allergens [11, 12]. Because of the lability of Bet v 1-related homologues, skin testing is frequently performed with fresh fruits instead of commercial extracts [13]. Of course, the disadvantage of this approach is that standardization is impossible. Immobilized extracts used for *in vitro* diagnostic approaches such as RAST or CAP are known not to be similarly labile. Apparently, immobilization protects Bet v 1-related allergens as well as profilins from rapid degradation. For this

reason, *in vitro* techniques are to be preferred over skin testing with commercial extracts. If skin testing is desired, fresh fruits are currently the only reasonable option. Recent developments, however, have created new opportunities.

The crossreactive allergens from birch pollen, Bet v 1 and profilin (Bet v 2), are now commercially available as purified recombinant proteins (Biomay, Linz, Austria) [14, 15]. The same is true for the Bet v 1 homologues from apple (Mal d 1) [16] and celery (Api g 1) [17]. These reagents are much more stable than food extracts, because the natural matrix responsible for degradation (by, e.g., proteolysis) is absent. Therefore, such recombinant allergens might very well be a good alternative to be used in skin testing and *in vitro* tests. As far as we know,

application of recombinant Mal d 1 or Api g 1 in diagnostic tests has not yet been evaluated on a larger scale [9]. First results in skin testing with the crossreactive pollen allergens, rBet v 1 and rBet v 2, are promising [18, 19]. The same is true for *in vitro* testing [19–23]. A prerequisite for the application of recombinant allergens is that their IgE-binding characteristics are equivalent to those of their natural counterparts. In a recent study, the performance of natural and recombinant Bet v 1 and Bet v 2 was compared in a RAST protocol with a large panel of sera [24]. Recombinant Bet v 1 turned out to be almost indistinguishable from its natural counterpart (see Figure 1A). In contrast, the mean IgE-binding capacity of recombinant Bet v 2 was only 43% of that of natural birch profilin (see Figure 1B). An explanation for this difference was not presented, but incorrect folding of the allergen during synthesis by *Escherichia coli* possibly plays a role. Eukaryotic expression systems like yeasts or plants might solve these problems. As long as recombinant proteins do not equal their natural counterparts, the use of purified natural allergens for application in diagnostic tests will be preferred.

Patterns of Crossreactivity

What is the significance of a positive RAST for Bet v 1 or for Bet v 2? This question can be answered from either a serologic or clinical perspective. If a serum contains IgE antibodies against Bet v 1 or Bet v 2, which foods are then recognized by the patient's serum? If a crossreactive structure is recognized in a vegetable food, does this imply that the patient has food-related symptoms? The degree of crossreactivity of IgE antibodies against Bet v 1 was investigated in a group of 90

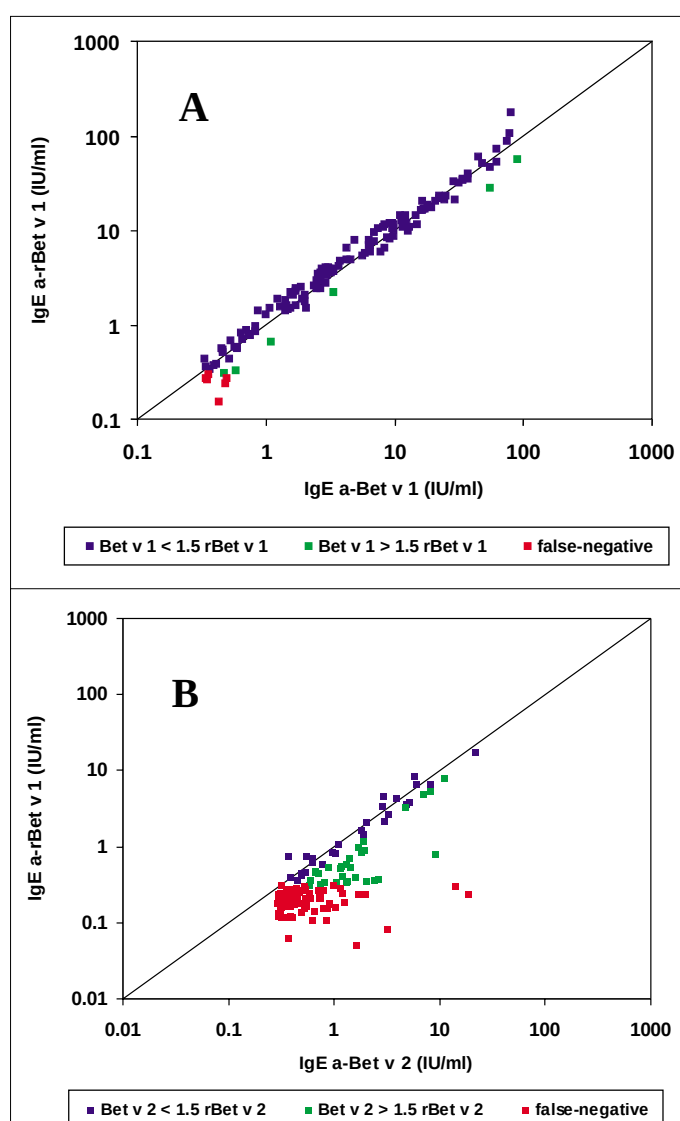


Figure 1. Comparison of IgE-binding capacity of natural and recombinant Bet v 1 and Bet v 2. Sera were tested in a RAST with purified natural and recombinant Bet v 1 ($n=136$) and with natural and recombinant Bet v 2 ($n=132$). Results are expressed in IU/ml. The majority of the sera (blue symbols) show similar responses to natural and recombinant Bet v 1 (A). This is not the case for Bet v 2, where the majority have a RAST that is > 1.5 times lower on the recombinant protein (B).

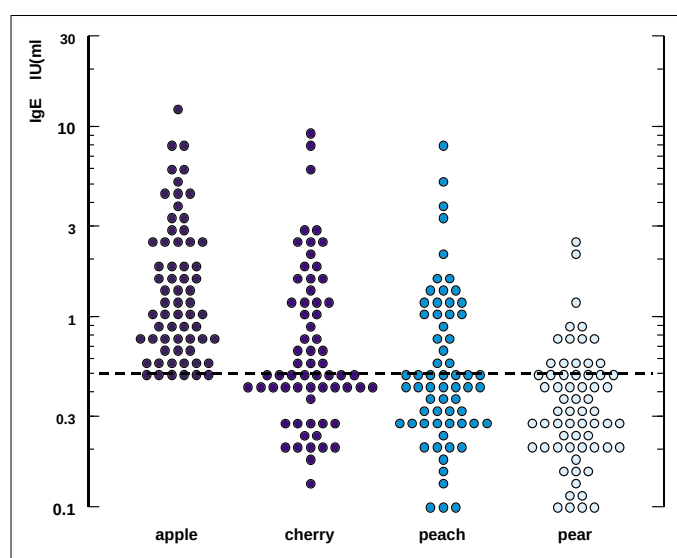


Figure 2. IgE reactivity of Bet v-positive sera with fruits. Sera ($n=90$) with a positive RAST for Bet v 1 (>1.0 IU/ml) and negative RAST for Bet v 2 (< 0.5 IU/ml) were tested in a RAST for apple, cherry, peach and pear. All sera ($n=64$) with a RAST > 0.5 IU/ml for one or more of the fruits are shown. Reactivity decreased in the following order: apple-cherry-peach-pear.

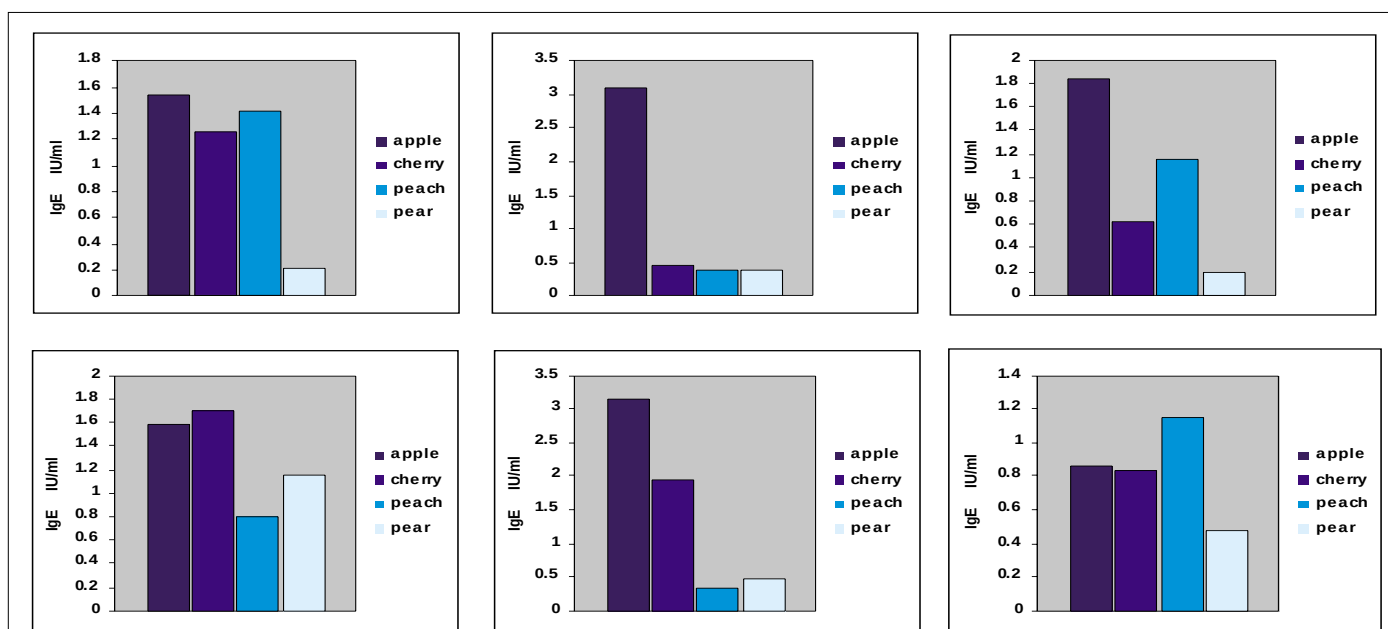


Figure 3. Different patterns of crossreactivity to fruits in Bet v 1-positive sera. Six individual sera are shown to illustrate the different patterns of crossreactivity that can be observed in Bet v 1-positive sera.

sera with > 1 IU/ml of specific IgE against Bet v 1 (mean 6.2; range 1.0–35.4), and < 0.5 IU/ml of IgE against Bet v 2. They were tested in a RAST for four fruits: apple, cherry, peach, and pear. Recognition decreased in this order (see Figure 2). There were no sera with IgE antibodies against cherry, peach or pear without IgE against apple. Different crossreactivity patterns were observed (see Figure 3). What is the clinical significance of such positive *in vitro* tests? Is a positive RAST always accompanied by clinical food allergy? In a study by Ebner and coworkers [7], approximately 75% of a group of birch pollen allergic patients with Bet v 1–Mal d 1 crossreactive IgE reported clinical allergy to apple. In an ongoing study focusing on the clinical relevance of crossreactive allergens, performed by our laboratory in collaboration with the Academic Hospital Utrecht, similar results are being found for apple and peach.

IgE antibodies against pollen profilins demonstrate an even broader spectrum of crossreactivity [25, 26], and have been indicated to play a role in the mugwort-celery-carrot-spice syndrome [27, 28]. In a group of 401 sera with IgE antibodies against pollen and/or vegetable foods, a RAST analysis was performed with purified profilin from birch pollen, grass pollen, and celery. Of these sera, 234 had a positive RAST (> 0.3 IU/ml) for birch profilin (mean 2.3; range 0.3–60.6), 178 for grass profilin (3.0; 0.3–46.0), and 74 for celery profilin (0.7; 0.3–3.9). No sera were found that selectively recognized celery profilin. Despite the high degree of homology between grass and birch profilin [29], clear individual differences in IgE reactivity were observed (see Figure 4). One hundred sera with a positive RAST for grass and/or birch profilin were also tested in a RAST for IgE to purified carrot profilin. IgE reactivity was about two times higher with carrot profilin than with celery profilin ($p < 0.001$). Responses to both profilins were closely correlated ($r_s = 0.7362$; $p = 2.5 \times 10^{-9}$). Again, marked individual differences were observed (see Figure 5). To compare spectra of crossreactivity, two sera with IgE antibodies against pollen

profilins were tested for IgE reactivity with a broad range of vegetable food profilins. Table 1 illustrates that clear individual differences exist. Both patients presented clinical food allergy (generalized urticaria). As in the case of Bet v 1-related positive food RASTs, however, positive RASTs for food profilins are no “guarantee” for food-related symptoms. Which factors influence the predictive value of the profilin RAST is currently under investigation in the above mentioned clinical study in collaboration with the Academic Hospital of Utrecht. At present, diagnostic tests for crossreactive pollen allergens like Bet v 1

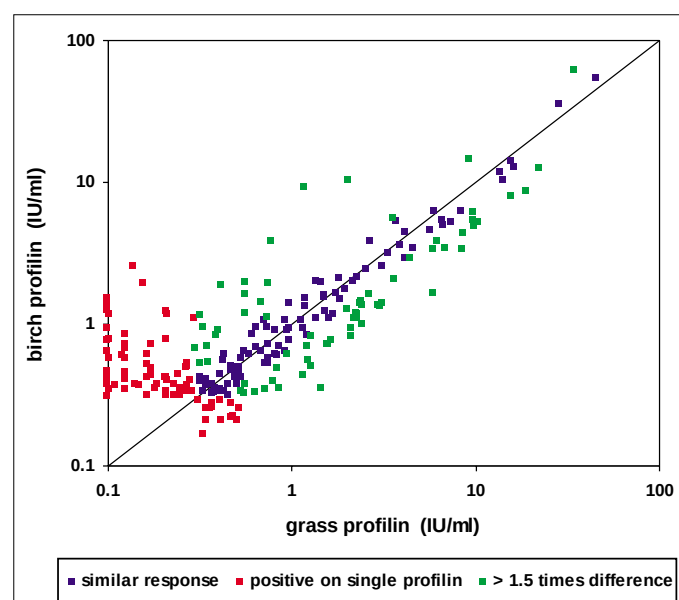


Figure 4. Comparison of IgE reactivity to purified profilin from birch pollen and grass pollen. RAST analysis ($n=249$) was performed with purified natural profilin from two different pollen: Birch (*Betula verrucosa*) pollen and grass (*Lolium perenne*) pollen. Differences up to almost a factor 10 were observed.

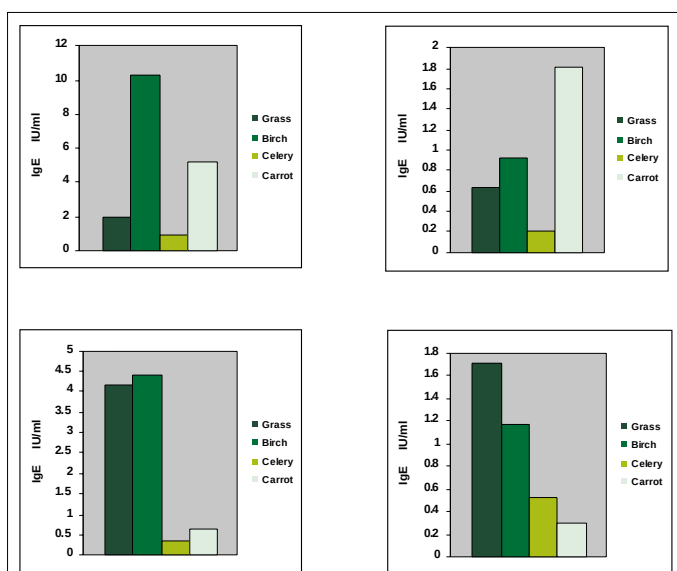


Figure 5. Different patterns of crossreactivity of sera reactive with pollen profilins. Four individual sera are shown to illustrate that different patterns of crossreactivity to pollen and food profilins exist.

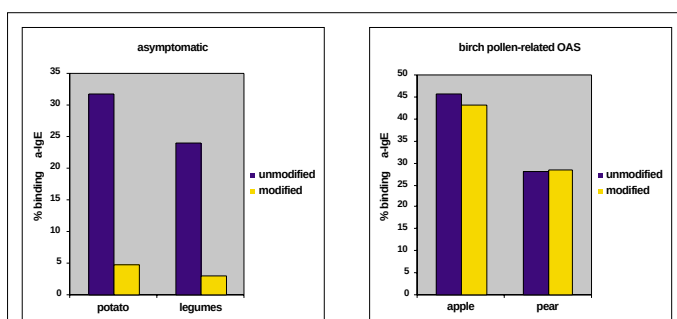


Figure 6. A modified RAST: No detection of anti-carbohydrate IgE antibodies. Grass-pollen extract was treated with proteinase K to destroy peptidic epitopes, but leaving carbohydrate determinants intact. Thus digested grass pollen extract was added during incubation of Sepharose-coupled allergen extract and serum (modified). In the left panel, a patient is shown who has grass pollen rhinitis and a positive RAST to a broad spectrum of vegetable foods (caused by anti-carbohydrate IgE) without clinical food allergy. It shows that the (false-)positive (unmodified) RAST becomes negative upon addition of digested grass pollen extract (modified). In the right panel, a patient with clinical food allergy (birch pollen-related OAS) is shown. The (true-)positive RAST remains unaffected.

Table 1
RAST Analysis of Two Sera for a Spectrum of Profilins

Food RAST	Serum A	Serum B
Carrot	29.1	9.2
Cucumber	23.1	3.1
Spinach	19.9	1.4
Avocado	18.6	14.9
Soy	16.0	4.3
Green pea	15.9	3.2
Peanut	15.2	5.1
Potato	14.3	5.6
Chervil	11.0	3.1
Apple	10.2	11.8
Tomato	8.1	3.2
Paprika	8.0	3.9
Orange	8.0	9.0
Almond	6.9	3.0
Hazelnut	6.7	2.0
Brussels sprouts	5.3	5.1
Coconut	4.5	6.0
Celery	2.9	4.2
Onion	2.5	6.8

RAST class 0 1+ 2+ 3+ 4+
blue shading

Sera were tested in a RAST with Sepharose-coupled poly L-proline incubated with food extracts. After pre-incubation of Sepharose and food extract, unbound material was washed out, resulting in different food-profilin Sepharoses. Results are expressed in IU/ml. It shows that crossreactivity spectra of both sera are different.

and profilin can be used for screening purposes. A positive test result serves as an indicator for follow-up investigations for the most likely foods to be involved.

Protease-Resistant Structures: Real Food Allergens or Harmless Structures?

In the early 1980s, the first reports appeared of IgE antibodies directed to carbohydrate structures on glycoproteins [30]. Such IgE antibodies are even more crossreactive than

those directed to profilin. Besides being found in vegetable foods, these crossreactive sugar determinants are also found in insect venoms and foods of invertebrate animal origin. IgE binding to carbohydrate determinants is periodate sensitive, but both protease and heat resistant. Resistance to proteases (stomach) and to heating (food-processing) are characteristics that would classify these IgE-binding structures as potentially severe food allergens. The opposite turns out to be true. Pollen allergic patients with anti-carbohydrate IgE have a positive RAST for a broad spectrum of foods, but do not present clinical food allergy [2, 3]. The explanation for this lack of biological activity of carbohydrate-specific IgE is still not known. Nevertheless, the observation has clear diagnostic implications: Sugar-specific IgE antibodies are the cause of many false-positive *in vitro* tests for food allergens. Avoiding detection in routine diagnostics would improve the positive predictive value considerably. A suitable method is the addition of a sugar-specific inhibitor during the incubation of allergen extract and serum. Protease-digested grass pollen extract inhibits the binding of carbohydrate-specific IgE to food extracts, but

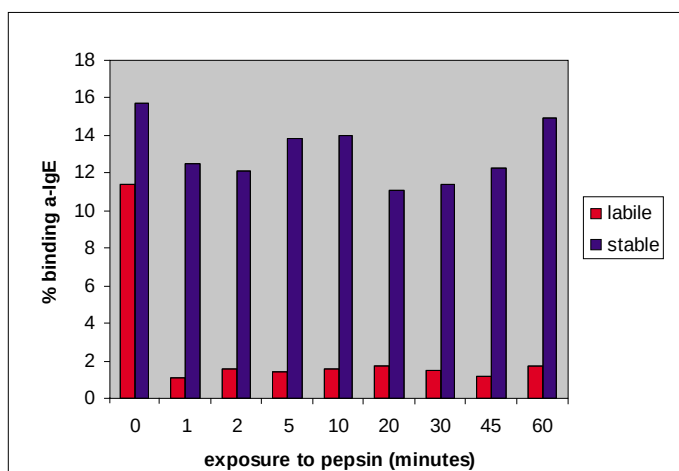


Figure 7. Different stability of food allergens during exposure to pepsin at low pH. Peach extract was exposed to 0.32% pepsin at pH 2. At different timepoints, a sample was taken and pH was adjusted to neutral to inactivate pepsin. All samples were coupled to Sepharose and tested in a RAST with two different sera, one reactive with Bet v 1 and homologous structures in fruits (red bars), the other with nonpollen-related allergen in peach (blue bars). The RAST analysis illustrates that the Bet v 1 homologue in peach loses its IgE-binding capacity within 1 min. The nonpollen-related allergen (causing more severe symptoms) is still fully capable of binding IgE after being exposed to pepsin at low pH for 1 h.

leaves the interaction with real (clinically relevant) food allergens untouched (see Figure 6).

Carbohydrate moieties are not the only IgE-binding structures that are protease resistant [31]. Pepsin is the main protease of the stomach. Bet v 1 homologues in fruits are digested in pepsin within seconds (see Figure 7). For this reason, patients with the classical birch-apple syndrome only suffer from OAS. The allergen just does not reach the intestines in an IgE-binding conformation. In contrast, allergens in foods that cause severe systemic symptoms [32] probably have a virtually unchanged IgE-binding capacity after prolonged exposure to pepsin at low pH (see Figure 7). Therefore, treatment of food extracts with pepsin is a way to distinguish mild (OAS) and potentially severe food allergens (see Figure 8). Introduction of pepsin-treated extracts to *in vitro* diagnostic tests might improve clinical relevance of these tests.

Concluding Remarks

In which directions will the development of food allergy diagnostics go? Purified (natural or recombinant) allergens will become increasingly important. They are more stable than food allergen extracts. Furthermore, the use of purified allergens will potentially increase the clinical significance of diagnostic tests. This can be illustrated by looking at the important food allergen peanut. Over the past decade, several major peanut allergens have been identified, some of which have been cloned and expressed: Ara h 1, Ara h 2, and Ara h 3 [33]. All three were

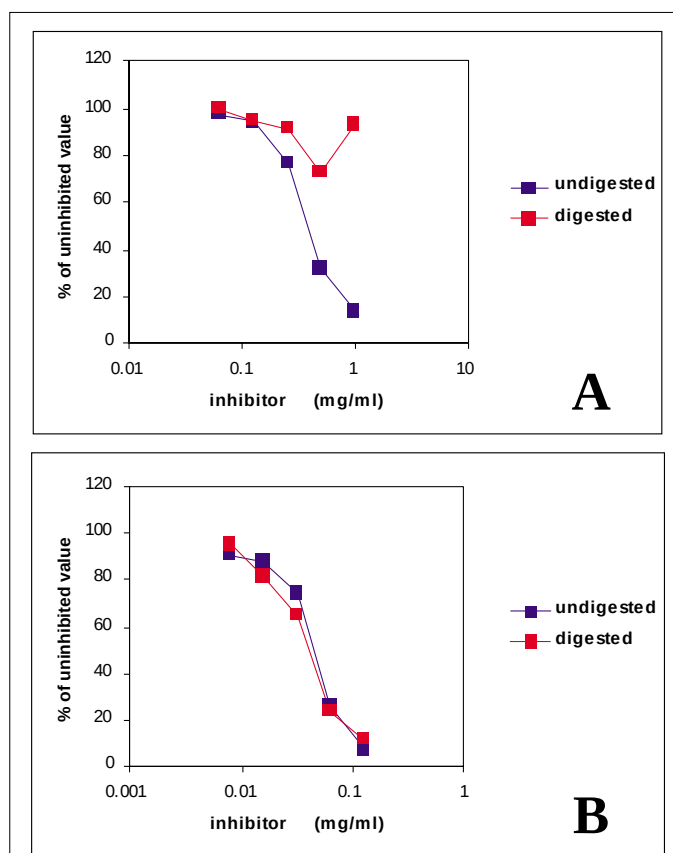


Figure 8. RAST inhibition with pepsin-digested food extract.

Serum of a patient with birch pollen-related OAS to apple and peach, and of a patient with nonpollen-related allergy to apple and peach (anaphylaxis) was tested in a peach RAST inhibition. Inhibition was performed with peach extract and with pepsin-digested peach extract (1 h digestion). It is shown that allergen recognized by the OAS patient has lost most of its inhibitory potency, whereas allergen recognized by the other patient is unaffected.

shown to be recognized by patients with “real” peanut allergy. In other words, these allergens are linked to a potentially life-threatening form of food allergy. Patients can, however, also be tested positive for peanut on the basis of the recognition of pollen-crossreactive structures [2, 26]. In these cases, less severe symptoms (OAS), or no symptoms at all, are to be expected. By testing on whole peanut extract, such different clinical presentations can not be distinguished. In the (near) future it should be possible to test sera on different purified allergens, each with their own (potential) clinical presentation. Detection of the clinically irrelevant IgE antibodies against carbohydrate structures (in whole extracts or on purified glycoproteins) can be avoided by addition of a soluble inhibitor to allergen reagents. The number of false-positive tests will decrease with this modification. If well-characterized purified (natural or recombinant) allergens are not (yet) available, discrimination between potentially severe and mild allergens in food extracts can be achieved by treating whole allergen extracts with pepsin prior to their application in diagnostic tests.

In conclusion, the current knowledge of food allergens opens several routes for the improvement of food allergy

diagnostics, both *in vivo* and *in vitro*. Whether the improvements will justify replacement of the DBPCFC as the golden standard for food allergy diagnosis remains to be seen.

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Ronald van Ree (to whom correspondence should be addressed), Jaap H. Akkerdaas, W. Astrid van Leeuwen, and Rob C. Aalberse are with the CLB and Laboratory for Experimental and Clinical Immunology, Department of Allergy, Academic Medical Centre, PO Box 9190, NL-1006 AD Amsterdam, The Netherlands (tel. +31 20 512-3242, fax +31 20 512-3170, e-mail r_van_ree@clb.nl). Montserrat Fernández-Rivas is with the Hospital Fundacion Hospital Alorcon, Unidad de Alergia, Alorcon, Spain. Riccardo Asero is with the Ospedale Caduti Bollatesi, Ambulatorio di Allergologia, Bollate, Italy. Vlasta Knul-Brettlova is with the Prinsengracht Ziekenhuis, Department of Allergology, Amsterdam, The Netherlands. André Knulst is with the Academic Hospital Utrecht, Department of Dermatology and Allergology, Utrecht, The Netherlands.