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Development of an epitope-specific analytical tool for the major peanut allergen Ara h 2 using a high-density multiple-antigenic peptide strategy

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Using the major peanut allergen Ara h 2 as an example, an analytical tool enabling the determination of immunoglobulin E (IgE)-epitopes in processed food allergens was developed. We synthesized a multiple-antigenic peptide (MAP) of the IgE-reactive linear epitope 3 (amino acid positions 27–36) of Ara h 2 and raised a monospecific antiserum against this epitope to obtain a positive control for future epitope resolved diagnostics. First, a MAP of epitope 3, having a molecular mass of 7770 Da, was synthesized, purified, and its structure confirmed by liquid chromatography-mass spectrometry (electrospray ionization) (LC-MS(ESI)), matrix assisted laser desorption/ionization-time of flight (MALDI-TOF), and Edman sequencing. The MAP was then used to raise high titer antibodies in rabbits using the adjuvant Titermax™ and to characterize the specificity of IgE from allergenic patients sensitized to Ara h 2. The antiserum exclusively detects Ara h 2 in crude peanut extract with a titer of 10^7 by Western blot and reacts specifically with epitope 3 shown by epitope mapping for a library of solid-phase-bound synthetic 15-mer peptides covering the entire sequence of Ara h 2. Such IgE-reactive epitopes are of high analytical relevance as they could constitute the basis for epitope-specific detection systems for use in quality control in the food industry or for forensic purposes in cases of fatal reactions to otherwise undetected peanut proteins.

Keywords: Anti Ara h 2 antibodies / Ara h 2 / Epitope mapping / Multiple-antigenic peptide / Peanut allergy

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1 Introduction

Among food allergies, peanuts and peanut-derived products elicit the most severe allergic reactions, often with fatal outcome. In most cases peanut allergies begin already in early childhood and are rarely outgrown in adult life, thus posing a lifelong health problem and a considerable loss in quality of life for the individuals affected. In order to gain further insight into this immune disorder, the allergenic components of peanuts have been studied and have recently been identified as the proteins or glycoproteins Ara h 1 [1–4], Ara h 2 [4–6], Ara h 3 [7], Ara h 4 [4], Ara h 5 [4], Ara h 6 [4], and Ara h 7 [4]. Among these, Ara h 1 and Ara h 2 are recognized by serum immunoglobulin E (IgE) in over 90% of peanut-sensitized patients and, therefore, are considered as the dominant allergens with diagnostic relevance [8]. The major linear IgE-binding epitopes of Ara h 2 were mapped using overlapping synthetic peptides among which epitope No. 3, a sequence representing amino acid residues 27 to 36, was recognized by all patient sera tested [6]. Such epitopes are of high diagnostic and clinical value as they could constitute the basis for epitope-specific immunoassays to be used for forensic purposes in the case of fatal reactions to otherwise undetected peanut proteins.

Identification and validation of candidate epitopes, however, is still impeded by the lack of rapid and reliable screening procedures and by the problem of generating epitope-specific immunochemical reagents by simple standard procedures. Even for monoclonal antibody technologies it is not possible to predict to which epitopes a laboratory animal might react when native allergen is used for immuniza-

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Abbreviations: AP, alkaline phosphatase; **BCIP**, 5-bromo-4-chloro-3-indoyl phosphate; **IgE**, immunoglobulin E; **MAP**, multiple-antigenic peptide; **NC**, nitrocellulose

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tion. Screening libraries of recombinant antibodies may be an alternative approach, but has the drawback of being a simple and too general lab method. Pure epitopes in the form of synthetic peptides, on the other hand, often are poorly immunogenic when monomeric and lack the desired T cell activation properties that are necessary for class switching and affinity maturation.

One approach to overcome this problem might be the use of multiple-antigenic peptides (MAPs) comprising several copies of the peptide antigen layered as dendrites around a core matrix molecule as first synthesized by Tam and co-workers [9]. MAPs are, thus, composed of a small peptidyl core matrix bearing radially oriented peptide epitopes in multiple copies as dendritic arms. MAPs have been used in experimental vaccines and for the production of anti-peptide antibodies [9, 10], but have thus far not been used in allergy research. These MAPs are not only able to cross-link surface Ig on B cells and induce clonal expansion, but also provide covalently linked epitopes in close proximity such that Ig molecules can bind with both Fab arms simultaneously. In order to introduce the map technology to the field of food allergen research, the aim of the following investigation was (i) to synthesize and purify a multiple antigenic peptide containing the immunodominant epitope 3 (aa 27–36) of Ara h 2, (ii) to produce epitope-specific antibodies by use of the adjuvant Titermax™, and (iii) to verify epitope specificity for Ara h 2 using an epitope mapping strategy.

2 Materials and methods

2.1 Chemicals

Reagents for solid-phase peptide synthesis were obtained from Applied Biosystems (Weiterstadt, Germany), except for $(Fmoc_2Lys)_2Lys$-β-Ala-*Wang*-resin and protected amino acid derivatives Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys-(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, and Fmoc-Ser(tBu)-OH which were from Novabiochem (Läufelfingen, Switzerland). Trifluoracetic acid and 1,2-ethanedithiol were purchased from Fluka (Buchs, Switzerland), and triisopropylsilane from Merck (Darmstadt, Germany). Organic solvents were either freshly distilled or of peptide synthesis- or HPLC-grade. Reagents for solid-phase peptide synthesis were obtained from Applied Biosystems (Weiterstadt, Germany).

2.2 Synthesis of MAP-3

Synthesis of the MAP of epitope 3 (MAP-3) (34 µmol scale) was accomplished on a peptide synthesizer (Applied Biosystems 431) using an $(Fmoc_2Lys)_2Lys$-β-Ala-*Wang*-resin as a tetra-branched matrix core and standard Fmoc technology. Briefly, after removing the terminal Fmoc groups of the resin by treatment with 20% v/v piperidine in *N*-methylpyrrolidone aa 27–41 of Ara h 2 covering the IgE-reactive Ara h 2 epitope number 3 were assembled by coupling the protected amino acids (1 mmol) step by step onto the tetra-branched matrix through activation with DCC/HOBt (1 mmol HOBt in 3.1 mL *N*-methylpyrrolidone and 1 mmol DCC in 1 mL *N*-methylpyrrolidone), followed by piperidine-induced removal of the Fmoc group. Deprotection of the amino acid side chains and final cleavage from the resin was achieved by treatment with a mixture of trifluoroacetic acid/water/1,2-ethanedithiole/triisopropylsilane (94/2.5/2.5/1 v/v/v/v, 10 mL/g resin) for 1.5 h at room temperature. After filtration, cleavage reagents were removed *in vacuo*, the crude MAP was precipitated with ice-cold *tert*-butyl-methyl ether, dried *in vacuo*, dissolved in water, and lyophilized. The crude MAP was analyzed and purified by RP-HPLC as described below. Fractions containing MAP-3 were collected and lyophilized affording pure MAP as a white powder (yield: 62%). LC/MS: *m/z* 1942.7 (23, $[M+4H]^{4+}$), 1555.1 (100, $[M+5H]^{5+}$), 1295.9 (38, $[M+6H]^{6+}$), 1110.8 (20, $[M+7H]^{7+}$), 972.0 (13, $[M+8H]^{8+}$), 864.5 (8, $[M+9H]^{9+}$), 778.8 (2, $[M+10H]^{10+}$). MALDI-TOF-MS: *m/z* 7770.8 $[M+H]^{+}$, 3886.1 $[M+2H]^{2+}$, 2592.6 $[M+3H]^{3+}$ (calc.: monoisotopic mass 7765.1; mass of main isotopomer 7769.1). Sequence analysis by automated Edman degradation showed the correct amino acid sequence within the branches.

2.3 Peptide spot library

The spot-synthesis technique [11] described by Frey and Schmidt [12] was used to generate an array of overlapping synthetic 15-mer peptides, offset from each other by two amino acids and covering the entire sequence of Ara h 2. The peptides were synthesized on β-alanyl-β-alanine spacers attached to acid-resistant Whatman 540 cellulose filter paper by using an ABIMED ASP 422 peptide synthesizer (ABIMED, Langenfeld, Germany) and were end-capped with acetic acid anhydride. The formation of inter-peptide disulfide bridges upon cleavage of the side chain protecting groups was prevented by use of Fmoc-Cys-(Acm)-OH from which the thiol-protecting group is not released under the conditions employed for side chain deprotection. The aminoterminally acetylated, deprotected cellulose-bound peptide libraries were used directly for binding assays with patient IgE and rabbit anti Ara h 2 sera.

2.4 HPLC

Analytical-scale separations (20–100 µL samples) were performed on a stainless steel column (4.6 × 250 mm; flow

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rate 1.0 mL/min) packed with RP-18 (Nucleosil 100 C-18 column, 5 µm, 10 nm; Macherey and Nagel, Düren, Germany) as stationary phase and a gradient of 1% v/v acetonitrile per min in 0.1% v/v aqueous trifluoroacetic acid as liquid phase (R_t MAP 29.4 min). For large-scale purification, the MAP was dissolved in water/1,2-ethanedithiol (1000/1 v/v), and separated isocratically by RP-HPLC using a mixture (22/78 v/v) of 0.1% trifluoroacetic acid and acetonitrile (R_t MAP 8.8 min).

2.5 Amino acid sequence analysis

Automated *N*-terminal sequence analysis was carried out on a Procise 492 instrument (Applied Biosystems) with 100 pmol peptide dissolved in 10 µL TFA (0.1% in water). Calibration was performed with a commercially available mixture of PTH-amino acids (10 pmol each; Applied Biosystems).

2.6 LC-MS and MALDI-TOF-MS

An analytical HPLC column (Nucleosil 100-C18) was coupled to an LCQ-MS (Finnigan MAT, Bremen, Germany) which was operated in positive electrospray ionization (ESI) mode. For MALDI-TOF-MS a Biflex 3 instrument (Bruker, Rheinstetten, Germany) was used with α-cyano-4-hydroxycinnamic acid as matrix.

2.7 Human sera and rabbit antisera

Patients sensitized to peanut were selected according to case history, skin test reactivity, and positive CAP tests (Pharmacia, Uppsala, Sweden). Sera were collected at the Medical Hospital Borstel from informed outpatients after giving their consent and used for detection of IgE binding to peanut proteins. Recombinant (r)Ara h 2 was expressed in *Escherichia coli* strain BL21(DE3)-RIL and purified as his-tag fusion protein as described previously [13]. 100 µg of either MAP-3 or rAra h 2 were dissolved in 250 µL 0.9% NaCl, emulsified with 250 µL adjuvant (Titermax™; Sigma, Deisenhofen, Germany) and administered i.m. without further booster injections to Chinchilla outbread rabbits (Charles River Wiga, Sulzfeld, Germany) which were fed with standard rabbit chow (Ssniff-Kaninchenmüsli, Soest, Germany). Antisera titers were defined as the serum dilution which just enabled clear detection of Ara h 2 by Western blot analysis of peanut extract. Antibody production was performed according to the legal requirements following an animal protocol approved by the state of Schleswig-Holstein.

2.8 Electrophoresis and immunoblotting

Discontinuous SDS-PAGE of peanut extracts was performed as reported earlier [14]. For separation of low-molecular-weight proteins (<25 kDa) ready-to-use NuPAGE™ Bis-Tris gels (Invitrogen, Groningen, Netherlands) were used according to the manufacturer's instructions. After electrophoresis, proteins were either visualized with Coomassie blue or blotted onto nitrocellulose (NC) membranes (0.45 µm; Schleicher & Schuell, Dassel, Germany) by semidry blotting [15] at 0.8 mA/cm^2 for 30 min or onto polyvinylidene difluoride (PVDF) membranes (Millipore, Eschborn, Germany) using a tank-blot system according to the manufacturer's instructions (Invitrogen). NC membranes were blocked by incubation in 0.1 M TBS (pH 7.4) containing 0.05% v/v Tween 20 (TBST) for 1 h under gentle agitation. Blocking of PVDF membranes was achieved by shaking in 5% w/v non-fat dry milk/TBS. The membrane were then incubated in an appropriate dilution of primary antibody. Human sera were diluted 1 : 10 in TBST. Rabbit antisera were applied in a dilution of $1:2\times10^7$. Specific binding of human IgE or rabbit antibodies was detected using either monoclonal mouse anti-human-IgE (1 : 2000; Allergopharma, Reinbek, Germany) or goat anti-rabbit-IgG antibodies (1 : 5000; Dianova, Hamburg, Germany) conjugated to alkaline phosphatase (AP), respectively. Bound antibodies were visualized after three washes with TBST by incubation with 0.033% w/v NBT and 0.017% w/v 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in AP buffer (0.1 M TBS, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) [16] .

2.9 Epitope mapping

Immunological detection was performed essentially as described by Frank and Overwin [11]. The Ara h 2 15-mer peptide array was blocked with blocking buffer (MBS; Sigma-Genosys) overnight and washed with TBST pH 7.0 for 30 min. Afterwards the membrane was incubated in an appropriate dilution of primary antibody. Human sera were diluted 1 : 10 in MBS. Both, the Ara h 2 specific rabbit serum and the MAP-3 anti-serum were used in a dilution of $1:10^5$. Specific binding of human IgE or rabbit antibodies was detected using either monoclonal mouse anti-human-IgE (1 : 2000; Allergopharma) or goat anti-rabbit-IgG (1 : 5000; Dianova) antibodies conjugated to AP, respectively. Antibodies were diluted in MBS. Bound antibody was visualized after two washes with TBST (pH 7.0), followed by two washes with citrate-buffered saline (CBS; 137 mM NaCl, 2.7 mM KCl, 10 mM citric acid; pH 7.0) by incubation with 0.03% w/v dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide (MTT; Sigma) and 0.024% w/v BCIP in CBS-substrate buffer (CBS + 5 mM $MgCl_2$, pH 7.0). The reaction was stopped with TBS and documented. For re-use of the peptide library the bound antibodies and

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the precipitated chromophore were stripped according to the protocol of Frey and Schmidt [12] where removal of bound ligands is achieved by sonication of the cellulose membrane in neat dimethylformamide and freshly prepared stripping buffer (8 M urea in TBS, 1% w/v SDS, 0.5% v/v mercaptoethanol adjusted to pH 7 with acetic acid) followed by several washing steps in distilled water.

2.10 Extracts and roasted peanut extracts

Peanuts (*Arachis hypogaea*), seeds of white lupine (*Lupinus albus*), or of soy (*Glycine max*) and raw peanuts (Virginia; Internut Handels GmbH, Hamburg, Germany), roasted for 40 min at 140°C or 160°C, respectively, were ground under dry ice and then extracted with an aqueous $(NH_4)_2CO_3$ solution (0.1 mol/L, pH 8) for 4 h at 4°C. A protein extract was obtained after centrifugation, filtration of the supernatant (0.2 µm pore size), followed by dialysis.

3 Results

3.1 MAP of epitope 3 of Ara h 2

Preparation of a MAP bearing copies of the epitope number 3 (*H_2N*-DRRCQSQLERANLRP-*COOH*) (underlined aminoacids represent the sequence of epitope 3) of Ara h 2 was performed by stepwise solid-phase synthesis of the peptide linked to a tetravalent $(Fmoc_2Lys)_2$Lys-β-Ala-modified *Wang*-resin, a polymeric benzyl alcohol, as outlined in Fig. 1. The structure of this synthetic MAP-3 was verified by means of LC-MS and MALDI-TOF experiments, the spectra of which are displayed in Fig. 2. The LC-MS spectrum showed the ion with *m/z* 1555.1 as the base ion, most likely corresponding to the 5-fold positively charged MAP-3 (Fig. 2A). In addition, the 4-fold (*m/z* 1942.7), the 6-fold (*m/z* 1295.9), the 7-fold (*m/z* 1110.8), the 8-fold (*m/z* 972.0), the 9-fold (*m/z* 864.5), and the 10-fold charged ions (*m/z* 778.8) were detected. Furthermore, MALDI-TOF-MS was performed revealing an isotopomer at *m/z* 7770.8 fitting well with the amino acid composition of the synthetic MAP-3 (Fig. 2B). Finally, amino acid sequencing of the synthetic MAP-3 by automated Edman degradation verified the sole presence of the DRRCQSQLERANLRP sequence in the four copies of the epitope peptide. Thus, MAP-3 could be successfully synthesized as a homogeneous entity carrying a tetramer of the intended sequence motif.

3.2 Immunization with MAP-3 and Ara h 2 holoprotein

100 µg MAP-3 or 100 µg Ara h 2 were dissolved in isotonic saline, emulsified with the adjuvant Titermax™, and administered i.m. without booster injections. This gave rise to

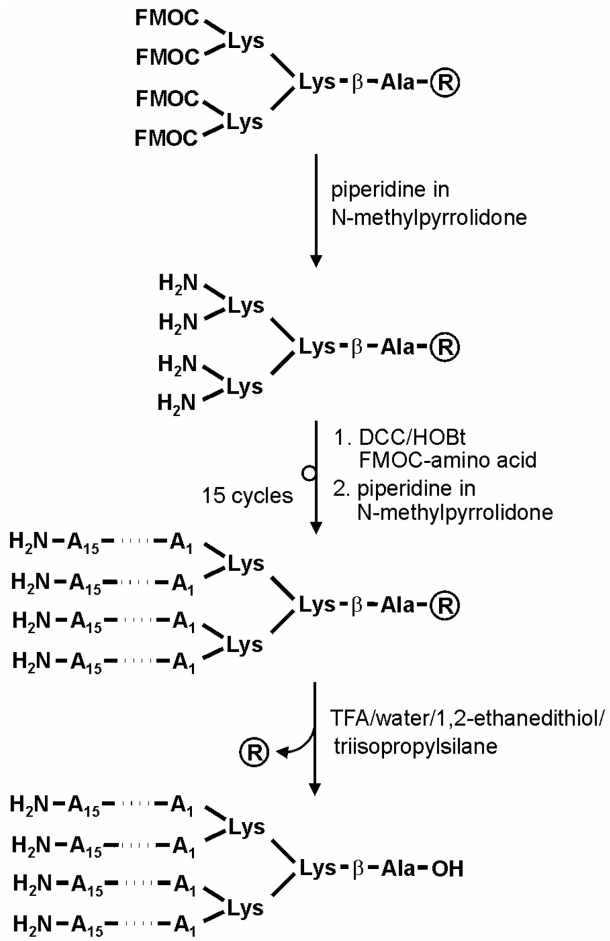

Figure 1. Strategy for the synthesis of the multiple antigenic peptide MAP-3 by a stepwise solid-phase technique.

high titer antisera that recognized Ara h 2 in crude peanut extracts. For both antigens the Western blot endpoint titer of the sera was $1:10^4$ three weeks after immunization, $1:6.4\times10^6$ after five weeks, and reached $1:10^7$ after nine weeks. As the curve appeared to flatten at this time point, the rabbits were exsanguinated. Thus, the onset of the immune response was rapid and the titer continued to increase without a need for further booster immunizations.

3.3 Specificity of rabbit anti MAP-3 and anti Ara h 2 sera to natural Ara h 2 in peanut extract

As the diet of the rabbits contained soy which is phylogenetically related to peanut, the pre-immune sera were tested against soy and peanut extract. Titers of $1:10^4$ to soy and peanut extracts were observed. Although this reactivity is adsorbable with immobilized soy extract, preadsorbtion was not necessary in our experiments as the antisera were used in dilutions greater than $1:10^5$. Despite this rather

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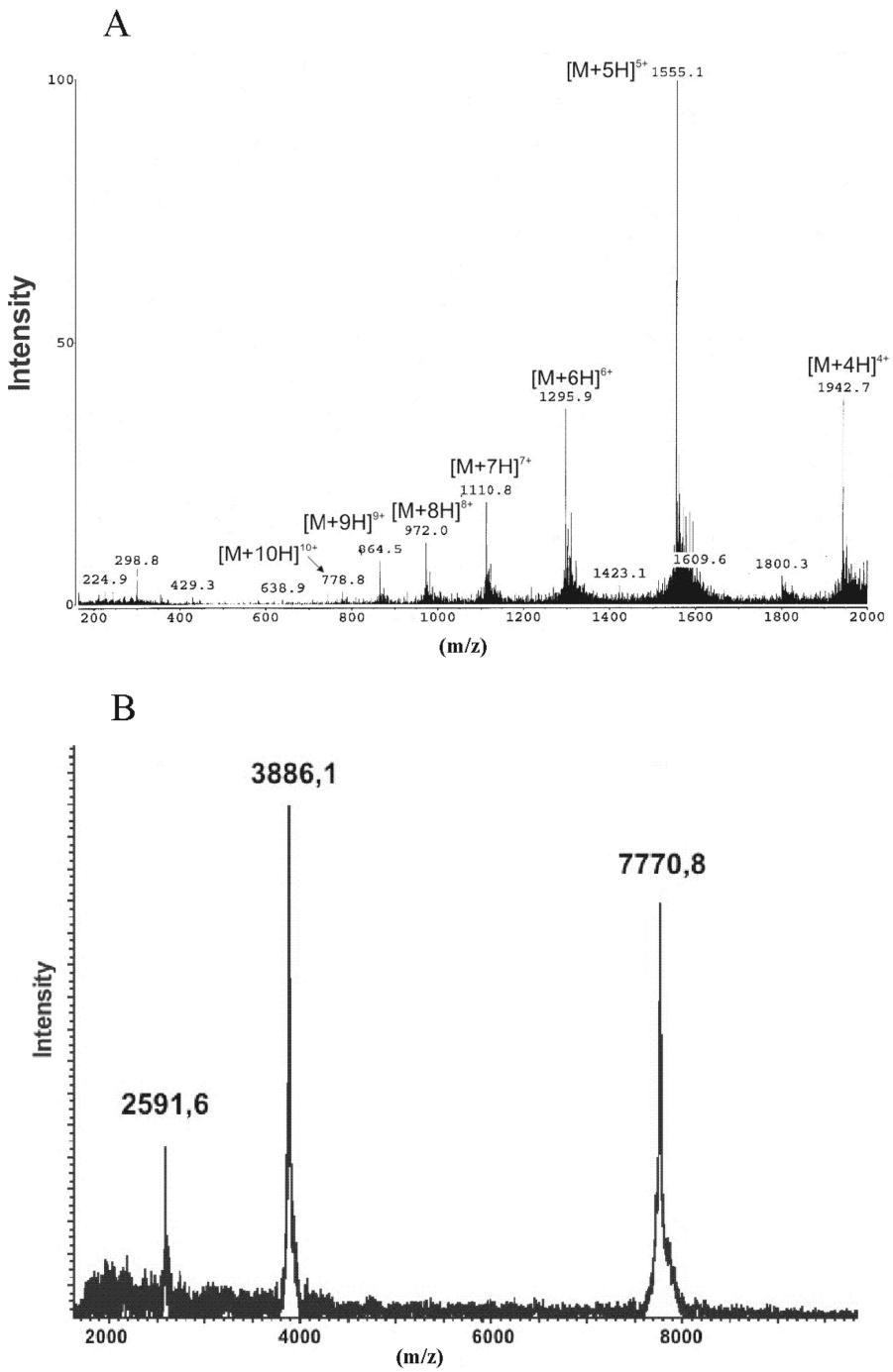

Figure 2. Mass spectroscopic analysis of MAP-3. (A) LC-MS (ESI) spectrum of MAP-3. (B) MALDI-TOF-MS spectrum of MAP-3.

immature pre-existing immunity to peanut protein in all experimental animals, clear differences were observed for the mature responses after systemic immunization with the two Ara h 2 antigens.

As depicted in Fig. 3 A (lane 5), the anti Ara h 2 serum reacts intensely with the double band of Ara h 2 in peanut extract and weakly with a third, lower band. The anti MAP-3 serum, on the other hand, reacts exclusively with the Ara h 2 double band (Fig. 3, lane 3). For comparison, lane 2 (Fig. 3) depicts the binding pattern of peanut allergic patient IgE. Thus, the synthetic MAP-3 antigen outperforms the recombinant antigen in terms of elicited specificity of the antisera. Moreover, the anti MAP-3 serum does not react to soy extract (Fig. 3 B, lane 2), or to white lupine extract (Fig. 3 C, lane 3).

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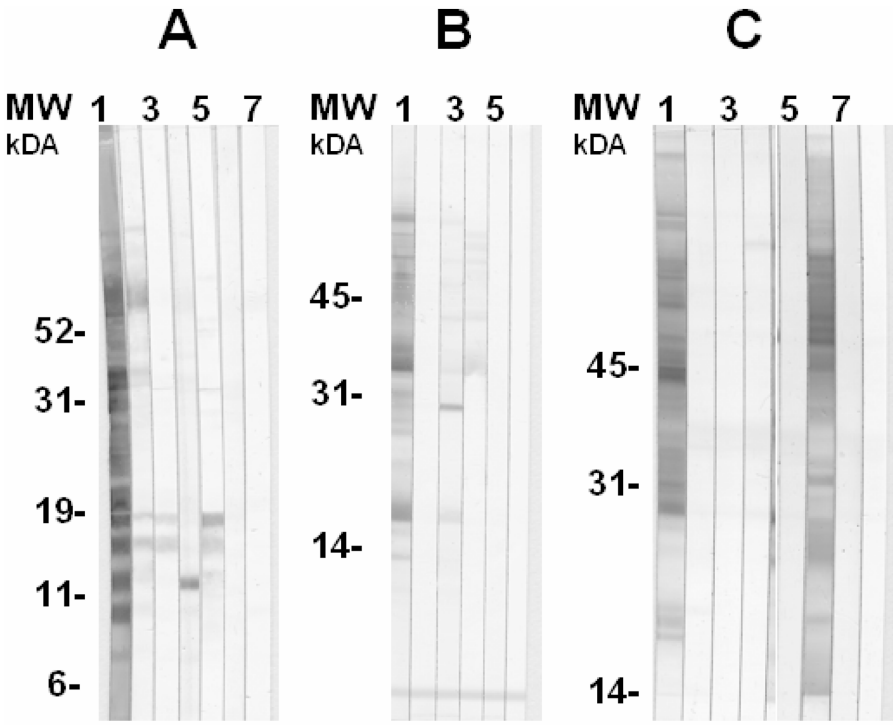

Figure 3. Western blot of (A) peanut extract (30 μg/cm) on PVDF under reducing conditions. Separation on 12% NuPAGE™. MW, molecular weight marker; 1, India ink protein staining; 2, IgE patient JG3; 3, rabbit anti MAP-3 serum; 4, rabbit anti rAra h 6 serum; 5, rabbit anti rAra h 2 serum; 6, goat anti rabbit IgG control; 7, mouse anti human IgE control. (B) soy extract (15 μg/cm) on NC under reducing conditions. Separation on 12% SDS-PAGE. MW, molecular weight marker; 1, India ink protein staining; 2, rabbit anti MAP-3 serum; 3, rabbit anti rGly m 4 serum; 4, IgE soy allergic patient TJ; 5, goat anti rabbit IgG control; 6, mouse anti human IgE control. (C) lupine extract (15 μg/cm) on PVDF under nonreducing conditions. Separation on 12% SDS-PAGE. MW, molecular weight marker; 1, India ink protein staining; 2, rabbit anti rAra h 2 serum; 3, rabbit anti MAP-3 serum; 4, rabbit anti rAra h 6 serum; 5, IgE peanut allergic patient L; 6, IgE lupine allergic patient H; 7, mouse anti human IgE control; 8, goat anti rabbit IgG control.

3.4 Linear epitope recognition of rabbit anti MAP-3 and anti Ara h 2 sera

A 15-mer peptide spot library with an offset of two amino acid residues was constructed resulting in 72 spots covering the whole Ara h 2 primary structure. As shown in Fig. 4A, the anti MAP-3 serum reacts only with peptides 11–18 containing the glutamic acid residue at position 9 of the original MAP-3 sequence (peptide 14). The anti rAra h 2 serum reacts with peptides 25 to 34 (Fig. 4D) covering the major epitopes 6 and 7 identified recently [6]. Peanut allergic patient serum IgE (JG3) shows binding to peptides 12–14 (epitope 3) sharing amino acids DRRCQSQLERA and peptides 25–34 which include the epitopes 5, 6, and 7 as indicated by the underlined amino acids (Fig. 4C).

3.5 Reactivity of the patient IgE and rabbit antisera to MAP-3

The MAP-3 preparation used for immunization was resolved on NuPAGE™ peptide gels and blotted onto nitrocellulose. As shown in Fig. 5 (lane 1), MAP-3 exhibits one band at 6 kDa upon staining with India ink. This band was immunostained with IgE of patient JG3 (lane 5) and the rabbit anti MAP-3 (lane2) serum, but binds rather weakly the anti rAra h 2 serum (lane 3) emphasizing the specificity of the anti-MAP-3 rabbit antisera. This finding confirms the result of the epitope map (Fig. 4D), since the anti Ara h 2 serum did not react with peptides 11–18. Moreover, 6 out of 11 peanut allergic patients react to MAP-3 (Fig. 5, lane 6–16).

3.6 Reactivity of rabbit anti MAP-3 serum towards roasted peanut extracts

In order to investigate the antibody reactivity of rabbit anti MAP-3 serum towards allergenic proteins in roasted peanuts, peanuts were roasted at 140°C and 160°C, respectively, and prepared extracts were analyzed by SDS-PAGE. Coomassie blue staining revealed a clear reduction in protein amount when peanuts were roasted at 160°C with a shift of the protein bands below 20 kDa (Fig. 6A, lanes II and III). Western blots of roasted peanut extracts showed that the IgG reactivity of the rabbit anti MAP-3 serum was nearly unchanged (Figs. 6B, C, lane 2). By comparison, Western blot analyses were performed with roasted peanut extracts and rabbit anti Ara h 6 and patient IgE (JG3), respectively. Reactivity of anti Ara h 6 diminished totally

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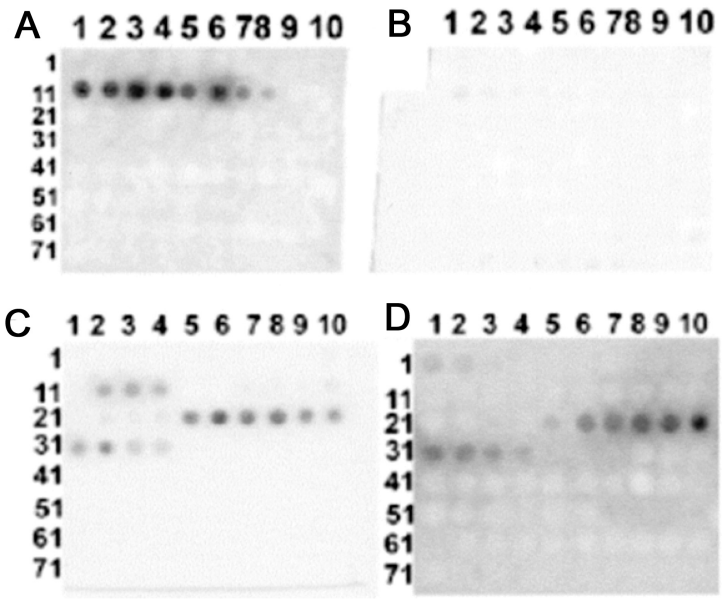

E

11	QWELQG**DRRCQSQLE**	
12	ELQG**DRRCQSQLERA**	
13	**QGDRRCQSQLERANL**	
14	**DRRCQSQLERANLRP**	epi-3
15	**RCQSQLERANLRP**CE	
16	**QSQLERANLRP**CEQH	
17	**QLERANLRP**CEQHLM	
18	**ERANLRP**CEQHLMQK	epi-4
19	ANLRPCEQHLMQKIQ	
20	LRPCEQHLMQKIQRD	
21	PCEQHLMQKIQRDED	
22	EQHLMQKIQRDEDSY	epi-5
23	HLMQKIQRDEDSYER	
24	MQKIQRDEDSYERDP	
25	**KIQRDEDSYERDPYS**	
26	**QRDEDSYERDPYSPS**	epi-6
27	**DEDSYERDPYSPSQD**	
28	**DSYERDPYSPSQDPY**	
29	**YERDPYSPSQDPYSP**	
30	**RDPYSPSQDPYSPSP**	epi-7
31	**PYSPSQDPYSPSPYD**	
32	**SPSQDPYSPSPYDRR**	
33	**SQDPYSPSPYDRRGA**	
34	**DPYSPSPYDRRGAGS**	
35	YSPSPYDRRGAGSSQ	

F

LTILVALALFLLAAHASARQQWELQG**DRRCQSQLERANLRP**CEQHLMQ**K**[49]**IQRDEDSYER**
DPYSPSQDPYSPSPYDRRGAGS[81]SQHQERCCNELNEFENNQRCMCEALQQIMENQSDRLQ
GRQQEQFKRELRNLPQQCGLRAPQRCDLDVESGGRDRY

Figure 4. Epitope mapping in a 15-mer peptide library of Ara h 2 (offset 2 aa). (A) Rabbit anti MAP-3 serum; (B) second antibody control; (C) IgE of patient JG3; (D) rabbit anti rAra h 2 serum; (E) primary structure of the peptides, reactive peptides in bold; identified epitopes according to the literature [6] are underlined; (F) primary structure of Ara h 2 according to [6].

after roasting the peanuts at 160°C (Fig. 6C, lane 3), whereas the IgE reactivity of patient JG3 was only abolished for the allergen bands above 20 kDa; in contrast, the reactivity to Ara h 2 was weak and nearly unchanged (Figs. 6B, C).

4 Discussion

Peanut allergies pose a considerable problem not only to the individuals affected, but also to the food producers who want to be able to detect minute contaminations of their

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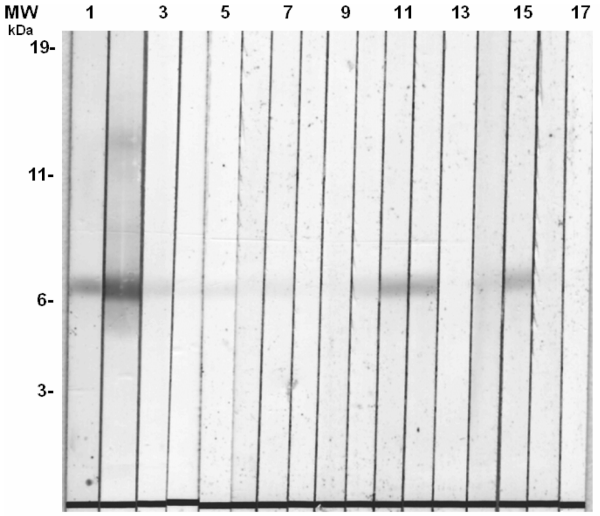

Figure 5. Western blot of MAP-3 (5 μg/cm) on NC under reducing conditions. Separation on 12% NuPAGE™. MW, molecular weight marker; 1, protein staining India ink; 2, rabbit anti MAP-3 serum; 3, rabbit anti rAra h 2 serum; 4, goat anti rabbit IgG control; 5, IgE reactivity of patient JG3; 6–16, IgE reactivity of peanut allergic patients; 17, mouse anti human IgE control.

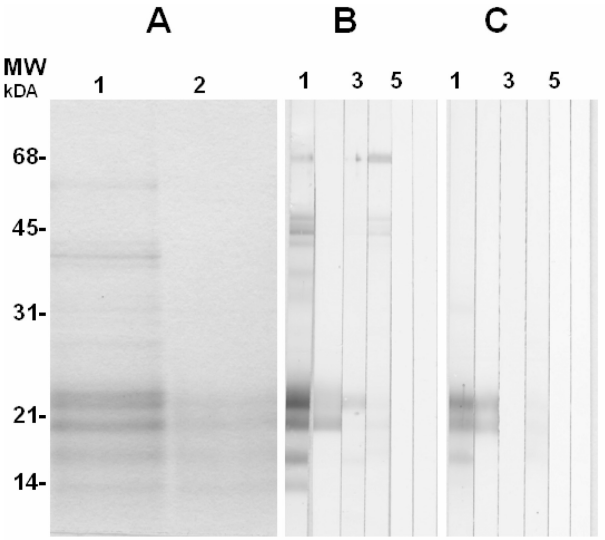

Figure 6. Antibody reactivity to proteins in peanuts roasted at 140°C (A, 1; B) and 160°C (AC); MW, molecular weight marker. (A) Coomassie blue protein staining; (B) Western blot of roasted peanut (140°C) extract (15 μg/cm); (C) Western blot of roasted peanut (160°C) extract (15 μg/cm). 1, India ink staining; 2, anti epitope 3 (MAP-3); 3, anti rAra h 6; 4, patient serum (JG3); 5, mouse anti human IgE control; 6, goat anti rabbit IgG control.

products with peanut protein in order to protect peanut sensitive customers. As classical chemical analytical regimens fall short for detecting these proteins in such protein mixtures, immunoassays which are able to specifically discriminate between certain proteins are the method of choice. Food processing, such as roasting, cooking, or fermentation, however, often denatures proteins and therefore changes or entirely degrades the determinant against which an immunoassay is directed. It may therefore prove to be an advancement in food analysis if the actual allergenic determinants could be detected or their absence demonstrated at significant detection levels. To meet this goal, robust epitope-specific reagents, as well as purified epitope materials which can serve as positive controls, are required. In this study, we successfully addressed both problems, thus providing a new tool to submolecular allergen research and diagnostics.

4.1 MAPs as immunogens

When complex antigens are used for immunization it is impossible to restrict the immune response to a distinct part of the antigen even if it is an immunodominant epitope. In order to obtain epitope-specific antibodies one must either generate monoclonal antibodies or the antigen must be reduced to the desired epitope by directed use of peptides as immunogens. However, peptides are often poorly immunogenic because they often lack T cell reactivity that is necessary for mature immune response. Polymerization of peptides is one means to solve this problem because repetitive epitopes are able to activate naive B cells for IgM production and certain oligomerization or polymerization strategies are able to foster the correct folding of the peptide monomers such that the resulting antibodies show higher reactivity with the native parent protein [17]. As a random polymerization procedure exhibits high batch-to-batch variability [17], we used the MAP strategy of Tam and coworkers [9, 10] to generate peptide polymers. With this procedure, a highly defined antigen was prepared which gave rise to a hyperimmune serum after a single innoculation only when coadministered with Titermax™ adjuvant. Interestingly, antibody class switching to IgG was also induced indicating the presence of a helper T cell determinant in the peptide sequence motif used. T cell reactivity also indicates that the carboxy terminus of the peptides on MAP-3 must be accessible to lysosomal proteases. This may be due to the rather open structure of the MAP where the core matrix is relatively small and the bulk of the molecule is formed by the four copies of the peptide antigen layered as dendrites around the core matrix (displayed by the grey lines in the energy-minimized model in Fig. 7).

In addition, the Titermax™ adjuvant which combines novel immunomodulators with a stable water in oil emulsion most likely produces a depot of antigen which continuously releases the MAP over time, thus not requiring additional boosting to get high-quality antisera. We thus obtained excellent results in producing more than 12 antisera with various antigens by means of Titermax™ coadministration

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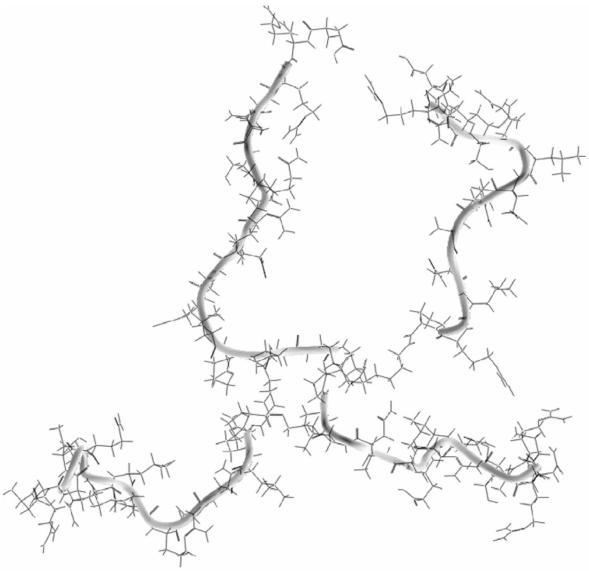

Figure 7. Energy-minimized structure of the synthetic MAP-3. Peptide epitopes are indicated by a grey lines. For energy minimization the program Sybyl 6.7 (Tripos, St. Louis, MO, USA) was used.

in rabbits. This is in contrast to our previous experience with Freund's adjuvant which normally requires a more time-consuming immunization schedule and is stressing to the animals. This, in turn, enabled us to neglect the preexisting antibodies against soy antigens. Although no further antibody pre-adsorbtion was necessary in this case, such a food-borne background should always be kept in mind.

4.2 Specificity of the rabbit anti Ara h 2 sera

Ara h 2, identified as a major peanut allergen, is a 2S seed storage protein that belongs to the conglutin family. It displays an identity to Ara h 6 of 53% and to Ara h 7 of 42%. The anti Ara h 2 serum raised against recombinant Ara h 2 reacted strongly to the typical Ara h 2 double band, but with less intensity to a peanut extract component at 11 kDa (Fig. 3 A, lane 5). However, this orphan band was not identical with Ara h 6 as judged by the binding pattern of an anti Ara h 6 serum (Fig. 3 A, lane 4) since the serum did not recognize the 11 kDa protein. The anti MAP-3 antiserum detects exclusively the Ara h 2 double band (Fig. 3 A, lane) and is not reactive to soy or white lupine extract (Fig. 3 B, lane 2 and Fig. 3 C, lane 3).

Interestingly, the serum IgE of patient JG3 and our anti Ara h 2 serum exhibited an almost identical binding pattern to linear epitopes of Ara h 2 between amino acids 49–81, but the patient serum also recognized amino acids 27–36 (DRRQCQSQLERA) of Ara h 2 which covers epitope 3. This demonstrates that epitope 3 must fold correctly on the peptide array and can be recognized by antibodies in this assay. The failure of the anti rAra h 2 serum to recognize the later sequence motif, in turn, indicates that no antibodies against this epitope were made in rabbits when the whole (recombinant) molecule was used as immunogen.

Our immunizations with MAP-3 illustrate that this shortcoming can be overcome when using polymeric peptide antigens since, in this case, antibodies against epitope 3 could be induced. Thus, using an IgE-reactive linear epitope as target for immunization improves the usefulness of the induced antibodies as universal tools for identification and purification of allergens from various sources. As proof of concept we investigated the hypothesis that a rabbit anti epitope 3 of Ara h 2 serum (anti MAP-3 serum) is able to detect this epitope in processed peanut proteins. This is exemplified with extracts of roasted peanuts. As depicted in Fig. 6 C (lane 2), the reactivity of the anti MAP-3 serum is nearly unchanged, whereas the IgE reactivity is rather weak. This demonstrates the anti MAP-3 serum as a useful tool for identifying a clinically relevant epitope under conditions where the IgE-reactivity begins to decline. Since peanuts processed at 160°C for 40 min are overroasted and not desirable, the sensitivity of the anti MAP-3 serum covers the relevant test degree of roasting-induced protein processing.

Such IgE-reactive epitopes are of great diagnostic and clinical relevance as they could constitute the basis for an epitope-resolved diagnostic. An epitope-specific detection system with clinical relevance may be used for quality control in the food industry to spot harmful carry over effects with relevance to peanut allergic patients. They will also aid in generating crops with reduced allergenic potential by eliminating or mutating the determinants in question, and might provide a basis for safe and efficient immunotherapeutic drugs to combat food allergy.

The IgE-reactivity to epitope 3 of Ara h 2 in the form of MAP proves its usefulness as a small antigenic peptide and paves the way as a novel concept for diagnostics, *i.e.*, an epitope-resolved diagnosis with the option to correlate the identified reactivity with the strength of clinical symptoms and/or to predict clinical reaction. As shown in Fig. 5, lane 5–16, seven out of twelve sera from Ara h 2 reactive patients recognized MAP-3.

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