

IgE Binding to Pepsin-Digested Food Extracts

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Key Words

Pepsin digestion · Food allergy · Stable allergens · Lipid transfer protein

Abstract

Background: Pepsin resistance of allergens like lipid transfer protein and 2S albumin has been suggested as explanation for the severity of symptoms often induced by these allergens. Component-resolved diagnosis with purified labile and stable allergens has therefore been proposed to better characterize the risk involved in a positive in vitro IgE test. However, for many foods, purified allergens are not (yet) available. **Objective:** It was the aim of this study to evaluate the potential of pepsin-digested whole-food extracts to distinguish between IgE responses to stable (potentially severe) and labile (mild) allergens. **Methods:** Sera (n = 143) from Italian, Spanish and Dutch patients with hazelnut and/or apple ingestion-related symptoms were analyzed for residual IgE binding to pepsin-resistant hazelnut and/or apple allergens. Control and pepsin-digested hazelnut and apple extracts were used for radioallergosorbent test analysis and immunoblot analysis. **Results:** Pepsin digestion of food ex-

tracts, like from hazelnut and apple used for in vitro diagnostic tests, provides a way to distinguish sensitization to pepsin-resistant allergens from that to pepsin-susceptible allergens. In this selected group of patients, IgE reactivity to pepsin-digested extracts correlated with sensitization to the stable allergen lipid transfer protein. The analysis further revealed that the use of soluble pepsin can result in false-positive in vitro tests (2/143). **Conclusion:** Pepsin-digested food extracts are a convenient tool to identify patients with IgE antibodies against potentially dangerous stable allergens, in particular for those foods where the relevant stable allergens have not yet been identified. This can increase the clinical prognostic value of food allergy serology.

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Introduction

Essentially, food allergies can have two appearances, i.e. a cross-reactive phenomenon linked to inhalant allergies or a true food allergy. The best characterized example of the former is allergy to fruits, nuts and some vegetables in patients with birch pollinosis [1, 2]. The characteristic

of true food sensitization is that the sensitization is caused by consumption of the food, i.e. independent from sensitization by the airways [3, 4].

A prerequisite for food allergens to be able to sensitize is that they are (at least partly) resistant to proteolytic attack in the gastro-intestinal tract. It is generally accepted that this stability also determines whether a food allergen is capable to cause severe systemic reactions. The most important and first attack that a food allergen has to endure is in the stomach by gastric fluid containing pepsin. Since allergen stability is a decisive factor in the process of sensitization and determines the potential of a protein to induce severe food allergy, pepsin resistance of food allergens has drawn a lot of attention [5]. So far, no convincing data are available indicating that pollen-derived pepsin-resistant allergens like lipid transfer proteins (LTP) [6] and thaumatin-like proteins [7] act as primary sensitizing agents and subsequently cause true food allergy.

Introduction of novel proteins into crops has become an important technique in agriculture to improve crop yields and quality. Before genetically-modified organisms can enter the market, an in-depth safety assessment is required [8]. One of the elements of such an assessment is a check for potential allergenicity of the transgene [9]. Evaluation of pepsin resistance is one of the requirements [10], because allergen stability clearly is a risk factor. In most cases, pepsin resistance of a novel food protein is only assessed by SDS-PAGE analysis. Persistence of IgE recognition is less commonly evaluated.

Since pepsin-resistant allergens have been implicated to play an important role in determining the severity of a food allergic reaction [4, 11], the use of pepsin-digested food extracts for in vitro diagnosis of food allergy has been proposed. IgE reactivity to pepsin-digested food extracts would be a risk factor for severe food allergy.

Theoretically, the digestion of proteins with pepsin might alter the tertiary structure of the protein from 'non-sensitizing' to 'sensitizing' and subsequently from 'non-IgE binding' to 'IgE binding'. In general, measurement of IgE against these so-called 'neo-epitopes' is hampered, since extracts or purified proteins used for in vitro diagnosis are not digested by pepsin.

In this study, we have evaluated the use of pepsin digestion of food extracts for application in radioallergosorbent test (RAST) analyses. We have confirmed that patients can indeed be subdivided into two groups according to the degree of recognition of stable allergens. Patients were predominantly identified with IgE against pepsin-susceptible allergens and with IgE against pepsin-resis-

tant allergens. The analysis has also revealed that in rare cases, IgE reactivity to porcine pepsin can be mistaken for IgE reactivity to stable food allergens. Therefore, the use of immobilized pepsin for digestion has preference over soluble pepsin.

Material and Methods

Serum Samples

Sera of 143 subjects sensitized to hazelnut and/or apple with hazelnut and/or apple ingestion-associated clinical symptoms were used to study the influence of pepsin digestion on IgE binding to these foods. Sera were retrospectively selected from serum panels still available from earlier studies on plant food allergies [12–14]. The selection included 118 serum samples from Utrecht (The Netherlands) of patients that had birch pollen-related hazelnut allergy (oral allergy syndrome; OAS), 20 serum samples from Bollate (Northern Italy) of patients with symptoms (OAS and/or more severe symptoms) after hazelnut and/or apple ingestion (14 positive for hazelnut and apple, 1 only for apple and 5 only for hazelnut), and 5 serum samples from Avila and Madrid (Spain) with apple-related symptoms (OAS and, in some cases, anaphylaxis). The studies were reviewed and approved by the local Ethics Committees. All patients gave written consent before enrolment in the study.

Two additional Dutch sera from patients with a clinical history of hazelnut allergy were selected as representative sera for immunoblot analysis. One was a typical birch pollen (Bet v 1) cross-reactive patient, the other had IgE antibodies against LTP.

Hazelnut and Apple Extracts

Hazelnut and apple extracts were prepared as described previously, essentially following the protocol described by Björkstén et al. [16]. In brief, grounded hazelnut or apple was extracted in a buffer containing polyvinylpyrrolidone (PVPP) and diethyldithiocarbamate. These reagents prevent loss of allergenicity by inhibiting oxidative processes mediated by polyphenoloxidases upon disruption of the plant tissue. Polyphenols adsorb proteins and thereby prevent oxidative processes by steric hindrance. In particular, Bet v 1-related food allergens (i.e. Cor a 1 in the case of hazelnut and Mal d 1 in the case of apple) have been shown to be lost due to these processes.

Hazelnut and apple extract were treated with pepsin, generally as described previously [5]. In brief, simulated gastric fluid containing 0.32% (w/v) pepsin (Sigma, St. Louis, Mo., USA) or an equivalent coupled to agarose (Sigma) was used to digest hazelnut or apple extract (1 mg/ml protein) at pH 2. After 1 h incubation at 37°C, the reaction was stopped by adjusting the pH to 7. In order to avoid interference of pepsin during subsequent analysis, the hazelnut protein was also digested with agarose-immobilized pepsin (Sigma).

RAST and RAST Inhibition

RAST was performed as described previously [17]. In brief, 100 µg recombinant carrot LTP (kindly provided by Dr. S.C. de Vries, Wageningen University and Research Centre, The Netherlands) or 4 mg hazelnut or apple extract (undigested or digested) was coupled to 100 mg Sepharose (Amersham Pharmacia Biotech,

Uppsala, Sweden). Fifty microliters serum was incubated overnight with 0.5 mg Sepharose in a final volume of 300 μ l PBS/0.3% BSA/0.1% Tween 20 (PBS-AT). After washing away unbound material with PBS/0.1% Tween 20, bound IgE was detected by overnight incubation with ^{125} I-radiolabeled sheep anti-human IgE. After washing, bound radioactivity was measured in a γ -counter. Results were expressed in international units (IU) IgE per milliliter using a standard curve of chimeric IgE anti-Der p 2 and Sepharose-coupled recombinant Der p 2 [18].

For RAST inhibition, 50 μ l serum was pre-incubated with 220 μ g (boiled) pepsin or 210 μ g hazelnut extract (in 50 μ l PBS-AT) for 2 h prior to the addition of Sepharose-coupled pepsin-digested hazelnut extract (1.5 mg Sepharose) in a final volume of 350 μ l PBS-AT. The rest of the procedure was as described for the RAST.

SDS-PAGE/Immunoblotting

For immunoblotting, hazelnut extract (pepsin digest) was separated under reducing conditions by SDS-PAGE (5 μ g/lane) on a Novex XCell II system using a 4–12% gel (Novex, San Diego, Calif., USA), followed by immunoblotting to nitrocellulose according to the manufacturer's instructions.

Basophil Histamine Release Bioassay

The stripped basophil histamine release bioassay was performed as described previously [19].

Skin Prick Test

Skin prick test with pepsin (range 0.002–1 mg/ml) was performed as described previously [2] on the flexor aspect of a patient's forearm with a standardized prick needle (ALK Lancet) according to Dreborg and Frew [20].

Results

Apple and hazelnut extracts were treated with pepsin for 1 h and subsequently coupled to Sepharose. Both were compared with untreated immobilized extracts by RAST with serum samples of subjects sensitized to apple and hazelnut, respectively. Both in the case of hazelnut RAST results (fig. 1a) and apple RAST results (fig. 1b), IgE reactivity was divided into sensitive (<40% of IgE binding retained) and resistant (>40% retained) to pepsin digestion.

Among the 11 Italian sera with sensitization to both apple and hazelnut, all but two retained >40% of their IgE reactivity to both pepsin-digested apple and pepsin-digested hazelnut extract.

Sera (n = 32) were also tested for IgE reactivity to LTP. Among sera that were classified as 'sera with IgE against allergens resistant to pepsin digestion', 17/32 tested were LTP positive. No sera with IgE against pepsin-sensitive allergens were found to be LTP positive. Only 2/25 Italian and Spanish sera lost >40% of IgE against pepsin-re-

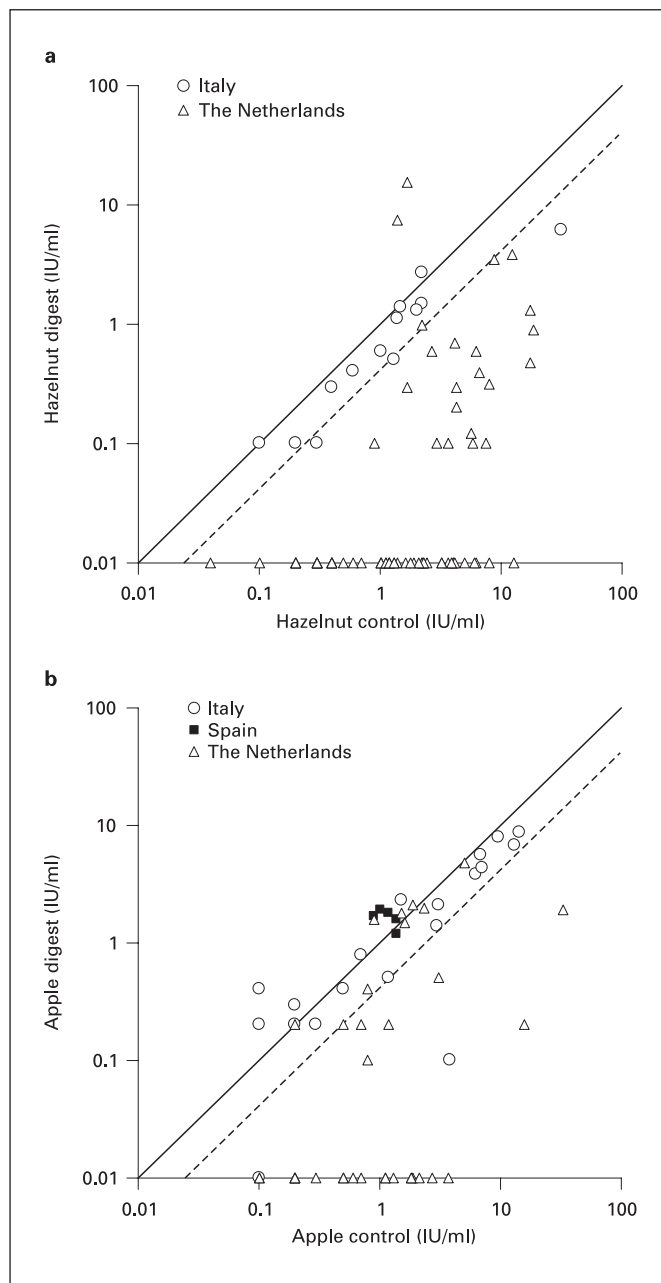


Fig. 1. **a** RAST results obtained with Italian sera (n = 15) and Dutch sera (n = 72). Hazelnut (control) or pepsin-digested hazelnut was coupled to Sepharose. The dotted line indicates a digest/control ratio of 0.4. A ratio <0.4 indicated the presence of IgE against predominantly pepsin-susceptible hazelnut allergens. IgE reactivity <0.01 IU/ml was set to 0.01 IU/ml. **b** RAST results obtained with Italian sera (n = 19), Dutch sera (n = 35) and Spanish sera (n = 5). Apple (control) or pepsin-digested apple was coupled to Sepharose. The dotted line indicates a pepsin digest/control ratio of 0.4. A ratio <0.4 indicated the presence of IgE against predominantly pepsin-susceptible apple allergens. IgE reactivity <0.01 IU/ml was set to 0.01 IU/ml.

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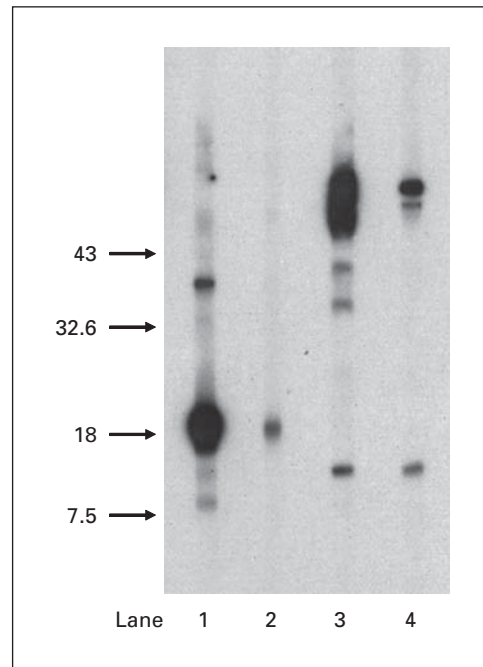


Fig. 2. Hazelnut PVPP extract (odd lanes) and immobilized pepsin-digested hazelnut PVPP extract (even lanes) were subject to SDS-PAGE (4–12%), followed by immunoblotting with serum No. 200 (lanes 1, 2) and serum No. 768955 (lanes 3, 4). Bound IgE was detected with ^{125}I -radiolabeled sheep anti-human IgE in conjunction with autoradiography.

Fig. 3. Hazelnut PVPP extract (lane 1) and (soluble) pepsin-digested hazelnut PVPP extract (lane 2) was subjected to SDS-PAGE (4–12%), followed by immunoblotting with serum 2 (1.4 IU/ml IgE anti-hazelnut, 15.6 IU/ml IgE anti-pepsin-digested hazelnut).

3

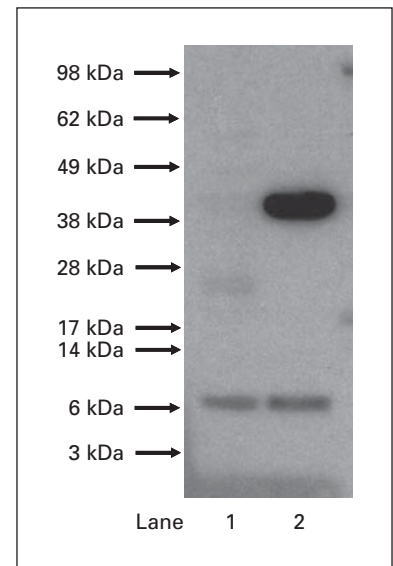


Table 1. Prevalence of IgE against pepsin-resistant allergens was studied by RAST with sera from Italy, the Netherlands and Spain

Sera		Hazelnut ratio digest/control		Apple ratio digest/control		Carrot LTP >0.4 IU/ml and any ratio ≥ 0.4
		<0.4	≥ 0.4	<0.4	≥ 0.4	
Italian	20	9/20 (45.0)	11/20 (55.0)	2/19 (10.5)	17/19 (89.5)	10/18 (55.6)
Dutch	118	74/78 (94.9)	4/78 (5.1)	31/40 (77.5)	9/40 (22.5)	4/9 (44.4)
Spanish	5	–	NT	0/5 (0.0)	5/5 (100.0)	3/5 (60.0)

Figures in parentheses are percentages. The ratio between residual IgE binding to pepsin-resistant hazelnut and apple allergens and IgE against undigested hazelnut and apple extract was calculated. Furthermore, 32 sera with IgE against stable allergens were analyzed for carrot LTP-specific IgE. NT = Not tested.

sistant apple allergens; for the Dutch sera, this was the case in 31/40 (table 1).

To illustrate the sensitivity of IgE binding to pepsin digestion of hazelnut extract for patients with IgE antibodies against Cor a 1 and the contrasting resistance for patients with IgE against Cor a 8 (LTP), an immunoblot was performed (fig. 2). Clearly, the strong band observed for Cor a 1 disappears almost completely upon pepsin digestion. The LTP band is unaffected.

Two serum samples from hazelnut-sensitized Dutch patients demonstrated low (1.7 and 1.4 IU/ml) IgE reac-

tivity to hazelnut extract and high IgE reactivity (7.5 and 15.6 IU/ml) to pepsin-digested hazelnut extract in RAST (fig. 1a). Immunoblot analysis (fig. 3) of the second serum revealed the appearance of a very strong band at approximately 40 kDa in the pepsin-digested hazelnut extract, close to the molecular mass of porcine pepsin. This band was not observed in the undigested extract. The results strongly suggested that this serum contained IgE against pepsin. This was confirmed by a hazelnut digest RAST inhibition which was completely inhibited with pepsin but not with hazelnut extract (data not shown). IgE against

porcine pepsin was biologically inactive, as was demonstrated by a negative basophil histamine release test and skin prick test.

Discussion

This study analyzed anti-food IgE responses against pepsin-resistant allergens and pepsin-susceptible allergens. The study focused on IgE responses to apple and hazelnut. Allergy to both foods belongs to the typical birch pollen-related food allergies that are common in Northern and Central Europe. The major birch pollen allergen Bet v 1 plays a dominant role in this cross-reactive syndrome. Food allergies linked to sensitization to Bet v 1 are characterized by mild reactions, almost without exception. This is explained by the nature of food homologues of Bet v 1, like Mal d 1 from apple or Cor a 1 from hazelnut, which are rapidly degraded in the gastrointestinal tract [21–24]. In contrast, fruit and tree nut allergy in Mediterranean countries is rarely linked to birch pollen allergy and its clinical presentation is often more severe. This has been explained by the important role of sensitization to the stable food allergen LTP [15, 25]. Although the retrospective selection of Italian and Spanish sera for this study was certainly biased by the interest of researchers in LTP sensitization, it is now well accepted that sensitization to LTP is much more common in Southern Europe [26].

The clinical background of many of the patients in this study was not completely documented. The retrospective character of inclusion also meant that the recording of symptoms lacks uniformity. However, it is now well accepted that sensitization to stable allergens is a risk factor for severe systemic reactions [3, 14]. For some of the Spanish and Italian patients, severe reactions were indeed reported. Some of the patients were also part of studies that were published earlier [13, 14]. The majority of the Dutch patients demonstrated only OAS [12], which is the typical presentation of fruit and tree nut allergy in patients with birch pollinosis. In line with this clinical background, the majority of serum samples from Dutch patients lost most of their IgE reactivity to apple and hazelnut after pepsin digestion. The relevance of pepsin digestion as a tool to identify IgE responses to stable allergens was confirmed by the association between a positive response to LTP and to pepsin digest of whole-food extracts. A correlation of $R_s = 0.896$ was found for IgE against carrot LTP and pepsin-digested apple extract. Of course, also pepsin-resistant allergens other than LTP can

be involved in IgE binding. When allergenic extracts are analyzed for stable allergens by means of pepsin digestion, it is important to realize that soluble pepsin might interfere with IgE binding. In that case, application of immobilized pepsin is preferred.

Where individual pepsin-resistant (e.g., LTPs like Cor a 8, Mal d 3) and pepsin-sensitive (e.g., Cor a 1, Mal d 1) allergens are available, the use of pepsin-digested food extracts is no longer of much interest. In that case, component-resolved diagnosis [27, 28] with purified (natural or recombinant) allergens is the correct method. However, pepsin-digested food extracts are very useful additions for food allergy diagnosis in cases where purified stable and labile major food allergens are not yet available at all or only partly.

In summary, this study has confirmed that the use of pepsin-digested food extracts is a good alternative to the application of purified allergens as long as these reagents are not available. It offers the possibility to make a better assessment of the risk of a positive diagnostic test for food allergy with respect to potential severity of clinical reactions.

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