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IgE epitopes of intact and digested Ara h 1: A comparative study in humans and rats

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ABSTRACT

Background: Allergen epitope characterization provides valuable information useful for the understanding of proteins as food allergens. It is believed that IgE epitopes in general are conformational, nevertheless, for food allergens known to sensitize through the gastrointestinal tract linear epitopes have been suggested to be of great importance.

Objective: The aim of this study was to identify IgE specific epitopes of intact and digested Ara h 1, and to compare epitope patterns between humans and rats.

Methods: Sera from five peanut allergic patients and five Brown Norway rats were used to identify intact and digested Ara h 1-specific IgE epitopes by competitive immunoscreening of a phage-displayed random hepta-mer peptide library using polyclonal IgE from the individual sera. The resulting peptide sequences were mapped on the surface of a three-dimensional structure of the Ara h 1 molecule to mimic epitopes using a computer-based algorithm.

Results: Patients as well as rats were shown to have individual IgE epitope patterns. All epitope mimics were conformational and found to cluster into three different areas of the Ara h 1 molecule. Five epitope motifs were identified by patient IgE, which by far accounted for most of the eluted peptide sequences. Epitope patterns were rather similar for both intact and digested Ara h 1 as well as for humans and rats. **Conclusions:** Individual patient specific epitope patterns have been identified for the major allergen Ara h 1. IgE binding epitopes have been suggested as biomarkers for persistency and severity of food allergy, wherefore recognition of particular epitope patterns or motifs could be a valuable tool for prevention, diagnosis, and treatment of food allergy.

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1. Introduction

Peanut allergy is one of the most persistent and severe forms of food allergy (Emmett et al., 1999; Sicherer et al., 2001, 1999)

Abbreviations: 3D, 3-dimensional; A, anaphylaxis; AP, alkaline phosphate; BN, Brown Norway; BO, bronchial obstruction; D, digested; EMT, epitope mapping tool; F, female; GI, gastro-intestinal symptoms; I, intact; M, male; OE, oral oedema; PBS-T, PBS Tween 20; RBL, Rat Basophilic Leukemia; RPB, reduced blood pressure; RT, room temperature; SMP, skimmed milk powder; U, urticaria.

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and is mediated by allergen-specific IgE molecules, which bind to high affinity receptors on mast cells or basophils. Cross-linking of receptor-bound IgE by specific allergen, leads to degranulation and mediator release of the effector cells (Holowka and Baird, 1996; Lin and Sampson, 2009). For a food protein to be a 'complete' allergen (Aalberse, 2000, 1997) it must therefore be multivalent, i.e. expressing a minimum of two IgE-binding epitopes.

Since most IgE binding epitopes are thought to be conformational (Aalberse, 2000; Barlow et al., 1986; Roggen, 2006; Van Regenmortel, 1996), the epitope profile of a globular protein is greatly influenced by the micro-environment. Thus, changes in e.g. pH, ionic strength, or binding to other molecules may affect the number of epitopes that are accessible for the antibodies by tightening or loosening the protein structure. In the latter case, new epitopes (neo-epitopes) may be observed. Such neo-epitopes are believed to be very prominent after proteolytic cleavage of the protein, as occur during digestion (Rich et al., 2001).

Different methods for identifying epitopes exist. Linear epitopes can be identified by analysis of IgE binding to peptides derived from the primary sequence (Burks et al., 1997; Lai et al., 2004; Roggen, 2006; Stanley et al., 1997). However, identification of conformational epitopes requires more elaborated methods, such as X-ray crystallography of antibody–antigen complexes (Niemi et al., 2007; Spangfort et al., 2003) or site-directed mutagenesis (Karisola et al., 2004; Lai et al., 2004; Spangfort et al., 2003). A promising alternative to these methods is competitive immunoscreening of a phage-displayed library of random oligopeptides to select epitope mimicking peptides followed by computer-based mapping of the identified peptide sequences (Mittag et al., 2006; Roggen, 2006). Epitope mimics are small peptides, which mimics binding sites of the protein and are able to compete with the native protein for antibody binding (Meloan et al., 2000).

The peanut protein Ara h 1 is a major allergen, which is recognized by serum IgE from more than 80% of peanut allergic patients (Burks et al., 1997, 1991). In its native form Ara h 1 is a 63.5 kDa protein that forms stable homotrimers maintained by hydrophobic interactions between amino acids at the monomer-monomer contact points (Maleki et al., 2000; Shin et al., 1998). Both linear and conformational IgE binding epitopes of Ara h 1 have been mapped. Ditto et al. (2010) presented a case report supporting the presence of conformational Ara h 1-specific IgE epitopes. In addition, Ara h 1 are suggested to consist of 23 independent linear binding sites ranging from 6 to 10 amino acids in length (Burks et al., 1997) and a single linear binding site consisting of 25 amino acids (Shreffler et al., 2004), that have no obvious sequence motifs (a sequence pattern of amino acids) shared by them. Individual patients with IgE antibodies to Ara h 1 were shown to have IgE that recognized multiple epitopes on the Ara h 1 molecule, and of these epitopes four of them appeared to be immunodominant in that they were recognized by sera from more than 80% of the patients tested and bound more IgE than any other Ara h 1 epitopes. The 24 linear epitopes were evenly distributed along the entire primary sequence of the molecule (Burks et al., 1997; Shreffler et al., 2004). However, a molecular model of the tertiary structure of the Ara h 1 protein showed that the IgE epitopes were clustered into two main regions, the same regions where Ara h 1 monomer-monomer contact points are located (Maleki et al., 2000; Shin et al., 1998). Such reports, that the majority of the 24 linear IgE binding epitopes were suggested to be located in these hydrophobic contact sites led to the assertion that they may be protected from digestion, and that the quaternary structure of the Ara h 1 protein may play a significant role in its allergenicity (Maleki et al., 2000; Shin et al., 1998). Additionally, it is suggested that the structure may be so compact that potential cleavage sites are inaccessible until the protein is denatured and that the formation of a trimeric complex and further higher order aggregation may also afford the molecule some protection from protease digestion and denaturation (Shin et al., 1998).

Studies on the in vitro digestibility of Ara h 1 have produced rather divergent results. Becker (1997) reported that Ara h 1 was completely resistant to in vitro digestion. In contrast, Maleki et al. (2000) and Vieths et al. (1999) reported that it was digested but to relatively large MW peptide fragments only. Finally, the protein was reported to be easily digestible producing peptide fragments with maximum sizes as small as 2 kDa (Bøgh et al., 2009; Eiwegger et al., 2006; Kopper et al., 2004). The contradicting reports appearing in the literature is probably a result of different digestion conditions. However, although Ara h 1 was broken down to small peptide fragments, IgE binding epitopes survived the digestion process (Bøgh et al., 2009; Eiwegger et al., 2006). Digested Ara h 1 was shown to retain both sensitizing and eliciting capacity, which was suggested to be a result of the small peptide fragments aggregating to complexes of larger sizes (Bøgh et al., 2009). This notion is supported by a recent study, further indicating that the way in which

the peptide fragments of digested Ara h 1 aggregate, is of major importance for the sensitizing capacity and suggesting that the peptide fragments is in an aggregated state resembling the intact Ara h 1 (Bøgh et al., 2012).

Susceptibility to digestion is an important issue since a characteristic feature of allergenic proteins in food is believed to be resistance to digestion (Astwood et al., 1996; FAO/WHO, 2001). Presently, it is recommended to include digestibility in risk assessment of novel proteins introduced into genetically modified food (FAO/WHO, 2001; EFSA, 2010).

This study was performed to investigate the impact of in vitro digestion on the IgE epitope profile of Ara h 1 as revealed by humans and rats. This was accomplished by competitive immunoscreening of a phage-displayed random peptide library using polyclonal IgE from individual patient and rat sera. The resulting peptide mimics were mapped on the surface of a model of the 3-dimensional (3D) structure of the Ara h 1-monomer using a computer-based algorithm.

2. Material and methods

2.1. Allergens

Ara h 1 was purified as described by Eiwegger et al. (2006), and digested as described by Bøgh et al. (2009). In short, purified Ara h 1 was digested in an in vitro gastro-duodenal model, designed to simulate the human digestion process, as described by Moreno et al. (2005a,b). Digestion was performed with immobilized enzymes to make enzyme-free digests suitable for animal sensitization. The digestion process was divided into two phases, a gastric phase where intact Ara h 1 was digested with immobilized pepsin for 120 min at pH 2.5 and a duodenal phase where the gastric digests were further digested with immobilized trypsin and chymotrypsin for 15 min at pH 6.5.

2.2. Allergen characteristics

Purity of Ara h 1 was confirmed by reverse phase high-performance liquid chromatography to be >99%.

Ara h 1 was digested to small peptide fragments, with sizes ≤ 2 kDa, of which more than 85% of the peptides had masses between 0.5 and 1.5 kDa, demonstrated by mass spectrometry. From gel permeation chromatography it was indicated that more than 50% of the peptide fragments occurred in aggregated complexes of up to M_r 20,000, as described in Bøgh et al. (2009).

2.3. Peanut allergic patient sera

Sera from five individual patients (patient no.: 2205, 2208, 2209, 2304, and 2305) with clear-cut peanut allergy diagnosed by an experienced allergologist were used. These patients had convincing history of peanut anaphylaxis (severe, potentially fatal, systemic allergic reaction that occurred suddenly after contact with peanut) or clear peanut exposure-related symptoms (urticaria, itching of the skin, gastrointestinal symptoms) within the last 12 months (Table 1). Due to severity and clear-cut exposure-related recent reactions a DBPCFC was not justified from an ethical point of view.

All sera were tested positive for peanut extract-specific IgE by ImmunoCAP and Ara h 1-specific IgE by ELISA (Eiwegger et al., 2006). The protocol was approved by the local Ethical Committee of the Medical University of Vienna and written informed consent was obtained (protocol no. EK 428/2008).

Table 1
Patients' characteristics.

ID	Anti-whole peanut IgE (IU/L)	Anti-Ara h1 IgE (IAU/L)	Total IgE (kU/L)	Clinical symptoms after peanut consumption	Age	Gender
2205	45	29	535	A, OE, BO, GI	25	M
2208	18	10	26.9	A, OE, GI	23	M
2209	250	91	>2000	A, RBP, BO	27	F
2304	327	262	1013	A, BO, GI	25	M
2305	155	129	>2000	A, RBP, OE, U, GI	18	F

A: anaphylaxis, BO: bronchial obstruction, GI: gastro-intestinal symptoms, OE: oral oedema, RBP: reduced blood pressure, U: urticaria, M: male, F: female.

2.4. Brown Norway rat sera

Sera from five individual Brown Norway (BN) rats immunized three times i.p. with either 200 µg purified intact Ara h 1 (rat no.: 49, 52, 53, and 54) or 200 µg gastro-duodenal digestion products hereof (rat no.: 19) without the use of adjuvant were used. The BN rats were kept on a diet free from *leguminosa* for at least three generations.

All sera were tested positive for intact and digested Ara h 1-specific IgE by ELISA and Rat Basophilic Leukemia (RBL)-assay. From the RBL-assay it was also evident that IgE was biologically functional being able to induce degranulation of RBL cells with both intact and digested Ara h 1 (Bøgh et al., 2009). Animal experiments were carried out at the National Food Institute (DTU, Denmark) facilities and performed under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

2.5. Selection of phage-displayed IgE epitope mimicking peptides

IgE epitope mimicking peptides were identified by competitive immunoscreening of phage-displayed libraries of random oligopeptides essentially described by Mittag et al. (2006), which allows for detection of both linear and conformational epitopes. In short, 4 × 500 µL aliquots of tosyl-activated M280 Dynabeads suspension (Dyna Biotech, Oslo, Norway) were each coated with 25 µg rabbit-α-human IgE (DAKO, Glostrup, Denmark) or mouse-α-rat IgE (Oxford Biotechnology, Kidlington, UK) for 48 h at 37 °C, and blocked with 0.5% (w/v) skimmed milk powder (SMP) in Phosphate Buffer Saline (PBS). Patient serum (1000 µL) or rat serum (500 µL) was diluted in PBS with 0.5% SMP (w/v) and 0.05% (w/v) Tween 20 (dilution buffer), and incubated overnight at 4 °C with 400 µL or 200 µL coated bead suspension, respectively. After extensive washing, 2 × 50 µL (for human) or 2 × 25 µL (for rat) of the coated beads were used immediately for the first round of selection, while the remaining 300 µL or 150 µL, respectively, were restored for later selection rounds.

2.5.1. First selection round

50 µL or 25 µL beads with immobilized IgE were blocked in 1 mL of 2% (w/v) SMP in PBS for 1 h at room temperature (RT) and washed extensively. The beads were then incubated overnight with 10 µL (~2 × 10¹¹) phages from a library of phages displaying linear random hepta-mer peptides (Ph.D.-7 Phage Display Peptide Library Kit, New England BioLabs, Beverly, MA, USA), followed by extensive washing and negative selection to remove unbound phages. For positive selection, phages were eluted by competitive immunoscreening, by adding 25 µg allergen (intact or digested Ara h 1). Eluted phages were amplified by direct infection of ER2738 *Escherichia coli* cells (provided with the phage library). Phage amplification was allowed overnight at 37 °C. Amplified phages were purified by PEG/NaCl-precipitation and tittered.

2.5.2. Second selection round

50 µL or 25 µL beads with immobilized IgE were incubated with ~10⁹–10¹⁰ amplified phages selected in the first round. All further steps were performed as described for the first selection round.

2.5.3. Third selection round

Third selection round took place as described for the second, besides that eluted phages were not amplified right away. Instead phages were tittered and single colonies were picked, isolated and amplified as described by the library manufacturer.

2.6. Phage-capture ELISA for determination of IgE-reactivity to phage-displayed peptides

For selection of positive phage clones microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 µL/well of 8.3 µg/mL rabbit-α-human IgE (DAKO, Glostrup, Denmark) or 100 µL/well of 1.0 µg/mL rabbit-α-rat IgE (Zymed, Berlin, Germany) in PBS. All following steps were performed at RT for 1 h. Plates were blocked with 200 µL/well of 2% (w/v) SMP in PBS. Between each step plates were washed 3 times in PBS with 0.05% (w/v) Tween 20 (PBS-T). One half of the plate was incubated with 100 µL/well of serum diluted 1:60 (human) or 1:20 (rat) in dilution buffer while the other half of the plate was incubated with 100 µL/well of dilution buffer (background). For plates with rat serum, plates were blocked once more, this time with 200 µL/well of naïve rat serum diluted 1:20 in dilution buffer. Subsequently, all plates were incubated with 100 µL/well supernatants from single colonies of amplified phages or wild type phage without an insert coding for a peptide (negative control), followed by incubation with 100 µL/well of mouse-α-M13 phage (Amersham Biosciences UK Limited, Buckinghamshire, UK) diluted 1:1000 in dilution buffer. For detection, the plates were incubated with 100 µL/well of alkaline phosphatase (AP)-conjugated goat-α-mouse Ig (DAKO, Glostrup, Denmark) diluted 1:1000 in dilution buffer. Phage-IgE interactions were visualized with 100 µL/well of 1 mg/mL *p*-NitroPhenyl Phosphate (Sigma, Berlin, Germany) in diethanolamine buffer as AP-substrate. Incubation was conducted at 37 °C for 30 min. Reaction was stopped by adding 100 µL/well 1.0 M NaOH. Absorption was measured at 405 nm with a microtiter reader (Molecular Devices, Sunnyvale, CA, USA).

The results were corrected for background, where OD values of greater than OD + 3 × S.D. of the negative control were regarded as a positive result.

2.7. Phage DNA precipitation and sequencing

Precipitation of DNA from the selected phages was carried out according to manufacturer instruction. DNA sequencing of the displayed hepta-mer peptides was performed using M13 phage specific primers.

2.8. Mapping of peptides on the Ara h 1 monomer surface using 3D-structure

Amino acids corresponding to the selected peptides were localized on the 3D structure of the Ara h 1 monomer (SwissProt no. P43237) amino acid 164–583. The homology based model of Ara h 1 amino acid 164–583, was constructed from the X-ray crystal structure of canavalin from jack bean (Ko et al., 2000), which have a sequence identity with Ara h 1 of 48%, and optimized to give lowest Gibbs free energy (G). The peptides obtained by immunoscreening of the phage-displayed peptide library were localized on the Ara h 1 surface using a computer-based algorithm (epitope mapping tool (EMT), Novozymes A/S, Bagsvaerd, Denmark) as described by Batori et al. (2006). The potential epitope mimics on the Ara h 1 molecule were subjected to structural analyses using Swiss-PdbViewer (<http://www.expasy.org/>) to remove those that were unlikely to occur due to severe structural constraints. By mapping the selected peptides on the 3D structure of industrial enzymes no localization of epitope mimics were possible.

3. Results

The IgE binding regions of intact and digested Ara h 1 were assessed by competitive immunoscreening of a phage-displayed random hepta-mer peptide library using polyclonal IgE from five individual peanut allergic patients and five individual BN rats immunized with either intact or digested Ara h 1. The resulting peptide mimics were mapped on the surface of a model of the 3D structure of the Ara h 1-monomer using a computer-based EMT. The obtained data were analyzed in order to identify quantitative and qualitative differences between the epitope profiles of intact and digested Ara h 1, as well as between the profiles for humans and rats.

3.1. Identification of IgE binding epitope mimics

3.1.1. Peanut allergic patients

A total of 123 IgE binding peptide mimics were selected for the five individual peanut allergic patients. The number of mimics was rather similar ($N=20-32$) for all patients, and ($N=8-17$) for both intact and digested Ara h 1. Some of the peptide sequences were eluted multiple times for an individual patient, some eluted with both intact and digested Ara h 1, and some shared by two or more peanut allergic patients (Table A.1).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2012.04.002>.

Each patient was found to have an individual pattern of eluted sequences, which was independent of the elution condition (i.e. if eluted using either intact or digested Ara h 1). However, five motifs (a sequence pattern of amino acids) were found to account for more than 65% of all eluted sequences; K*PAF*L, PRG[I/L/V]F, SPI*LY, DRGLF and PYTL[D/S]K (Table 2). As can be seen from Table 2, the recognition of these five motifs was not equally distributed between the five peanut allergic patients. While the motif K*PAF*L and motif PYTL[D/S]K were only eluted for a single patients the motif DRGLF was eluted for four patients. While the patients 2208, 2209 and 2305 recognized three of the five motifs, patient 2205 only recognized one motif. Also the proportion of eluted sequences corresponding to a motif varied between the five patient, where all eluted sequences for patient 2205 corresponded to a single motif, less than half of all eluted sequences corresponded to a motif for patient 2305. This demonstrates heterogeneity of the Ara h 1-specific IgE antibody response between patients, which yet seems to overlap to a significant degree for some of the patients. However,

the absence of a motif recognition for a patient does not exclude that such motif could not be recognized by the patient but only implies that the IgE antibody-affinity for this motif was not high enough to be selected for sequencing.

Importantly from Table 2 it is shown that all five motifs were eluted with both the intact and the digested Ara h 1, revealing the survival of these epitope motifs after simulated gastro-duodenal digestion.

3.1.2. BN rats

A total of 138 IgE binding peptide mimics were selected for the five individual rats. The number of mimics was rather similar ($N=23-38$) for all rats, as well as was the number ($N=11-22$) for intact and digested Ara h 1, respectively. (Rat no. 52 only had sequences eluted with intact Ara h 1 because of serum shortness) (Table. A.2.) Some of the peptide sequences were eluted multiple times for an individual rat, some eluted with both intact and digested Ara h 1, and some shared by two or more rats. However, each rat was found to have an individual pattern of epitope mimics, which was irrespective of the immunization and eluting condition (intact vs. digested Ara h 1).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2012.04.002>.

3.2. Amino acid distribution of the epitope mimics

To identify if any qualitative differences in amino acid composition of the found epitope mimics existed between intact and digested Ara h 1 and between humans and rats, an analysis of the amino acid distribution was performed. No apparent difference in overall physico-chemical characteristics of the amino acids involved in the epitope mimics between intact and digested Ara h 1 and between humans and rats was observed (data not shown). Hydrophobic amino acid residues occurred most frequently, responsible for over 50% of all amino acids in the identified epitope mimics, with polar residues being the next most frequent type of amino acids, responsible for around 30%, and charged residues representing the least frequent amino acid group containing around 16%. This was applicable for both eluting conditions as well as both immunization conditions (intact and digested Ara h 1), and for both human and rat specific epitope mimics. This amino acid distribution according to physico-chemical characteristics, is not a picture of the general amino acid distribution of the Ara h 1 molecule (SwissProt no. P43237; amino acid 79–614, which is the full length of the purified Ara h 1 molecule after cleavage of the N-terminal (Bøgh et al., 2012)), where the hydrophobic amino acids account for 40.6%, the polar for 24.6%, and the charged amino acids for 34.6% of all amino acids. This indicates that the epitope mimics are clustered into the more hydrophobic and less charged areas of the Ara h 1 molecule.

Differences in amino acid distribution, according to single amino acids, between sequences eluted with intact and digested Ara h 1 and between humans and rats (Fig. 1A–F), are small, especially when compared to the general amino acid distribution for the Ara h 1 molecule (Fig. 1G). The most common amino acids in the eluted sequences were Leucine (L), Proline (P), and Serine (S) for both humans and rats. In contrast the most common amino acids in the entire Ara h 1 molecule are Glutamic acid (E) and Arginine (R). In fact Glutamic acid, which are the most common amino acid in the Ara h 1 molecule, occurred only seldom in the eluted sequences. Comparing amino acid distribution according to immunization condition in the rats (intact vs. digested Ara h 1) (Fig. 1E–F) small differences are evident. These are most pronounced for the amino acid Alanine (A) and Serine (S).

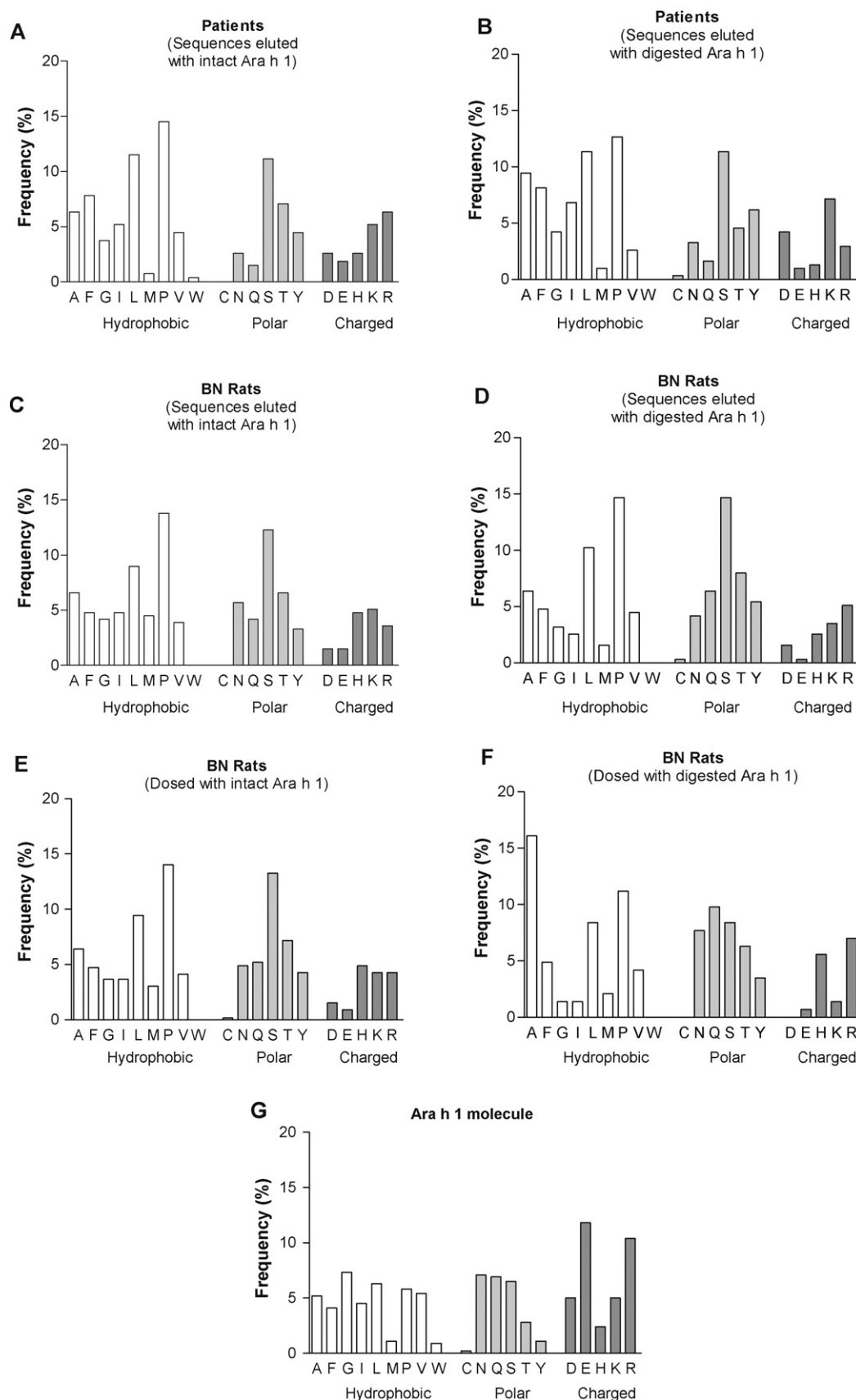


Fig. 1. Amino acid distribution in IgE binding sequences. The bars represent the total number of a particular type of amino acid residue found in all of the eluted IgE binding peptides. Amino acid distribution is represented as overall frequency of a single amino acid residue for a given condition: Amino acid distribution based on patient sera for peptide sequences eluted with intact (A) or digested (B) Ara h 1, or amino acid distribution based on sera from BN rats for sequences eluted with intact (C) or digested (D) Ara h 1, or amino acid distribution based on BN rats immunized with either intact (E) or digested (F) Ara h 1, and the general amino acid distribution for the expressed Ara h 1 molecule (G). The amino acids are grouped according to their physico-chemical feature.

Table 2
The five motifs found in the epitope mimics for the five peanut allergic patients.

Motifs	Localization of the Ara h 1 monomer molecule	Proportion of sequences eluted with either intact (I) or digested (D) Ara h 1, belonging to the given motif for each individual patients				
		2205	2208	2209	2304	2305
K*PAF*L ^(a)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹	17/17 (I)	0/8 (I)	0/9 (I)	0/10 (I)	0/14 (I)
		15/15 (D)	0/12 (D)	0/11 (D)	0/13 (D)	0/14 (D)
PRG[I/L/V]F	P ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² L ⁴⁹⁹ F ⁵⁰⁵	0/17 (I)	1/8 (I)	2/9 (I)	0/10 (I)	1/14 (I)
		0/15 (D)	2/12 (D)	0/11 (D)	0/13 (D)	0/14 (D)
SPI*LY	S ²⁶² P ²⁶¹ T ²⁶⁰ XL ²⁶⁹ Y ²⁶⁷	0/17 (I)	2/8 (I)	2/9 (I)	0/10 (I)	5/14 (I)
		0/15 (D)	6/12 (D)	5/11 (D)	0/13 (D)	3/14 (D)
DRGLF	D ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶	0/17 (I)	0/8 (I)	1/9 (I)	2/10 (I)	0/14 (I)
		0/15 (D)	2/12 (D)	1/11 (D)	2/13 (D)	1/14 (D)
PYTL[D/S]K	P ²²¹ Y ²⁶⁷ T ²³⁸ L ⁵²⁴ XK ³⁷⁹	0/17 (I)	0/8 (I)	0/9 (I)	5/10 (I)	0/14 (I)
		0/15 (D)	0/12 (D)	0/11 (D)	6/13 (D)	0/14 (D)
Total		17/17 (I)	3/8 (I)	5/9 (I)	7/10 (I)	6/14 (I)
		15/15 (D)	10/12 (D)	6/11 (D)	8/13 (D)	4/14 (D)

^aAmino acids are indicated with their one-letter code. * and X represent any given amino acid and indicate that no single amino acid was found to match on the basis of the eluted peptide sequence (*) and no specific amino acid residue was therefore to be assigned.

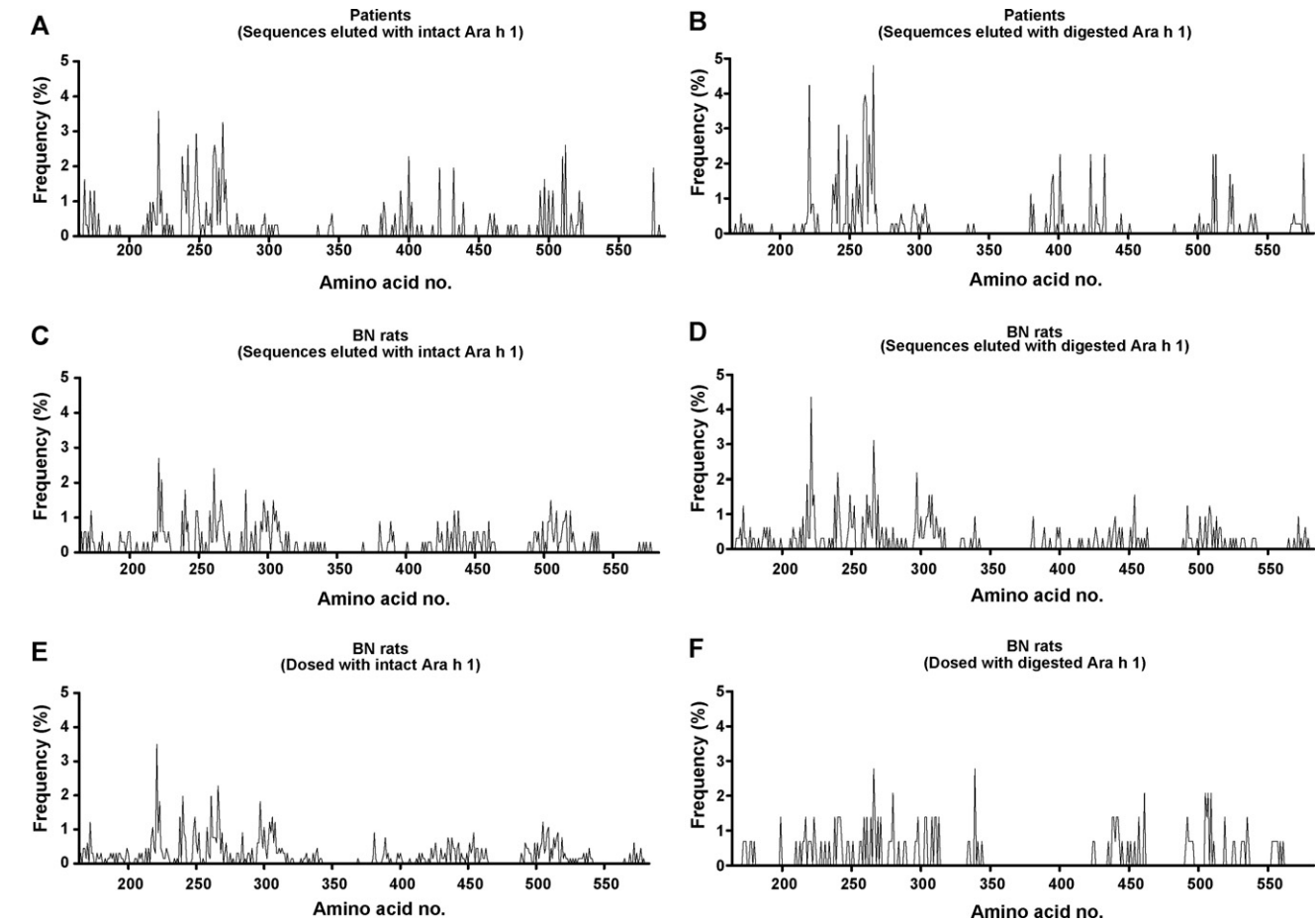


Fig. 2. Ara h 1 IgE binding epitope fingerprinting profiles. Epitope frequency is represented as sums of each amino acid residue along the primary protein sequence (amino acid 164–583) found in all suggested mapped epitope mimics in a given condition: For peanut allergic patients, where sequences were eluted with intact (A) or digested (B) Ara h 1, for rats where sequences were eluted with intact (C) or digested (D) Ara h 1, and for rats immunized with intact (E) or digested (F) Ara h 1.

3.3. Ara h 1 IgE binding fingerprinting profile

The Ara h 1 epitope fingerprinting profile, defined as the frequency distribution of amino acid residues identified as being involved in IgE binding along the primary protein sequence is shown in Fig. 2. From the IgE binding epitope fingerprinting profiles it is evident that some amino acid residues along the primary sequence of the Ara h 1 molecule appear more often than others in the suggested mapped epitope mimics. This indicates that some regions of the primary structure is more likely than others to be involved in IgE binding and that amino acid residues being part of the IgE epitopes cluster into specific areas. Some amino acid residues account for nearly 5% of all specific amino acids found in IgE binding sequences and other amino acid residues did not occur in any mapped epitope mimics at all.

When comparing the IgE binding profile for Ara h 1 according to eluting condition (intact vs. digested Ara h 1), it is seen that the profiles are very similar, for both patients and rats, respectively (Fig. 2A–D). A similar pattern is seen for both peaks and gaps, which are lying in the same areas of the Ara h 1 primary structure. It was also seen that most amino acids occurring in the epitope mimics are clustered, according to primary structure, between amino acid residues 220 and 270.

When comparing the Ara h 1 IgE binding fingerprinting profile according to immunization condition (intact vs. digested Ara h 1) for rats (Fig. 2E–F), it is seen that the profiles are different from each other. This is primarily shown by the larger gaps in the fingerprinting profile obtained for the rat immunized with digested Ara h 1. Any conclusions should however be taken with caution, since the results are based on a single rat immunized with digested Ara h 1.

Comparing the Ara h 1 IgE binding fingerprinting profiles for humans and rats (Fig. 2A–D) it is seen that the profiles are very similar, with peaks in the same areas of the primary structure of Ara h 1, though differences in frequency for single amino acid residues occur.

3.4. Mapping of epitope mimics on the surface of Ara h 1

3.4.1. Peanut allergic patients

Computer-based mapping of the eluted sequences on the 3D structure of the monomer Ara h 1 (SwissProt no. P43237, amino acid 164–583) revealed that all sequences corresponded to conformational epitopes, although short stretches of juxtaposed amino acids were found in some mimics.

Irrespective of whether the sequences were eluted with intact or digested Ara h 1, the mapped epitope mimics clustered into three areas when based upon those amino acid residues which occurred in at least 3% of all mapped IgE epitope mimics. This cut-off corresponds to specific amino acids occurring four or more times in all epitope mimics. Most epitope mimics were thus clustered in the yellow area (Fig. 3). So in consistency to identification of the localization along the primary sequence of Ara h 1 (Fig. 2), it is also here identified that the epitope mimics are not distributed equally throughout the entire molecule but seems to cluster into some areas.

Mapping of the eluted heptameric sequences on the surface of Ara h 1 showed that the epitope mimics were well exposed on the molecular surface of the 3D model of an Ara h 1 molecule and that no significant differences was evident between the elution with intact or digested Ara h 1 (data not shown).

3.4.2. BN rats

As was the case for the peanut allergic patients, only peptide sequences corresponding to conformational epitope mimics emerged from the selection. For a few of these sequences more

than one good match could be found on the surface of the Ara h 1 model.

Even though, each rat was found to have an individual pattern of epitope mimics, most IgE selected peptides were shown by the EMT to be localized into the same three surface areas of the Ara h 1 molecule, as were the peptides selected with the patient sera (see Fig. 3). However it was observed that no motifs was shared by the eluted peptide sequences, which was in contrast to the results found for the peanut allergic patients.

With regard to the accessibility of the epitope mimics on the surface of an Ara h 1 molecule, also for the rats no significant differences were seen between the elution with intact and digested Ara h 1, respectively (data not shown).

4. Discussion

This study supports the notion that most IgE binding epitopes are conformational and not linear in nature (Aalberse, 2000; Barlow et al., 1986; Roggen, 2006; Van Regenmortel, 1996). Although we used a method able to detect both linear and conformational epitopes, we only identified sequences (regardless of human or rat) mimicking epitopes on Ara h 1 which were composed of amino acid residues put together by the tertiary structure of the molecule or by two or more short stretches of juxtaposed amino acid residues. This is in contrast to most of the previous studies of Ara h 1-specific IgE binding epitopes showing that these are linear in origin. Burks et al. (1997) identified a total of 23 IgE binding epitopes consisting of 6–10 amino acids and Shreffler et al. (2004) identified yet another consisting of 25 amino acid, using the methods of overlapping peptides, which allows for detection of only linear epitopes, to identify the IgE binding sites. Additional studies confirmed the importance of such linear Ara h 1-specific IgE binding epitopes (Beyer et al., 2003; Flinterman et al., 2008; Shin et al., 1998). This is in agreement with the suggestion that linear IgE epitopes could be of special importance for food allergens known to sensitize through the gastrointestinal tract (Bannon and Ogawa, 2006; Lin and Sampson, 2009).

The present study indicates that there could be five important conformational IgE binding epitopes on Ara h 1, since more than 65% of all eluted sequences, based on patient sera, could be categorized as belonging to one of only five motifs. The five motifs showed patterns of a minimum of five amino acids, which is also the number of amino acids suggested to be involved in binding between antibody and antigen. Even though epitopes are suggested to consist of at least 8 amino acids, energy calculations suggest that a smaller subset of 5–6 amino acids are the key contributors to the binding between antibody and epitope (Bannon and Ogawa, 2006; Laver et al., 1990; Van Regenmortel, 1996). When choosing the use of short heptameric peptides for competitive immunoscreening and selecting only the eluted sequences showing the highest affinity in ELISA, we are in favor of selecting only those peptides containing the essential amino acids contributing to the binding between IgE molecules and epitopes, and deselect those which could simply be a result of non-specific binding. Also we are in favor of selecting only those peptides representing epitopes with the highest affinity to the specific IgE antibodies, while deselecting those with lower affinity. Therefore, the absence of an epitope mimic for one of the patients does not necessarily imply that this epitope could not be recognized by the patient. However, that 65% of all eluted sequences could be adjusted to belong to only five motifs and that these five motifs were identified not only with one patient and with one eluting condition, indicate that these motifs could be very important for the allergenicity of Ara h 1. Indeed all five motifs were identified by both intact and digested Ara h 1, and three of them were identified by three or four patients.

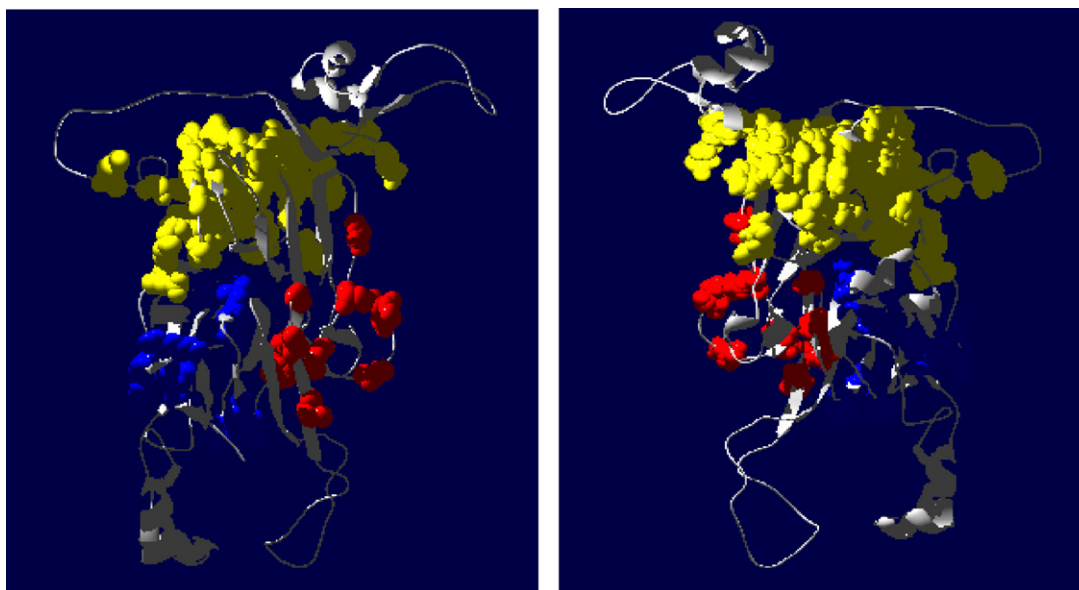


Fig. 3. Ribbon diagram of the 3D structure of the Ara h 1 monomer. Localization of the IgE-binding areas characterized by the polyclonal IgE response of the five peanut allergic patients. When cut-off level for frequency of single amino acid residues in the suggested epitope mimics was set to four, it was seen that the epitope mimics were clustered into three epitope areas on the surface of the Ara h 1 molecule, indicated by the yellow, red, and blue regions. By far the majority of the epitope mimics were clustered into the area indicated by yellow color. The figures are views of the Ara h 1 molecule by means of 180° rotation about a vertical axis.

Motifs were only identified by humans and not rats. Rats showed a much more heterogeneous pattern and only few eluted sequences were shared by two or more rats, and a greater proportion of the complete allergen surface seemed to be involved in mimicking IgE binding sites. This indicated that in contrast to humans no or only little affinity-maturation had occurred in rats. This, however, seems reasonable since the allergic history of rats consisted of only 5 weeks in contrast to patients which had a long and consistent history of peanut allergy. Arnon and Van Regenmortel (1992) and Van Regenmortel (2009) states that epitopes are not an intrinsic characteristic of an allergen that exists independently of its antibody partner. It is estimated that by immunization of an individual with a protein–antigen, more than 100 different antibodies will be formed, which differ in their epitope-specificity and may be directed against partly overlapping epitopes, so that the entire accessible surface of an allergen harbors many overlapping epitopes (Kawamura et al., 1984; Schroer et al., 1983). Upon reexposure to the same allergen affinity-maturation occurs, where progressively higher affinity B cell clones are generated, as a result of somatic hypermutation and antigen-specific selection of high affinity B cells (Griffiths et al., 1984; Kocks and Rajewsky, 1988; Neuberger et al., 2000; Siskind and Benacerraf, 1969). Another explanation for the many eluted sequences seen for the rats, could be a result of ‘true’ cross-reaction. As the relationship between an antibody and an epitope is not of an exclusive nature, ‘true’ cross-reactivity may occur, where an antibody react with other epitopes (here, eluted sequences) that is different in structure, but related to the epitope, which the particular antibody was originally raised against (Arnon and Van Regenmortel, 1992). This could in particular be relevant in situations where affinity maturation have not been fully established. Only a limited degree of resemblance is suggested to be sufficient to allow cross-reactivity (Arnon and Van Regenmortel, 1992; Van Regenmortel, 2009).

Besides the occurrence of motifs the epitope mapping profile of humans and rats were very similar. Both the IgE binding fingerprinting profile, amino acid distribution and the localization and clustering of epitopes on the Ara h 1 molecule were similar, indicating that humans and BN rats direct their antibody-response against the same areas of the allergen. This is one important issue

in identifying a suitable animal model (Knippels and Penninks, 2005), and has also been shown in an earlier study comparing human and BN rat allergic responses (Miller et al., 1999).

The definition of epitope seems to be a little unclear, so instead of talking about specific epitopes, it would make more sense to talk about epitope areas. The same amino acid residues in a protein can be part of different overlapping epitopes recognized by different antibodies, which is also evident from this study. Therefore, as Van Regenmortel (2009) states, it is not possible to draw clear boundaries between individual epitopes and there is no clear definition of an epitope on e.g. the atomic composition of binding interface to the antibody or the binding affinity that can be used as a benchmark to decide what is an epitope or if two epitope are the same or not. In light of this it seems even more important for the validation of the BN rat model that epitopes were clustered into the same areas of Ara h 1. Earlier studies have also indicated that Ara h 1-specific IgE epitopes could be clustered into areas of the monomeric Ara h 1 molecule (Maleki et al., 2000; Shin et al., 1998). Clustering of IgE epitopes have also been shown for Bos d 4 (Hochwallner et al., 2010), Phl p 1 (Flicker et al., 2006), Bet v 1 (Fedorov et al., 1997), and Phl p 5 (Flicker et al., 2000), and have been suggested to be a general phenomenon for allergens determining the allergenic activity (Hochwallner et al., 2010; Flicker et al., 2006).

In the present study we found that the IgE epitope profiles of intact and digested Ara h 1 were very alike, as the fingerprinting profile, amino acid distribution, localization and clustering of epitopes on the monomeric Ara h 1 molecule came out similar when elution was made with intact or digested Ara h 1, respectively. It seems especially important that all five identified motifs were identified when elution was made with both intact and digested Ara h 1. This indicates that even though Ara h 1 is digested to very small peptide fragments IgE binding epitopes survived the digestion process. This is in agreement with our earlier studies, showing that both intact and digested Ara h 1 were able to sensitize BN rats and elicit a biological relevant response (Bøgh et al., 2009, Bøgh et al., 2012). Ara h 1 is a labile protein digested to peptides of ≤ 2 kDa, which correspond to around 17 amino acids. However, more than 50% of these peptides were aggregated to complexes of sizes up to 20 kDa. Aggregation of Ara h 1-digests is suggested to

be the reason for the retained allergenicity of the digested Ara h 1 (Bøgh et al., 2009). That the peptides are in aggregated form under physiological conditions make the survival of conformational epitopes possible. Further, it is suggested that the digested Ara h 1 are kept in a conformation resembling the native structure of Ara h 1 (Bøgh et al., 2012), probably by non-covalently interactions, such as hydrophobic interactions. It has been shown that the hydrophobic amino acids of the Ara h 1 molecule are important for the IgE binding (Maleki et al., 2000; Shin et al., 1998). This is consistent with our findings showing that the amino acid residues composing the epitopes are relatively hydrophobic compared to the general Ara h 1 monomeric molecule. That conformational epitopes of a labile food allergen are able to survive digestion has not been shown before. Since most B cell epitopes are thought to be conformational and the fact that we find a significant cross-reactivity between intact and digested Ara h 1, supports the hypothesis that the peptides in digested Ara h 1 are in an aggregated form which represents the original folding of the intact Ara h 1.

The eluted epitope mimics are localized on a 3D model of Ara h 1 (amino acid 164–583), which is made by homology modeling of canavalin (Ko et al., 2000), and is therefore shortened in both N- and C-terminal ends. This makes it possible that some epitopes are missed. Also sequences obtained with peptide libraries are not necessarily real epitopes but only a mimic of epitopes. However, the level of mimicry is significant since an assessment of binding activity is the foundation for the selection of epitope mimics in the study. Since the epitope-specificity of an antibody is never of completely unique nature, considerable cross-reactivity may occur and inherent multispecificity of antibodies may exist (Arnon and Van Regenmortel, 1992; Van Regenmortel, 2009). The identified epitope matches on the Ara h 1 surface is not a precise identification but more an attempt to indicate the most likely location of the epitopes. Identification of IgE binding epitopes is an important issue in allergic diseases, where it can be used for a better understanding of the immune response. Epitope profiles can be useful for studying potential cross-reactivity between homologous proteins (Pomes, 2010), for design of recombinants for immunotherapy (Karisola et al., 2004), for design of peptide vaccines (Hochwallner et al., 2010), in a predictive manner when assessing allergenic potential of novel foods and genetically modified foods, and as a predictive biomarker for persistency, severity or diagnostic treatment of an allergic disease (Lin and Sampson, 2009; Huang and Honda, 2006). Indeed, different IgE epitope patterns have been found to be associated with the allergic phenotype, where e.g. epitope specificity correlated with persistency and diversity correlated with severity (Lin and Sampson, 2009). The present described epitope mapping technique could be a useful tool in allergic diseases for studying motif identification and pattern recognition by IgE.

Conflict of interest

The authors declare no conflict of interest.

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Table A.1: IgE binding epitope mimics, based on sera from five peanut allergic patients

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence	Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence
Patient no. 2205			
KAPAFDL (4)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ D ²²⁷ L ⁴²¹	KAPAFDL (3)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ D ²²⁷ L ⁴²¹
KAPAFNL	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹	KAPAFNL	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
KLIAFDL (4)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ D ²²⁷ L ⁴²¹	KEPAFML	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
KLPAFML (2)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹	KLPAFML (2)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
KQPAFNL (5)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹	KLPAFQL	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
KSPAFLN	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹	KQPAFNL (2)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
		KSPAFLD (3)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ XL ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ D ²²⁷ L ⁴²¹
		KTAAFLN (2)	K ⁴³¹ XP ⁵¹² A ⁵¹⁴ F ⁵⁷⁷ N ⁴⁰¹ L ³⁹⁹ K ²⁴⁸ XP ²²¹ A ²⁴² F ²⁶⁴ XL ⁴²¹
Patient no. 2208			
ASPKSLL	A ³³⁵ S ³⁰⁰ P ²⁹⁷ K ²²² S ²⁶⁶ L ²²⁰ L ²¹⁸	AKPASWA	A ²²⁴ K ²²² P ²²¹ A ²⁹⁸ S ³⁰⁴ XA ³³⁵
FHTARPW	F ²⁹⁵ H ²²³ S ²⁶⁶ A ²⁴² R ²⁴⁷ P ³⁸⁹ X	DSPRGVF	D ¹⁹⁴ S ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² V ⁴⁴⁹ F ⁵⁰⁵
FSPIVLY	XS ²⁶² P ²⁶¹ I ²⁶⁰ V ²³⁹ L ²⁶⁹ Y ²⁶⁷	MSDRGIF (2)	XXD ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶
SPIMDYF	S ²⁶² P ²⁶¹ I ²⁶⁰ M ²⁸⁴ D ²²⁵ Y ²⁶⁷ F ²⁹⁶	SPIINYY	S ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ XY ²⁶⁷ X
SPPRGIF	S ¹⁶⁴ P ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² L ⁴⁹⁹ F ⁵⁰⁵	SPIISHY (2)	S ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ S ²⁶⁶ H ²²³ Y ²⁶⁷
TSRADTL	S ⁵¹⁹ S ⁵¹⁸ A ⁵¹⁴ E ⁴⁵⁸ T ⁴⁹⁶ L ⁴⁴⁹	SPILAHY (2)	S ²⁶² P ²⁶¹ I ²⁶⁰ L ²⁵⁸ A ²⁵⁷ H ²⁵⁶ Y ²⁶⁷
TTSVRNT	T ²¹⁷ T ²³⁸ S ²⁴⁹ V ²⁴¹ R ²⁴⁷ N ²⁴⁶ S ²⁶⁶	SPITLYY	S ²⁶² P ²⁶¹ I ²⁶⁰ T ²⁴⁰ L ²⁶⁹ Y ²⁶⁷ X
VTLAPLR	V ⁴⁰⁰ T ⁴⁹⁶ L ⁴⁵⁷ A ⁵¹⁴ P ⁴³⁸ L ⁴³⁵ R ⁵⁸¹ V ²³⁹ T ²³⁸ L ²⁶⁹ A ²¹³ P ²¹⁵ L ²⁷⁷ R ²⁷⁸	VPPRGFLF	S ¹⁶⁴ P ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² L ⁴⁹⁹ F ⁵⁰⁵
		YDVSSLP	Y ¹⁸⁰ D ³⁰² XS ³⁰⁴ S ³⁰⁵ L ³⁰⁷ P ²⁹⁷
Patient no. 2209			
ATETSYR	A ²⁴² T ²⁴⁰ E ³⁸² T ²³⁸ S ²⁴⁹ N ²⁴⁶ R ²⁴⁷	DLTWAPK	D ²⁵³ L ²⁵² T ²⁴⁰ XA ²⁴² P ²²¹ K ²²² D ³⁹⁴ L ⁴²⁵ T ⁴²⁵ XA ⁵⁰⁹ P ⁵⁰⁸ K ⁴⁴³
HAPRGVF	XP ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² V ⁴⁴⁹ F ⁵⁰⁵		
LPPRGFLF	XP ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² L ⁴⁹⁹ F ⁵⁰⁵	FSPIIAF	XS ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ A ²⁵⁷ F ²⁹⁶
MSDRGIF	XXD ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶	GLAGYPP	G ⁵²⁵ L ⁴²⁵ A ²⁵⁷ G ²⁵⁵ L ³⁹² P ³⁸⁹ P ⁴¹⁶
SIPYPAP	S ¹⁷³ I ⁴⁴⁷ P ⁵⁰⁸ Y ¹⁷⁰ P ¹⁶⁸ F ¹⁹³ P ²⁸⁹	LSPIIVY	XS ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ V ²³⁹ Y ²⁶⁷
SNATWVP	S ⁵¹⁸ N ⁴⁵⁶ A ⁴⁹⁷ T ⁴⁹⁶ XV ⁴⁶⁰ P ⁵¹²	MSDRGIF	XXD ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶
SPIINYY	S ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ XY ²⁶⁷ X	NLVLFSY	N ²¹⁶ L ²¹⁸ V ³⁸¹ L ¹⁶⁸ F ²⁹⁶ S ²⁶⁶ Y ²⁶⁷
STIMSSR	S ²⁶⁶ T ²⁴⁰ F ²⁶⁸ S ³⁸¹ S ²⁴⁹ R ²⁴⁷	QWAFaip (2)	Q ³⁰³ A ¹⁷⁶ F ²⁶⁴ A ²²⁴ I ²⁶⁰ P ²⁶¹ Q ³³⁹ XA ²²⁴ F ²⁹⁵ A ²⁴² I ²⁶⁵ P ²⁹⁷
WSPIVHP	XS ²⁶² P ²⁶¹ I ²⁶⁰ V ²³⁹ H ²²³ X		
		SPILAHY	S ²⁶² P ²⁶¹ I ²⁶⁰ L ²⁵⁸ A ²⁵⁷ H ²⁵⁶ Y ²⁶⁷
		SPIVLYF	S ²⁶² P ²⁶¹ I ²⁶⁰ V ²³⁹ L ²⁶⁹ Y ²⁶⁷ F ²⁹⁶
		SPIVNNY	S ²⁶² P ²⁶¹ I ²⁶⁰ V ²³⁹ XXY ²⁶⁷

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence	Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence
Patient no. 2304			
AKQTD ~~TM~~	A ²⁸⁰ K ²⁸¹ Q ²¹⁰ T ¹⁷⁸ D ³⁰² S ³⁰⁴ S ³⁰⁵	ADM ~~RGLF~~	X ~~D~~ ³⁹⁴ X ³⁹³ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶
DIS ~~RGLF~~	D ³⁹⁴ X ~~XR~~ ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶	AKG ~~TDNW~~	A ⁵³⁹ K ⁵⁷⁰ G ⁵⁴⁰ S ⁵⁷⁵ D ⁵⁴³ N ⁵⁴⁴ X
EWS ~~R~~ GIF	E ⁴⁰⁸ X ~~XR~~ ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶	AKQ ~~TD~~ TM (2)	A ²⁸⁰ K ²⁸¹ Q ²¹⁰ T ¹⁷⁸ D ³⁰² S ³⁰⁴ S ³⁰⁵
IPY ~~T~~ LDK (3)	I ²⁶⁸ P ²²¹ Y ²⁶⁷ T ²³⁸ L ⁵²⁴ S ³⁸¹ K ³⁷⁹	GSA ~~FSAF~~	G ³⁰⁹ S ³⁰⁴ A ⁵¹⁷ F ²⁶⁴ S ²⁶⁶ A ²²⁴ F ²⁹²
LHR ~~P~~ LHP	L ²⁶⁹ H ²⁷² R ³⁴⁵ P ²¹⁵ L ²¹⁸ X ^{P221}		G ⁴³³ S ²⁹⁹ A ²⁹⁸ F ²⁹⁵ S ⁵⁷⁹ A ⁵⁸⁰ F ²⁶⁴
	L ⁵²³ H ⁵²² R ⁴⁰⁵ P ³⁹⁷ L ³⁹⁹ X ^{P438}	GVY ~~S~~ LSK	XXY ²⁶⁷ T ²³⁸ L ⁵²⁴ S ³⁸¹ K ³⁷⁹
TPY ~~T~~ LDK (2)	T ²⁴⁰ P ²²¹ Y ²⁶⁷ T ²³⁸ L ⁵²⁴ X ^{K379}	IPY ~~T~~ LDK	I ²⁶⁸ P ²²¹ Y ²⁶⁷ T ²³⁸ L ⁵²⁴ S ³⁸¹ K ³⁷⁹
VTG ~~P~~ AKT	V ²¹⁹ T ²¹⁷ G ³⁴³ P ²¹⁵ A ²¹³ R ¹⁸⁶ T ¹⁷⁸	LPY ~~T~~ LSK (3)	L ²²⁰ P ²²¹ Y ²⁶⁸ T ²³⁸ L ⁵²⁴ S ³⁸¹ K ³⁷⁹
		SFPA ~~RFY~~	S ³⁰⁰ F ²⁹⁵ P ²²¹ A ²⁴² R ²⁴⁷ F ²⁵⁰ Y ²⁶⁷
		TPY ~~T~~ LDK	T ²⁴⁰ P ²²¹ Y ²⁶⁷ T ²³⁸ L ⁵²⁴ X ^{K379}
		WP ~~D~~ R ^G IF	XXD ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶
Patient no. 2305			
FSPIV ~~LY~~ (2)	X ~~S~~ ²⁶² P ²⁶¹ I ²⁶⁰ V ²³⁹ L ²⁶⁹ Y ²⁶⁷	ACCR ~~SIP~~	A ²⁵⁷ G ²⁵⁵ C ⁴²⁷ R ⁴⁰⁵ S ⁴⁰⁰ I ⁴³⁰ P ³⁹⁷
KSIYHQW	K ²⁴⁸ S ²⁴⁹ I ²⁶⁸ Y ²⁶⁷ H ²²³ N ²⁸⁷ X	AVES ~~NDK~~	A ⁵⁸⁰ V ⁵⁷⁸ E ⁵⁷⁴ S ⁵⁷⁵ N ⁴⁸¹ D ⁵⁴¹ K ⁵⁷⁰
LPS ~~R~~ G ~~L~~ F	X ~~P~~ ¹⁶⁸ P ¹⁷² R ¹⁷⁵ G ⁵⁰² L ⁴⁹⁹ F ⁵⁰⁵	FPPK ~~P~~ KL	F ⁴⁴⁰ P ⁵¹² P ⁵⁰⁸ K ⁴⁴³ E ⁵³¹ K ⁴¹⁰ L ⁴²⁵
LTDLTQK	L ²⁶⁹ T ²³⁸ D ²⁵³ L ²⁷⁷ T ²¹⁷ Q ³⁴⁴ R ³⁴⁵	KNVYHQ ~~T~~ (2)	K ²⁴⁸ N ²⁴⁶ V ²⁴¹ Y ²⁶⁷ H ²²³ N ²⁸⁷ T ²⁸⁸
QSLTRL ~~P~~	Q ¹⁹¹ S ²⁷³ L ⁴⁹⁹ T ⁴⁹⁶ R ⁴⁹⁴ L ⁴⁵⁷ P ⁴³⁸	LINQ ~~A~~ LK	L ⁵⁶⁸ I ⁵⁶⁹ N ⁵⁷¹ Q ⁵⁷² A ⁵³⁹ L ⁵³⁸ K ⁵⁴²
SPI ~~L~~ AHY	S ²⁶² P ²⁶¹ I ²⁶⁰ L ²⁵⁸ A ²⁵⁷ H ²²³ Y ²⁶⁷	MSD ~~R~~ GIF	XXD ³⁹⁴ R ³⁹³ G ²⁵⁵ L ⁵²⁴ F ⁵²⁶
SPI ~~T~~ E ~~F~~ Y (2)	S ²⁶² P ²⁶¹ I ²⁶⁰ T ²⁴⁰ E ³⁸² F ²⁵⁰ Y ²⁶⁷	SPI ~~I~~ SHY	S ²⁶² P ²⁶¹ I ²⁶⁰ I ²⁶⁵ S ²⁶⁶ H ²²³ Y ²⁶⁷
TFKLHPI	T ²³⁸ F ²⁵⁰ K ²⁴⁸ V ²⁴¹ H ²²³ P ²²¹ I ²²⁹	SPI ~~T~~ LYY	S ²⁶² P ²⁶¹ I ²⁶⁰ T ²⁴⁰ L ²⁶⁹ Y ²⁶⁷ X
TPPRVHL	T ³⁸⁷ P ³⁸⁹ P ⁴¹⁶ R ³⁹³ V ³⁶⁶ H ³⁶⁷ L ³⁶⁹	SPI ~~T~~ YY	S ²⁶² P ²⁶¹ I ²⁶⁰ T ²⁴⁰ T ²³⁸ Y ²⁶⁷ X
VLP ~~G~~ SKS	V ²³¹ L ²⁵⁸ P ²⁶¹ G ²⁶³ S ²⁶⁶ K ²⁴⁸ S ³⁸¹	TPFS ~~N~~ SP	T ²⁴⁰ P ²²¹ F ²⁶⁴ S ²⁶² N ²⁸⁷ S ²⁸³ P ²⁸⁹
WES ~~R~~ G ~~V~~ F	W ⁴⁷⁶ E ⁴⁷⁵ S ⁴⁸⁸ R ⁴⁷² G ⁴⁷⁰ V ⁴⁶² F ¹⁶⁹	VM ~~P~~ GSKP (3)	V ²⁸⁶ M ²⁸⁴ P ²⁶¹ G ²⁶³ S ²⁶⁶ K ²⁴⁸ P ³⁸⁹
YPPK ~~P~~ LH	Y ³⁰⁶ P ²⁹⁷ P ²²¹ K ²²² X ^{L269} Y ²⁶⁷		