Epitope Mapping of Major Ragweed Allergen Amb a 1

Abida Zahirović,1 Borut Štrukelj,1 Peter Korošec2 and Mojca Lunder1,*

1 Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia
2 University Clinic of Respiratory and Allergic Diseases Golnik, Golnik, Slovenia

* Corresponding author: E-mail: mojca.lunder@ffa.uni-lj.si
Tel.: +386 1 47 69 570; Fax.: +386 1 42 58 031

Received: 06-01-2018

Dedicated to the memory of Prof. Dr. Igor Kregar

Abstract

Ragweed is a prominent cause of seasonal allergies. Thus far, information on IgE-binding sites of major allergen in ragweed pollen, Amb a 1, is very limited. A powerful experimental method to gain insights on the allergen epitopes is the selection of peptides from biological libraries that bind to anti-allergen antibodies. In this work, we aimed to map IgE epitopes of Amb a 1 using epitope-mimicking short peptides – mimotopes that were affinity-selected from phage-displayed random peptide libraries. The peptides weakly aligned with the Amb a 1 primary sequence, thus suggesting that the epitopes are conformational. When the peptides were mapped onto the surface of Amb a 1 homology model, the EpiSearch analysis predicted the location of four potential epitopic sites on surface patches centred at residues K104, S110, H214, and W312. The peptides matching to the predicted epitopes bound selectively to the IgE from pool of ragweed-allergic patients’ sera and therefore represent mimetics of Amb a 1 IgE epitopes. The knowledge of IgE epitopes is a prerequisite for the rational design of molecular-based approaches to diagnosis and immunotherapy of allergic diseases.

Keywords: Ragweed allergy; Amb a 1; epitope mapping; phage display; mimotopes

1. Introduction

Short ragweed (Ambrosia artemisiifolia) is one of the most important allergen source in North America.1 Because of its fast spreading, sensitization rates are also increasing in Central and Southeastern Europe, ranging from 15% to ~80%.2 Current therapeutic options for ragweed allergy involve symptomatic treatment and allergen-specific immunotherapy. Conventional immunotherapy with crude pollen extracts is the only available curative treatment. However, it may induce undesired IgE-mediated side effects and long-term therapy is required, which often hampers patient compliance.3,4 Therefore, new approaches to immunotherapy that include well-defined therapeutic molecules with reduced or abolished IgE binding capacity are being investigated.

Ragweed pollen allergy is especially suited for molecule-based immunotherapeutic strategies due to the dominance of one allergen. Molecule-based vaccines are based on individual allergen proteins, allergen-derived peptides containing relevant epitopes or epitope-mimicking peptides (mimotopes).5 Among 14 allergens described in ragweed pollen, Amb a 1 has been identified as the major disease-causing agent, which reacts with IgE of more than 90% of the ragweed-sensitized patients.6,7 It is a non-glycosylated 38 kDa protein that belongs to the family of pectate lyases and accounts for up to 15% of total proteins in the ragweed pollen.8,9 It has been demonstrated that Amb a 1 in a form of a conjugate with toll-like receptor agonist can replace the whole pollen extract in immunotherapy.10 Five different isoforms of Amb a 1 with about 80% sequence identity have been found.11 They display distinct patterns of IgE binding and immuno-

...
sign of molecule-based reagents for diagnosis and immunotherapy.

Investigation of T-cell response to Amb a 1 revealed multiple dominant T-cell epitopes (Amb a 1 176–191, 200–215, 280–295, 304–319, 320–335, and 344–359). However, the data on the conformational B-cell epitopes is still scarce. Screening of random peptide phage libraries against anti-allergen antibodies is a fast and relatively inexpensive alternative compared to other methods for epitope mapping and in combination with computer-based algorithms can lead to the identification of conformational allergen epitopes. Successful application of this technology provides peptide mimotopes that are able to bind IgE antibodies and are not necessarily identical to original epitope, but rather mimic its essential features. Mimotopes are considered to have similar physicochemical characteristics and spatial organization as their corresponding epitopes. As such, they may be employed for the development of safer immunotherapies either based on carrier-bound peptide vaccines or based on hypoallergenic recombinant allergens.

In this study, we screened phage display libraries of random peptides using Amb a 1-specific IgG to define peptides mimicking Amb a 1 epitopes. Best mimotope candidates were tested for their IgE reactivity with sera of ragweed-allergic patients. Using computational epitope mapping tools affinity-selected peptide sequences were analyzed to predict the location of epitopes on Amb a 1.

2. Experimental

2.1. Purification and Evaluation of Target Antibodies

Anti-Amb a 1 IgG were affinity-purified from rabbit antiserum (Indoor Biotechnologies, Cat# PA-AM1, RRID:AB_2728628) using natural allergen Amb a 1 (INDOOR Biotechnologies) immobilized on Dynabeads M-280 Tosylactivated (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer’s instructions.

2.2. Biopanning of Phage Display Libraries

Affinity-purified rabbit IgG specific for Amb a 1 were immobilized alternatingly onto 0.45 mg of protein G or protein A coupled magnetic beads (Dynabeads, Thermo Fisher Scientific) by incubation in PBS/0.05% Tween 20 for 30 min at room temperature and used as a target in biopanning. Three phage display libraries (New England Biolabs, Ipswich, Massachusetts, USA) of linear heptamer, linear dodecamer, and cyclic heptamer random peptides were panned as described in the manufacturer’s manual. The libraries contain approximately $10^8$ unique peptide sequences fused to the pIII minor coat protein of the M13 filamentous phage. Bound phages were eluted from the target antibodies either with 0.1 M glycine-HCl (pH 2.2) for 10 min followed by immediate neutralization with 1 M Tris (pH 8.0) or competitively with Amb a 1 at the final concentration of 12 μg/ml. After three rounds of affinity selection, 24 individual clones from each elution method (total 144 clones) were amplified and screened for binding to target antibodies by monoclonal phage ELISA.

2.3. Monoclonal Phage ELISA

MaxiSorp microtiter plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 2 μg/ml of anti-Amb a 1 IgG (RRID:AB_2728628) in PBS overnight at 4 °C. Plates were blocked with 5% skimmed milk in PBS for 1.5 h at room temperature and washed three times with PBS/0.1% Tween 20. A separate set of wells was covered with blocking buffer only, to determine background binding. The amplified phage clones or control (wild-type phage clone with no peptide displayed on its surface) in LB were then loaded into the wells. After 60 min of incubation, the wells were washed five times with PBS/0.1% Tween 20. For detection, HRP-conjugated anti-M13 monoclonal antibodies (GE Healthcare Cat# 27942101 RRID: AB_2616587) diluted 1:5000 were added to the wells and incubated for 1 h. The colour was developed with TMB Super Tracker substrate (ImmunoO4, Westminster, UK) supplemented with 0.006% H₂O₂. After terminating the reaction with 2 M H₂SO₄ absorbance was measured at 450 nm with the microtiter plate reader (Tecan Safire, Tecan Group AG, Männedorf, Switzerland). Phage clones with the highest target to background absorbance ratio were subjected to DNA sequencing (GATC Biotech, Konstanz, Germany).

2.4. Characterization of Phage-displayed Peptides Binding to Target Antibodies

Seventeen phage clones displaying unique peptides were purified by PEG-precipitation, resuspended in PBS and quantified by spectrophotometry. Binding of the phage-displayed peptides to the target antibodies was assessed by semiquantitative ELISA. The suspensions containing $2 \times 10^8$ pfu of individual phage clones or control phage in PBS were loaded into the wells. To detect possible binders to antibody constant regions, the wells were coated with control rabbit antiserum raised against procathepsin X (Biogenes, Berlin, Germany) diluted 1:1000 in PBS overnight at 4 °C. For competition ELISA selected clones were added to the wells together with the allergen in three different concentrations (1 μg/ml, 5 μg/ml, 10 μg/ml) and incubated with target antibodies for 1 h at room temperature. The following steps in the assays were as described above for monoclonal phage ELISA. All experiments were carried out in triplicates.
2. 5. Linear Alignment of Peptides with Amb a 1 Sequence and Mapping to the 3D Homology Model of Amb a 1

Obtained amino acid sequences were checked for the presence of target-unrelated peptides using the MimoDB 2.0 database. Peptides were compared among themselves and aligned with Amb a 1 sequence using multiple sequence alignment program, Clustal Omega, to find a consensus pattern of amino acids.24 The 3D model structure of Amb a 1 was generated using a protein fold recognition server Phyre.25 The EpiSearch method was used to define the potential epitope sites on the surface of Amb a 1.26

2. 6. Isolation of pIII-Fused Peptides from E. coli Periplasm

Six peptides showing the highest specific binding to target antibodies were extracted from the periplasm of E. coli ER2738 as fusions with pIII phage coat protein. Host bacteria were infected with individual peptide-displaying phage clone and grown for 2 h at 37 °C with agitation. Bacterial pellets were spun down at 5000 ×g for 10 min and resuspended in 1 ml of an ice-cold solution consisting of 20% sucrose, 200 mM Tris-HCl (pH 8.0) and 1 mM EDTA supplemented with protease inhibitor cocktail (EZBlock®, BioVision, San Francisco, USA) at a dilution of 1:200. Following 1 h incubation on ice with occasional stirring, supernatants were harvested by centrifugation at 12000 ×g for 20 min at 4 °C. The resulting periplasmic extracts were concentrated (4–5 fold) and the extraction buffer was exchanged for PBS by ultrafiltration using 10 kDa cut-off membranes (Microsep Advance Centrifugal Device, Pall Corporation, New York, USA). Extracts of non-infected bacteria and bacteria infected with phage clone carrying unrelated pIII-fused control peptide (linear GTFDHPQ targeting streptavidin) were prepared in the same way and used as negative controls.27

2. 7. Sera of Ragweed-Allergic Patients

Serum samples from ragweed-allergic patients with positive sIgE to Amb a 1 were collected at the University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia prior to starting immunotherapy. Patients’ characteristics are shown in Table 1. The study was approved by the National Medical Ethics Committee of Republic of Slovenia (No. 35/06/14). All patients willingly donated their blood for research.

2. 8. Immunodot Assay: Binding of pIII-Fused Peptides to Patients’ IgE

Two microliters of each sample of pIII-fused peptides were spotted onto 0.45 µm nitrocellulose membrane (GE Healthcare). The membrane was blocked with 5% skimmed milk in Tris-buffered saline/0.05% Tween 20 (0.05% TBST) for 3 h at room temperature and then incubated overnight at 4 °C with a pool of sera from ragweed allergic-patients (patients 1 to 8, Table 1) diluted 1:10 in 0.05% TBST. After triple washing with 0.1% TBST, membranes were incubated with HRP-conjugated goat anti-human IgE antibodies (Thermo Fisher Scientific Cat# A18793 RRID: AB_2535570) diluted 1:2000 in 1% BSA/0.1% TBST for 2 h at room temperature. The reactive dots were visualized with CCD image analysis system (G-Box, Syngene, United Kingdom) using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

3. Results and Discussion

In this study, we sought to map epitopes of major ragweed allergen Amb a 1 by panning phage-displayed random peptide libraries. A thoughtful choice of biopanning conditions is crucial to overcome the limitations of commercially available peptide libraries and to improve the selection success rate.28,29 To avoid recovery of target-unrelated binders we used protein A or protein G coupled beads alternatingly for immobilization of target antibodies. Biopanning of three phage displayed-libraries was carried out with affinity-purified rabbit IgG specific for Amb a 1 using specific and non-specific elution. Given that the antibodies of IgG isotype were used as target, the reactivity of selected peptides with serum IgE was tested in order to evaluate whether a conserved epitope specificity between the IgG and IgE exist and thus to determine whether the identified peptides are also mimetics of IgE epitopes in ragweed-allergic patients. Forty-two phage clones reactive with Amb a 1-specific target antibodies but not with the components of background buffer in preliminary phage ELISA were selected for sequencing. Peptides RVVELMDWTVLH, CLFSQGNRC, MRTDMVI, and CIMSIVGTC were the most strongly enriched (number of isolated identical sequences is shown in brackets alongside these peptides in Figs. 1 and 2). Overall, sequencing yielded 17 different peptides.

### Table 1. Characteristics of ragweed-allergic patients.

| Patient | sIgE* Amb a 1 (kU/L) |
|---------|---------------------|
| Patient 1 | 0.77 |
| Patient 2 | 41.4 |
| Patient 3 | 1.51 |
| Patient 4 | 0.61 |
| Patient 5 | 0.62 |
| Patient 6 | 0.5 |
| Patient 7 | 1.6 |
| Patient 8 | 1.71 |

sIgE* – specific immunoglobulin E were measured by using CLIA Immulite (Siemens, Erlangen, Germany).
Binding of individual peptides displayed on phage to target antibodies was ranked in semiquantitative ELISA assay (Fig 1, A). In contrast to control phage (with no peptide displayed), six peptide-displaying phage clones demonstrated at least two-fold higher binding to the target antibodies compared to the background. Other 11 peptide-displaying phage clones showed lower binding to target antibodies. Potential non-specific interaction with antibody constant region was checked by ELISA with control antibodies that were produced in the same species as target antibody and thus contain identical Fc fragment. Binding to control rabbit antisera was low and comparable between peptide-displaying phage clones and control phage (Fig 1, B). Therefore, the interaction of peptides with antibody constant region was excluded. Six phage-displayed peptides that exhibited the best binding to target antibodies (RV-...)

**Figure 1:** Characterization of the phage-displayed peptides affinity-selected with anti-Amb a 1 rabbit IgG. (A) Binding of phage-displayed peptides to the immobilized target antibodies and background (5% milk). Values in brackets represent the frequency of the selected peptides. (B) Binding of phage-displayed peptides to the immobilized control rabbit antiserum (anti-procathepsin X). Wild-type phage with no displayed peptide served as negative control. The data are presented as the mean ± standard deviation of three individual experiments. (C) Displacement of six selected phage-displayed peptides from target antibodies with 1 μg/ml, 5 μg/ml and 10 μg/ml of natural Amb a 1.

---

Zahirović et al.: Epitope Mapping of Major Ragweed ...
Isoforms of Amb a 1 display distinct patterns of IgE binding. In previous study, Amb a 1.01 showed higher binding to target antibodies. For example, motif SQGNR appeared in 12 peptides from the cyclic library but only one peptide sequence (CLFSQGNRC) bound significantly to the target antibodies (Fig. 1A). This indicated that other residues outside the motif were important for target antibody binding as well.

Fig. 3 depicts linear alignment of the peptides with Amb a 1.01 sequence. The peptides were either aligned with shorter segments consisting of only a few matching residues within Amb a 1 sequence or not aligned at all (Fig. 3). Matching amino acids were also not the same as those included in common motifs within individual groups. This suggests that the epitopes of Amb a 1 are conformational. Indeed, it has been previously shown that inhalational allergens contain mainly conformational epitopes consisting of amino acids that are distributed over the protein sequence and come into close contact upon protein folding.30–32

For in silico mapping of conformational epitopes on Amb a 1, we created the 3D structural model of Amb a 1 since its crystal structure is not yet available. Using a protein fold recognition server Phyre2, we generated a high confidence close homology model of Amb a 1 with sequence coverage of 92%.25 The model is based on structural template d1pxza of Jun a 1, the major allergen from cedar pollen, as the closest homolog with known structure that contains a single-stranded right-handed beta-helix fold. Jun a 1 is a member of the pectate lyase family of allergens and provides a reliable homology model since it has a sequence identity of 47% with a FFAS score −93.1 with Amb a 1.33 EpiSearch method was used to reveal the location of epitopes on surface of Amb a 1 model.26 This approach uses patch analysis and solvent accessible surface area of amino acids to map peptides obtained from phage display experiments onto the 3D structure of a protein. The best match between the amino acid composition of the peptides and surface-exposed areas on the 3D model of allergen is predictive of epitope.

We used six representative peptides from groups 1–4 that showed the highest binding to target antibodies (Fig. 1C, Fig. 2; underlined peptides) as input sequences for Episearch. The natural allergen Amb a 1 is composed of two non-covalently associated subchains, the N-terminal β chain (amino acids 26–180) and the C-terminal α chain (amino acids 181–396). Two representatives from group 1 were mapped to the loop on β chain at the N terminus containing residues V72, A73, N74, L102, K104, V107, G127, V148, N149, P150, G151, G152, L153, S156, A161, A162, P163, A165, G166, and, S167 with center residue at K104 (score: 1.000) (Fig. 4A).

Two representatives from group 2 were mapped to the loop on β chain at the C terminus containing residues R233, H234, A236, S237, T239, L242, G248, G250, K252, H253, G255, E264, A265, A266, F268, S270, N271, W312, R315, V328, A329, V330, and, G331 with center residue at W312 (score: 0.950) (Fig. 4B). A representative from group 3 was mapped to the loop on β chain at the N terminus containing residues Q126, N127, R128, L129, N130, S131, A132, G133, N135, S135, C136, C137, S138, and Q179 with center residue at S110 (score: 1.000)
Figure 4C). A representative from group 4 was mapped to the beta strand on α chain at the C terminus containing residues D_{145}, D_{170}, D_{192}, L_{210}, Q_{213}, H_{214}, Q_{215}, F_{216}, D_{243}, D_{246}, Q_{247}, and D_{265} with center residue at H_{214} (score: 1.000) (Fig. 4D). β chain was suggested to contain IgE binding sites based on its higher IgE reactivity compared to α subchain in the previous study. In this study, Epi-search analysis predicted two epitopes to be located on β chain at the N terminus (mapped by peptides from groups 1 and 3) and also predicted two epitopes on α chain at the C terminus (mapped by peptides from groups 2 and 4). Predicted epitopes are located on the solvent-exposed loops and beta strands of Amb a 1 model structure. These results agree with the known fact that conformational B-cell epitopes are usually associated with turns or loops and exposed regions protruding from protein surfaces and suggest that residues in these areas are involved in antibody binding.

Given that the biopanning was carried out with antibodies of IgG isotype as target, in order to determine the relevance of deduced epitopes in ragweed-allergic patients we tested the conserved epitope specificity between IgG and IgE by evaluating the reactivity of identified peptides with patient sera. Six representative peptides from groups 1–4 that showed the highest binding to target (Fig. 1C, Fig. 2; underlined peptides) were isolated as fusions with pIII phage coat protein from Escherichia coli and tested for binding to IgE from sera pool of ragweed-allergic patients (patients 1–8, Table 1). Extract from noninfected bacteria and an unrelated pIII-fused peptide (linear GTFDHPQ targeting streptavidin) were used as controls. Sera pool showed IgE binding to the six pIII-fused peptides (Fig. 5). Protein pIII used as a carrier of peptides ensured the correct conformation of the peptides during the assay and allowed efficient immobilisation on the membrane. Signals were not detected with the control samples (Fig. 5). Therefore, binding was attributed only to the IgE epitope-mimicking peptides (mimotopes). Thus, the mimotopes showed binding with target IgG as well as with patients’ IgE. This indicates that the identified epitopes are relevant for both anti-
4. Conclusions

In biopanning experiments against the polyclonal Amb a 1-specific rabbit IgG, we enriched peptides and arranged them into six groups according to their degree of similarity. The peptides weakly matched with shorter segments of Amb a 1 sequence, thus suggesting that the epitopes of Amb a 1 are conformational. Conformational mapping of the six representative peptides to the surface of the structural model of Amb a 1 predicted the location of four epitopic sites on surface patches centred at residues K_{104}, S_{110}, H_{214}, and W_{312}. The representative peptides bound to IgE from ragweed-allergic patients and are therefore mimetics of Amb a 1 IgE epitopes. These results pave the way towards the identification of conformational epitopes of Amb a 1. The structure and location of each new epitope increase our knowledge and, hence, the probability of identifying common features of cross-reactive allergen-antibody recognition sites, which would ultimately help to reveal the characteristics of the cross-reactive allergens and their underlying mechanism of action. In the context of immunotherapy, the identification of allergen epitopes and their mimotopes provides the basis for the rational design of immunotherapeutic constructs either based on recombinant allergen derivatives with reduced allergenic activity or mimotope-based carrier-bound vaccine for more defined and safer allergen-specific immunotherapy.

Conflict of interest
Authors declare no conflict of interest.

5. References

1. F. Ihler, M. Canis, *J. Asthma Allergy* 2015, 8, 15–24. DOI:10.2147/JAA.S47789
2. M. L. Oswalt, G. D. Marshall, *Allergy Asthma Clin. Immunol.* 2008, 4, 130–135. DOI:10.1186/1710-1492-4-3-130
3. H. Lowenstein, *Chem. Immunol. Allergy* 2014, 100, 323–332. DOI:10.1159/000359989
4. M. Turkalj, I. Banic, S. A. Anzic, *Patient Prefer. Adherence* 2017, Volume 11, 247–257. DOI:10.2217/paa.s70411
5. B. Linhart, R. Valenta, *Curr. Opin. Immunol.* 2005, 17, 646–655. DOI:10.1016/j.coi.2005.09.010
6. M. Wallner, U. Pichler, F. Ferreira, *Immunotherapy* 2013, 5, 1323–1338. DOI:10.2217/imt.13.114
7. G. Gadermaier, N. Wopfner, M. Wallner, M. Egger, A. Didier-laurent, G. Regl, F. Aberger, R. Lang, F. Ferreira, T. Hawranek, *Allergy* 2008, 63, 1543–1549. DOI:10.1111/j.1398-9995.2008.01780.x
8. V. Bordas-Le Floch, R. Groeme, H. Chabre, V. Baron-Bodo, E. Nony, L. Mascarell, P. Moingeon, *Curr. Allergy Asthma Rep.* 2015, 15, DOI:10.1007/s11882-015-0565-6
9. T. Rafnar, I. J. Griffith, M. C. Kuo, J. F. Bond, B. L. Rogers, D. G. Klapper, *J. Biol. Chem.* 1991, 266, 1229–1236.
10. P. S. Cretics, J. T. Schroeder, R. G. Hamilton, S. L. Bali...
Povzetek
Ambrozijska je pomemben vzrok sezonskih alergij. Do sedaj imamo zelo malo informacij o IgE vezavnih mestih na glavnem alergenu iz cvetnega prahu ambrozijske, Amb a 1. Afinitetna selekcija pepitidov iz bioloških knjižnic z uporabo specifičnih protitela proti alergenu je uporabna laboratorijska metoda za določevanje epitopov. V tej raziskavi smo s pomočjo mimotopov, kratkih pepitidov, ki posnemajo epitope, izoliranih iz bakteriofagnih knjižnic naključnih pepitidov, določali IgE epitope Amb a 1. Izbrani pepitidi so se le šibko ujemali s primarnim zaporedjem Amb a 1, kar je nakazovalo, da so epitopi konformacijski. Da bi jih določili, smo izdelali homologni model tridimenzionalne strukture Amb a 1, na podlagi sekvence, konformacijskih IgE epitopov Amb a 1. Poznavanje IgE epitopov je predpogoj za racionalno načrtovanje molekularnih pristopov v diagnostiki in immunoterapiji alergijskih bolezni.

Zahirović et al.: Epitope Mapping of Major Ragweed ...