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Characterization and identification of allergen epitopes: recombinant peptide libraries and synthetic, overlapping peptides

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Abstract

For the understanding of the relationship between protein structure and allergenicity, it is important to identify allergenic epitopes. Two methods to characterize primarily linear epitopes are compared using the major allergen from brown shrimp (*Penaeus aztecus)*, Pen a 1, as an example. A recombinant peptide library was constructed and synthetic, overlapping peptides, spanning the entire Pen a 1 molecule, were synthesized and tested for specific IgE reactivity. Both methods identified IgE-binding of Pen a 1, however, the SPOTs procedure resulted in the identification of more epitopes of the major shrimp allergen Pen a 1 than the usage of the recombinant peptide library. For detection of specific IgE antibodies, the usage of ^{125}I-labeled detection antibody seems to be superior over enzyme-labeled anti IgE antibodies. The regeneration of SPOTs membranes is possible, but it is prudent to test regenerated membranes for residual activity. If a given food allergen contains significant linear epitopes, which seems to be true for stable major allergens such as those of peanut and shrimp the SPOTs system may be more advantageous than the use of recombinant peptides libraries. However, if allergens are studied that contain more conformational epitopes, recombinant peptide libraries may help to identify the relevant epitopes. It has to be emphasized that no system for epitope identification will detect all epitopes and that the relevance of identified epitopes has to be confirmed with other methods such as inhibition studies, crystallographic analysis or the immunological evaluation of modified whole allergens.

Keywords: Food allergens; Recombinant peptide library; Epitopes; Overlapping peptides

1. Introduction

To understand the pathophysiology of allergy, it is important to study the interaction between allergen and immune system on the molecular level. Although most features and structural properties that are responsible for the allergenicity of a protein are generally still poorly defined; some very broad characteristics of food allergens have been identified, these include abundance [1–6] of a given protein in a particular food, physicochemical properties, such as a molecular mass (10–70 kD) [1,2], acidic isoelectric point, glycosylation, and resistance to heat and digestion [1,2,7]. However, many of these properties characterize a vast number of non-allergenic proteins as well and thus, are not unique for food allergens. One approach to study the relationship between protein structure and allergenicity is to identify

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allergenic epitopes; and over the years, epitopes of several food allergens have been studied [8–16]. However, only recently, with the advent of molecular biological techniques and new methods to synthesize and test large numbers of synthetic peptides has it been possible to systematically identify major epitopes of food allergens.

Two types of epitopes, conformational or discontinuous epitopes and continuous, linear epitopes, are differentiated. Conformational epitopes are made up by different sections of the primary structure and/or depend on the three-dimensional structure whereas linear epitopes depend only on the primary amino acid sequence. It is difficult to characterize conformational epitopes and the experimental effort can be substantial including X-ray crystallography [17–23] of allergen-antibody-complexes or the generation of mutants carrying amino acid substitutions [24–29]. In this manuscript, two methods to identify primarily linear epitopes are compared using the major allergen from shrimp, Pen a 1, as an example.

2. Material and methods

2.1. Recombinant peptide library

In order to characterize Pen a 1 epitopes, a recombinant peptide library (Novatope epitope mapping system, Novagen) was constructed. The Pen a 1-coding plasmid was randomly cleaved by DNase I in the presence of Mn^{2+}, causing double strand cleavage. Electrophoretically separated fragments, averaging 50–150 bp in size, were eluted (QIAEX II Agarose Gel Extraction Kit, Qiagen), treated successively with T4 DNA polymerase and Tth DNA polymerase, ligated into the pTOPE T vector, and transfected into NovaBlue (DE3) cells. The library was screened with a sera pool of shrimp-allergic subjects and positive clones sequenced.

2.2. Synthetic, overlapping peptides

Forty-six overlapping peptides (length: 15 amino acids, offset: six amino acids, Fig. 1) spanning the entire Pen a 1 molecule, were synthesized using the SPOTs system (Genosys, The Woodlands, TX). The SPOTs system cellulose membrane contains 96 blue spots which are derivatized with a dimer of β-alanine-NH_2 groups that provide six atom linkers (anchor) between the membrane and the peptide. Fmoc-OPfp esters of the amino acids are coupled by repeated amino acid pipetting and washing. Amino acids are linked together by a condensation reaction between the C-terminal and N-terminal groups of two amino acids in a C-terminal to N-terminal direction from the membrane. The coupling reaction is monitored visually by staining the free amines after each coupling cycle with bromophenol blue. The resulting peptides are covalently bound to the membrane at their C-terminus. Each synthesis cycle begins with esterifying the appropriate Fmoc amino acid to the cellulose membrane or the previous amino acid. The coupling reactions are followed by acetylation with acetic anhydride in *N,N*-dimethylformamide to render the peptides unreactive during subsequent cycles. The Fmoc protective groups are removed by adding piperidine to activate the nascent peptides. To add the remaining amino acids the same cycle of coupling, blocking, and deprotection is repeated until the desired peptides are generated. The side chains are then deprotected with a 20:20:1 mixture of dichloromethane, trifluoroacetic acid, and triisobutylsilane and washed with methanol. The membranes are stored at −20°C until used. The synthesis schedules can be calculated using the software provided by Genosys or using the graphing calculator HP48GX (Fig. 2, software can be obtained by the author). The advantage of the HP48GX printouts is that the positions of a particular amino acid are provided as graphs rather than lists of position numbers.

For immunodetection, the membranes were blocked and incubated with 1:5 diluted serum pool or individual sera of shrimp-allergic subjects overnight. IgE reactivities were detected, either using 10 ml ^{125}I-labeled horse-anti-IgE (0.08 μCi/ml; Sanofi Diagnostics Pasteur, Inc.) or monoclonal alkaline phosphatase-labeled anti-human-IgE (Southern Biotechnology Associates, Birmingham, AL, USA) and autoradiography. The exposure time for ^{125}I-labeled anti-IgE was 72 h. For the detection of IgE antibodies using the alkaline phosphatase-conjugated

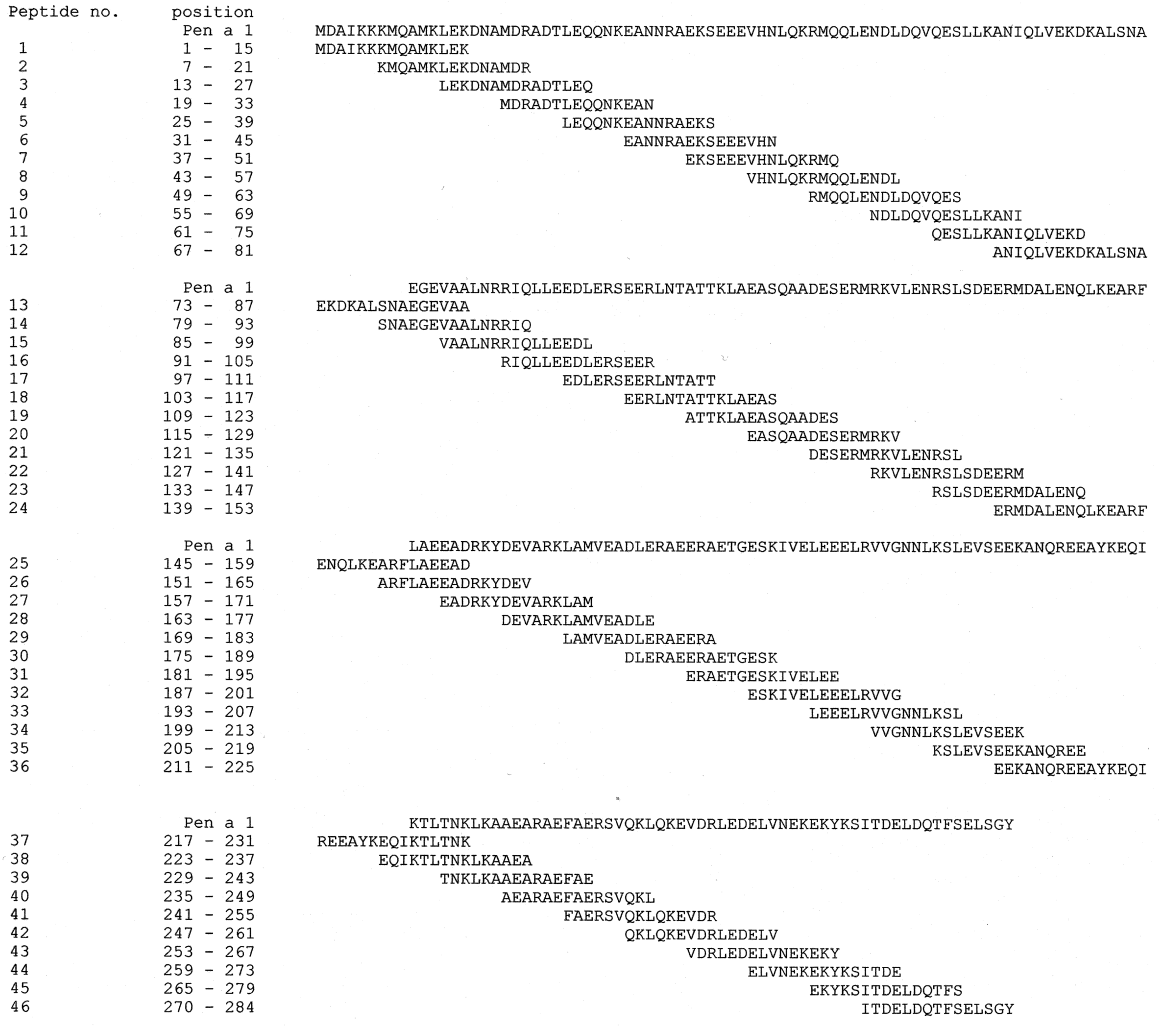

Fig. 1. Amino acid sequences of the 46 overlapping, synthetic peptides (length: 15 amino acids, offset: 6 amino acids) used for the identification of IgE-binding regions of the major shrimp allergen Pen a 1 from brown shrimp, *Penaeus aztecus*.

monoclonal antibodies blots were washed with freshly prepared assay buffer (100 m*M* diethanolamine/HCI, 1.0 m*M* $MgCl_2$ pH 10.0), incubated in 1:50 diluted Nitroblock® chemiluminescence enhancer (Tropix, Bedford, MA) for 5 min and incubated in a 1:1000 dilution of CSPD (disodium 3-(4-methoxyspiro{dioxetane-3,2′-(5′chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl)phenyl phosphate; Tropix) for 5 min. Excessive liquid was drained, and the blots were sealed between transparencies and exposed to autoradiography film for 15, 30, 60 and 120 s.

3. Results

3.1. Comparison IgE reactivities to recombinant and synthetic, overlapping peptides

Four recombinant peptides and nine synthetic peptides bound Pen a 1-specific IgE, respectively and are located at the N-terminus, center, and C-terminus of the Pen a 1 molecule. No reactivity was detected in the N-terminal part of Pen a 1 using the recombinant peptide library. In general, the SPOTs system

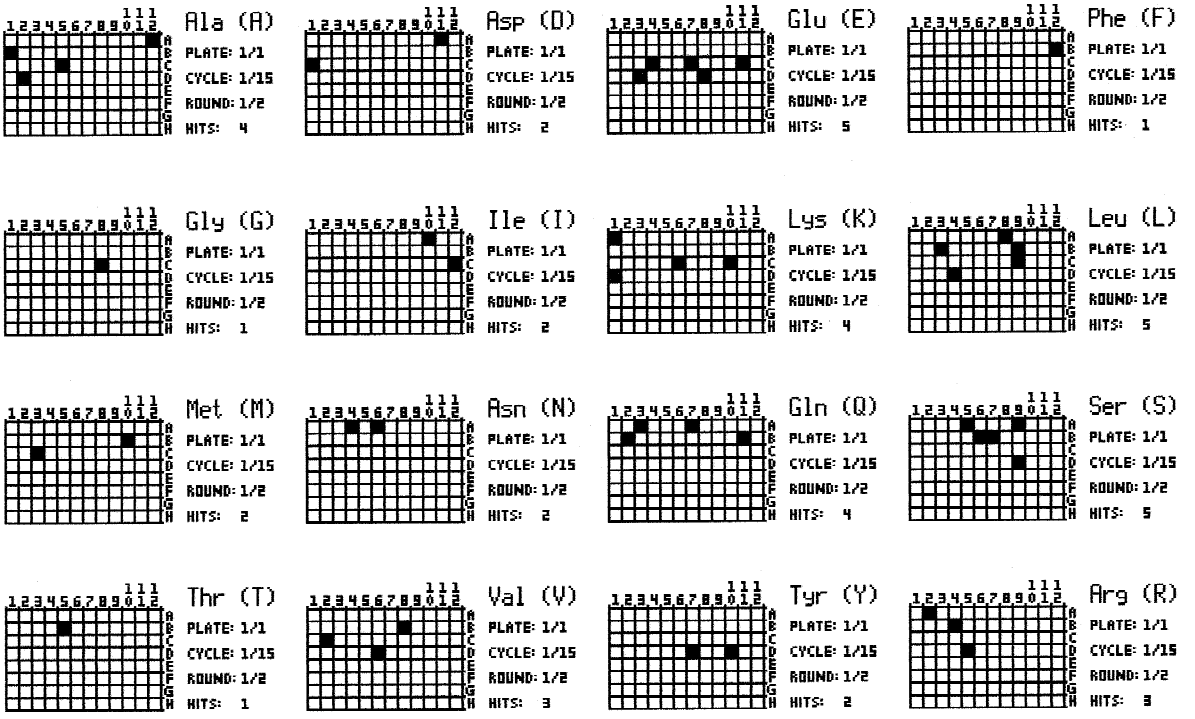

Fig. 2. Synthesis schedule for the C-terminal amino acid position of the overlapping peptides (Fig. 1) spanning the entire Pen a 1 sequence. PLATE: membrane identification number; CYCLE: current cycle, $CYCLE_{max}=15$ since the peptides are 15 amino acids long; ROUND: $ROUND_{max}=2$ since each amino acid is added twice per spot and cycle; HITS: number of spots to which the amino acid is added.

detects more IgE-binding sequences. However, two IgE-binding sequences, Pen a 1 157–169 and Pen a 1 167–179 that were detected using the recombinant peptide library were not detected using synthetic, overlapping peptides even though the entire sequence Pen a 1 157–169 is part of a synthetic peptide. In contrast Pen a 1 167–179 is part of two overlapping peptides (Fig. 3).

3.2. Comparison of ^{125}I and alkaline phosphatase-labeled anti-IgE as detection system for IgE reactivities to synthetic, overlapping peptides

To avoid the use of ^{125}I-labeled detection antibody but detect IgE antibody reactivity with high sensitivity, an alkaline phosphatase-labeled anti-IgE in combination with the chemiluminescence substrate

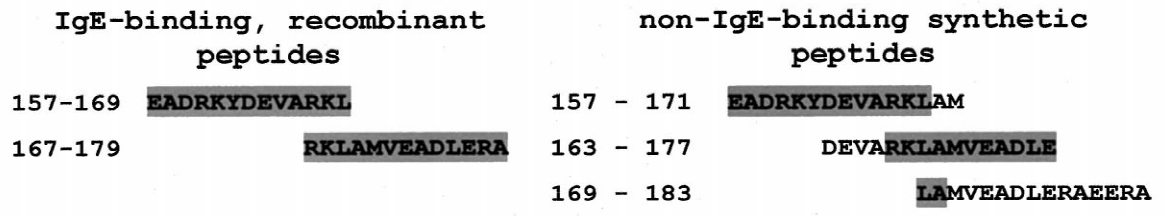

Fig. 3. Sequence comparison of IgE-binding, recombinant peptides and non-IgE-binding synthetic peptides: Identical sequences are shaded.

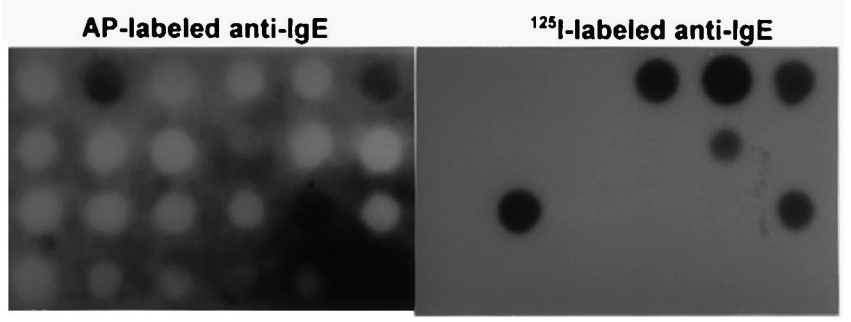

Fig. 4. Comparison of alkaline phosphatase-(AP)-labeled anti-IgE and ^{125}I as detection system for IgE reactivities to synthetic, overlapping peptides.

CSPD was initially used for the detection of IgE reactivities to synthetic, overlapping peptides. However, in comparison with the ^{125}I-labeled antibody, the enzyme-based detection system produced a higher background (Fig. 4) and ^{125}I-labeled anti-human IgE is now the routinely utilized.

3.3. Regeneration of SPOTs membranes

Since SPOTs membranes may be regenerated according to manufacturer's instruction the protocol was tested for efficiency. Briefly, the membranes are washed three times with MilliQ water, three times

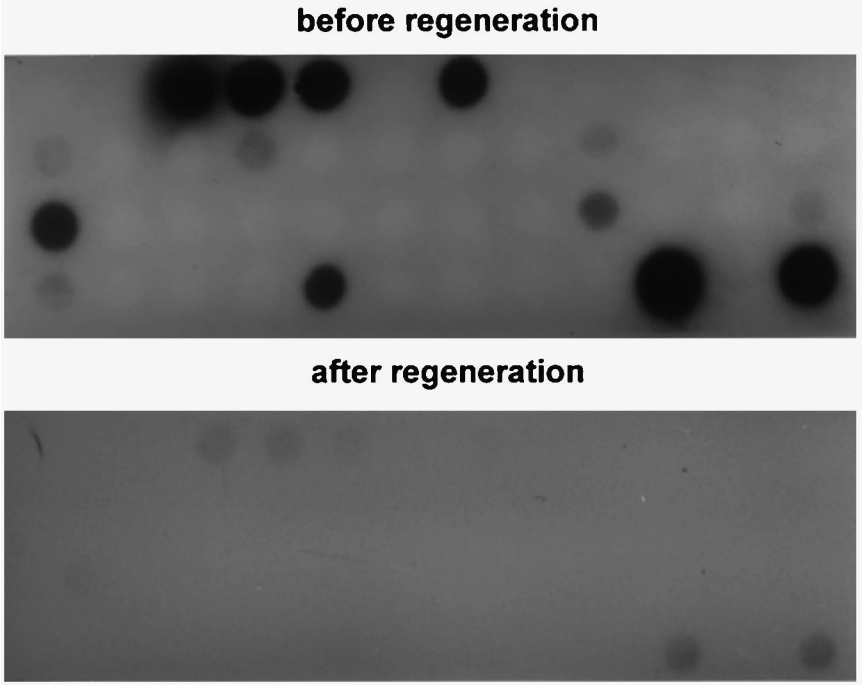

Fig. 5. Regeneration of SPOTs membranes.

with *N,N*-Dimethylformamide (DMF), and two times with MilliQ followed by three incubations in regeneration solution A (8 *M* urea, 1% (w/v) SDS, 0.1% (v/v) β-mercaptoethanol) and regeneration solution B (50% (v/v) ethanol, 10% (v/v) acetic acid). After two washes with methanol the membrane is dried between filter papers with a hair dryer. Five membranes were regenerated and re-exposed to autoradiography for 72 h. Three membranes were completely stripped of antibodies whereas two showed residual activity (Fig. 5). In general, the most intense spots were detectable after regeneration indicating that only some but not all membranes can be regenerated.

4. Discussion

In general, both methods may be used to identify IgE-binding sequences of food allergens, and, in our hands, the SPOTs procedure resulted in the identification of more epitopes of the major shrimp allergen Pen a 1. However, since the sequences of the synthetic, overlapping peptides have a defined offset, epitopes that are located on two peptides overlapping may not be detected. In regard to IgE detection, the usage of ^{125}I-labeled detection antibody seems to be superior over enzyme-labeled anti IgE antibodies. The regeneration of SPOTs membranes is possible, but it is prudent to test regenerated membranes for residual activity. As a consequence of our experience, we synthesize new peptides for each experiment. Besides the technical aspects other factors may influence the technique used for epitope identification of a given food allergen. First, to create a recombinant peptide library, it is necessary to have an expressed full-length allergen or fragments that span the entire length of the allergen and have significant overlaps. The sequences for synthetic, overlapping peptides can be deduced from information available through data bases such as GenBank or SwissProt. Second, its is not possible to ensure that entire allergen sequence is represented in the peptide library whereas overlapping peptides guarantee systematic coverage of the entire allergen sequence. Third, an advantage of the recombinant library method is that the peptide length is not limited to 15 residues as it is the case for the SPOTs system which may allow the identification of at least some conformational epitopes. An additional advantage of the Novatope system that it is easy to test additional patients' sera by simply growing more peptide-expressing *E. coli* and use lysates in a dot blot or grid blot. Synthetic peptides have to be resynthesized which requires in comparison a much higher experimental effort. Fifth, a major advantage of synthetic peptides is the ease in which the impact of amino acid substitutions have on the IgE binding of epitopes [12,30,31]; the side-by-side comparison of unmodified and mutated epitopes allows an easy quantification of changes of protein structure on the allergenicity of proteins [30]. This approach may be used to produce foods and other allergens with reduced allergenicity.

Another, very similar method for epitope mapping with overlapping peptides is the Multipin method according to Geysen [32,33]. The advantage of this method is that peptides may be cleaved from their solid-phase if appropriate linkers are used. These cleaved peptides can then be used to characterize T cell epitopes [34–37]. Linkers that would permit the synthesis of cleavable peptides were described for the SPOTs system [38], however, this modification is not currently available commercially.

Since the synthesis conditions are not optimized for each amino acid or peptide the question arises whether the synthesized peptides have the correct sequence. The synthesis protocol uses an acetylation step at the end of each cycle to acetylate any unreacted free amines with acetic anhydride. This prevents them from coupling to any subsequent amino acids and virtually eliminates the synthesis of deletion sequences. The purity of the peptides synthesized varies for each peptide and is dependent upon sequence and length even though the peptide purity is typically larger than 70% (Genosys, personal communication). As a consequence it is essential to verify the results obtained with overlapping peptides with highly purified peptides when peptides are designed for critical applications such as allergen-specific immunotherapy.

In conclusion, if a given food allergen contains significant linear epitopes, which seems to be true for stable major allergens such as those of peanut and shrimp the SPOTs system may be more advantageous than the use of recombinant peptides libraries.

However, if allergens are studied that contain more conformational epitopes, recombinant peptide libraries may help to identify the relevant epitopes. It has to be emphasized that no system for epitope identification will detect all epitopes and that the relevance of identified epitopes has to be confirmed with other methods such as inhibition studies, crystallographic analysis or the immunological evaluation of modified whole allergens.

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