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RESEARCH ARTICLE

Screening and identification of mimotopes of the major shrimp allergen tropomyosin using one-bead-one-compound peptide libraries

Nicki YH Leung[1], Christine YY Wai[1], Marco HK Ho[2], Ruiwu Liu[3], Kit S Lam[3], Jin Jun Wang[4], Shang An Shu[4], Ka Hou Chu[1] and Patrick SC Leung[4]

The one-bead-one-compound (OBOC) combinatorial peptide library is a powerful tool to identify ligand and receptor interactions. Here, we applied the OBOC library technology to identify mimotopes specific to the immunoglobulin E (IgE) epitopes of the major shellfish allergen tropomyosin. OBOC peptide libraries with 8–12 amino acid residues were screened with serum samples from patients with shellfish allergy for IgE mimotopes of tropomyosin. Twenty-five mimotopes were identified from the screening and their binding reactivity to tropomyosin-specific IgE was confirmed by peptide ELISA. These mimotopes could be divided into seven clusters based on sequence homology, and epitope mapping by EpiSearch of the clustered mimotopes was performed to characterize and confirm the validity of mimotopes. Five out of six of the predicted epitopes were found to overlap with previously identified epitopes of tropomyosin. To further confirm the mimicry potential of mimotopes, BALB/c mice were immunized with mimotopes conjugated to keyhole limpet hemocyanin and assayed for their capacity to induce tropomyosin-specific antibodies. BALB/c mice that received mimotope immunization were found to have an elevated level of tropomyosin-specific immunoglobulin G, but not mice that received an irrelevant mimotope. This study pioneers the successful application of the OBOC libraries using whole sera to screen and identify multiple shrimp allergen mimotopes and validates their mimicry potential using in vitro, in vivo, and in silico methods.
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INTRODUCTION

Mimotopes are short peptides resembling epitopes of an antigen and could serve important applications in immunotherapies. First coined in 1986,[1] they can induce epitope-specific antibodies when coupled to an immunogenic carrier, which are crucial in numerous therapeutic treatments in neutralizing pathogens. Increasing number of studies have demonstrated the success of mimotope-based therapy in various diseases.[2–6]

Inspired by the unique property of mimotopes to induce epitope-specific antibodies, we investigated the capacity of mimotopes to inhibit immunoglobulin E (IgE)-allergen binding.[2,7,8] Due to their monovalent properties, they are safer than natural extracts/recombinant allergens in allergen-specific immunotherapy (SIT), and can prevent anaphylaxis caused by cross-linking of IgE and degranulation of mast cells. Another key advantage of mimotope-based allergy treatments is the absence of T-cell epitopes which means that T-cell mediated late-phase anaphylactic reactions, which commonly occur in the course of SIT, are minimized.[9]

Despite such advantages, studies on mimotopes in allergies are mostly limited to epitope mapping of allergens[10–15] and their applications in immunotherapy are lacking. Biopanning with phage-displayed peptide libraries is the most common platform used in identifying mimotopes, but this process requires highly purified antibodies or, more often, monoclonal antibodies (mAbs). Moreover, developing SIT by identifying

[1]School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China; [2]Department of Pediatrics and Adolescent Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong SAR, China; [3]Department of Biochemistry and Molecular Medicine, University of California, Davis, CA 95616, USA and [4]Division of Rheumatology/Allergy, School of Medicine, University of California, Davis, CA 95616, USA
Correspondence: PSC Leung, Division of Rheumatology/Allergy, School of Medicine, University of California, Davis, CA 95616, USA.
E-mail: psleung@ucdavis.edu;
KH Chu, School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China.
E-mail: kahouchu@cuhk.edu.hk
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mimotopes of allergens with multiple IgE-binding epitopes could potentially be problematic and laborious, as biopanning with polyclonal antibodies does not always reveal a single consensus sequence or motif.[16] As a result, multiple rounds of biopanning using different mAbs may be required.

To circumvent the limitations of biopanning of phage-displayed libraries, we applied one-bead-one-compound (OBOC) combinatorial peptide library technology for high throughput screening of mimotopes. The OBOC library is a non-biological synthetic library that has gained much success in drug discovery and isolating cancer-specific peptides.[17,18] A key advantage of the OBOC library over the phage-display is the power of a quantitative estimation throughout the screening process, which allows the use of less stringent screening agent such as whole cells[19] in cancer-specific peptides or whole serum.[20] Here, we have identified mimotope sequences that are specific to dominant IgE epitopes of the major shellfish allergen tropomyosin from the shrimp *Metapenaeus ensis* (Met e 1). Our group was the first to clone and identify Met e 1 as the major shrimp allergen[21] and, recently, to define the IgE-binding epitopes using *in vitro* and *in silico* methods.[22] Since it contains well-characterized multiple immunodominant regions, tropomyosin is an ideal allergen to use to investigate the potential for applying OBOC library in isolating allergen-associated peptides, i.e. mimotopes.

METHODS

Human serum

Serum samples from 10 subjects with documented clinical shrimp hypersensitivity (Supplementary Table 1) were used for screening the OBOC combinatorial peptide libraries. The presence of Met e 1-specific IgE was confirmed by immunoblotting and enzyme-linked immunosorbent assay. Serum samples from patients 1–5 (age: 4–19) were pooled in equal volume to generate the adolescent/children serum pool and the adult serum pool was generated similarly by mixing serum samples from patients 6–10 (age: 29–54). This study was approved by the Joint Chinese University of Hong Kong (CUHK) – New Territories East Cluster Clinical Research Ethics Committee.

Design and synthesis of OBOC combinatorial peptide libraries

Five OBOC linear peptide libraries with length from 8-mer to 12-mer were designed. The libraries were prepared as previously described[23] using TentaGel S NH2 beads (Rapp Polymere, Tuebingen, Germany) as the solid support. TentaGel S NH2 beads are copolymer microspheres consisting of low cross-linked polystyrene matrix with grafted polyethylene glycol (3000–4000 daltons) linkers which have an amino group located at the end for direct peptide synthesis. The standard solid-phase peptide synthesis and the split- and mix-method[24] (Supplementary Figure 1) was adopted to synthesize the OBOC peptide libraries. In brief, the TentaGel S NH2 beads were evenly distributed into 19 polypropylene vials, where each vial only contained a single type of natural eukaryotic amino acid. The coupling reaction was initiated by adding threefold excess of amino acids, diisopropylcarbodiimide and N-hydrobenzotriazole. The vials were capped and mixed gently at room temperature for 1 h and the completion of the coupling reaction was confirmed by the Kaiser test. All the beads from the 19 vials were then transferred to a siliconized glass vessel and drained under vacuum. The beads were then washed with N,N-dimethylformamide (DMF) and methanol. Fmoc-deprotection was achieved with 20% piperidine (in DMF) twice, first 5 min and then 15 min. After washing, the beads were evenly distributed into 19 aliquots for another cycle of coupling. The coupling step was repeated 7–11 times so that peptide libraries of 8–12 amino acids in length were synthesized. After the last coupling step, the Fmoc group was removed with 20% piperidine, and the side-chain protecting groups were removed with reagent K (trifluoroacetic acid/phenol/water/thioanisole/ethanedithiol, 82.5:5:5:5:2.5, v/w/v/w/v) at room temperature for 4 h. After the liquid was removed by filtration, the library beads were then thoroughly washed with DMF, methanol, dichloromethane, DMF, 50% water/DMF, water, phosphate-buffered saline (PBS), and stored in 0.05% sodium azide/PBS at 4 °C until use. Two beads from each library were sequenced to ensure the libraries were of high quality before screening. The library permutation for 8-mer and 12-mer libraries would be $19^8 \approx 1.698 \times 10^{10}$ and $19^{12} \approx 2.213 \times 10^{15}$, respectively. Redundancy of the library is negligible as the possibility of two beads having the same sequence in a 8-mer and 12-mer library would be $(1/19)^8 \approx 5.889 \times 10^{11}$ and $(1/19)^{12} \approx 4.518 \times 10^{16}$, respectively.

Two-stage subtraction screening of OBOC libraries

We adopted a modified two-stage subtraction screening approach[25] to screen OBOC libraries (Supplementary Figure 2). For each round of screening, 80 μL of the library (approximately 60 000 beads) was used and the incubation process was carried out in a polypropylene disposable column (Qiagen, Hilden, Germany). In the first stage, the beads were blocked with 3% bovine serum albumin/PBS solution in a revolving mixer at 4 °C overnight. The beads were then washed with 0.05% Tween/PBS (PBST) and incubated with alkaline phosphatase (AP)-conjugated anti-human IgE (Southern Biotech, Birmingham, USA) at 1:20 000 dilution for 1 h at room temperature. The beads were then incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Roche, Mannheim, Germany) for 30 min at room temperature. The reaction was stopped by incubating with 100 μL 0.1 M HCl for 5 min. All beads were plated out onto a petri dish and those that turned in a blue color (false positives) were removed with a pipette under a dissecting microscope (Supplementary Figure 3). The rest of the beads were treated with 6 M guanidine-HCl pH 1.0 solution to remove all proteins bound to the beads followed by washing with water and PBS, and the same procedure used in the first stage was repeated but with incubation in pooled human serum (1:20 dilution) for 1 h at room temperature. True positive

Table 1 Epitope mapping of Met e 1 by EpiSearch using mimotopes obtained through screening OBOC combinatorial libraries with pooled serum from shellfish allergy patients. Only the predictions with the highest (a) and second highest (b) ranking score for each cluster were shown and included in this study. Cluster 6 has only one prediction above the threshold score (ranking score >0.800) and none for cluster 7. A total of 11 patches were predicted from the seven mimotope clusters

Cluster	Mimotope sequence	Ranking score	Patch range
Cluster 1			
(a)	RIWVGHFML[a] MRIMHLNWMYWK	0.875	43-57
(b)	RIWVGHFML MRIMHLNWMYWK	0.800	83-95
Cluster2			
(a)	DIHEESPD[a] HDGIPDWSM	1.00	269-283
(b)	DIHEESPD HDGIPDWSM	0.855	131-145
Cluster 3			
(a)	PTDVERKTSYTL TKYERGGRVRKI KRLFERDG[a]	0.933	252-267
(b)	PTDVERKTSYTL TKYERGGRVRKI KRLFERDG	0.900	186-201
Cluster 4			
(a)	KGHTKAHHGKNT GTKLQHFRQ[a] VTWERTTKHQHW YKTPHQVFQ	0.892	221-236
(b)	KGHTKAHHGKNT GTKLQHFRQ VTWERTTKHQHW YKTPHQVFQ	0.802	224-239
Cluster 5			
(a)	RTIPTMHWIH LHTIPVMI[a] IKALSRLQTIYG	0.917	224-239
(b)[b]	RTIPTMHWIH LHTIPVMI	0.900	88-101
Cluster 6	TFVDDRRFMS MHVLLMRRD HWSSTRRFPP KLAYMHVRV*	0.890	123-137
Cluster 7	MVGWPPKHRKDK RPWPQAHPNL	/	/

[a] Mimotopes selected for *in vivo* verification in each cluster.
[b] The mimotope IKALSRLQTIYG is solely responsible for mapping to a.a. 224-239. We chose to adopt the prediction without mimotope IKALSRLQTIYG for cluster 5 which yielded the second highest ranking score.

beads were collected and washed with freshly made 6 M guanidine-HCl and decolorized by DMF for another round of screening. Both stages were repeated with antibodies of lower dilution (1:50–1:100) and shorter incubation time (30–45 min) to identify beads with the highest affinity to antibody. Only one or two beads with the deepest color were selected from each round of screening. Amino acid sequences of the peptides on the beads were determined via Edman sequencing as described.[26] Each of the five libraries (8-mer to 12-mer) was screened by the adolescent/children serum pool twice and by the adult pool five times.

Multiple sequence alignment

Mimotope sequences were aligned using Clustal Omega.[27] Mimotopes that shared at least three amino acids at the same position were clustered as a group. All mimotope sequences were compared to the peptide sequences in MimoDB 2.0,[28] an information portal to biopanning results of random libraries, in order to exclude any target-unrelated peptide sequences.

Enzyme-linked immunosorbent assay

To determine the binding reactivity of the mimotopes to Met e 1-specific IgE, commercially synthesized mimotope peptides (GenScript, Piscataway, USA) from each of clusters 1–6 (Table 1) were coated at 37 °C for 3 h onto MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) using the peptide ELISA Coating Kit (Takara, Dalian, China). The plates were then washed with PBST and blocked with 10% fetal bovine serum (FBS)/PBS (Gibco, New York, USA) for 2 h at room temperature. After blocking, the plates were incubated overnight with pooled serum samples from subjects with shrimp allergy (Supplementary Table 1) at 1:20 dilution in 10% FBS/PBS at 4 °C. The plates were then washed three times with PBST and incubated with biotinylated anti-human IgE (Southern Biotech) at 1:1000 dilution for 1 h at room temperature, washed and then incubated with Horseradish Peroxidase Avidin D (Vector, Burlingame, USA) diluted at 1:1000 for 30 min at room temperature. After washing five times with PBST, 100 μL/well of TMB substrate (BD Biosciences, San Diego, USA) was added for color development. The reactions were terminated by addition of 100 μL of 0.1 M sulfuric acid to each well and the optical density at 450 nm was measured using a microplate reader (BioRad Model 680). Similarly, for the detection of Met e 1-specific IgG_1 and IgG_{2a} antibodies in mimotope-immunized mice, recombinant Met e 1 (0.5 μg/100 μL $NaHCO_3$, pH 9.6 coating buffer per well) were coated onto Maxisorp microtiter plates, blocked, and incubated overnight with serum samples from mimotope-immunized mice at 1:100 dilution at 4 °C. IgG_1 and IgG_{2a} reactivity were detected using biotinylated goat anti-mouse IgG_1 and IgG_{2a} (Southern Biotech) diluted at 1:2000. IgG_1 titers were also determined as described above using pooled serum diluted 1:100, 1:200, 1:400, 1:800, and 1:1600 in 10% FBS/PBS.

Immunization of BALB/c mice with mimotope-keyhole limpet hemocyanin conjugates

The mimotopes validated with peptide ELISA were conjugated to keyhole limpet hemocyanin (KLH) using an Imject EDC mcKLH Spin Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Female BALB/c

Cluster	Sequence
Cluster 1	R I W V G H F M L
	M R I M H L N W M Y W K
Cluster 2	D I H E E S P D
	H D G I P D W S M
Cluster 3	P T D V E R K T S Y T L
	T K Y E R G G R V R K I
	K R L F E R D G
Cluster 4	K G H T K A H H G K N T
	G T K L Q H F R Q
	V T W E R T T K H Q H W
	Y K T P H Q V F Q
Cluster 5	R T I P T M H W I H
	L H T I P V M I
	I K A L S R L Q T I Y G
Cluster 6	T F V D D R R F M S
	H W S S T R R F P P
	K L A Y M H V R V
	M H V L L M R R D
Cluster 7	M V G W P P K H R K D K
	R P W P Q A H P N L
Non-clustering	H W H A K H A Q R
	W Q R H M V H T W R W M
	A S S R W L G K V H D V
	F F E A G F G N K
	R A V F F R N D H

Figure 1 Mimotopes sharing at least three identical amino acid residues at the same position were clustered into a group after multiple alignment by Clustal Omega. Twenty mimotopes were divided into seven clusters, with 2–4 mimotopes each. The clustering of mimotopes is not related to the serum pool used for screening. Five 'non-clustering' mimotopes could not be grouped with any of the clusters and thus were not included for epitope mapping in this study.

mice (n = 4; 5–6 weeks) were immunized subcutaneously with a mimotope conjugate (50 μg in 200 μL PBS) emulsified in complete Freund's adjuvant on day 0 and incomplete Freund's adjuvant on days 14 and 21. Blood samples were collected on day 28 from the tail vein of the mice. A Der p 1 mimotope[13] (KGIPNTKAP) and an IgE epitope of Met e 1 (KEVDRLEDELVNEKEKYKSI; a.a. 251-270) were also included as negative and positive controls, respectively. All animal protocols were approved by the Animal Experimentation Ethics Committee, CUHK, in accordance with the Department of Health (Government of the Hong Kong Special Administrative Region, HKSAR) guidelines in Care and Use of Animals. All experiments were performed under licenses granted from the HKSAR Government.

Homology modeling

Tropomyosin from *Metapenaeus ensis* (Met e 1) was used as the model allergen in this study. The tropomyosin allergen Pen a 1 from another shrimp species, *Penaeus aztecus*, was also included for comparison.[29] Pen a 1 and Met e 1 are almost identical in amino acid sequence except for a single substitution at position 79. The 3-D structure of Met e 1 was constructed by the Swiss-Model Protein Modeling Server[30] using the wild boar *Sus scrofa* (PDB 1C1G) tropomyosin as modeling template.

Epitope mapping by EpiSearch

Epitope mapping of Met e 1 was carried out with EpiSearch[31] using the clustered mimotopes as data input. The EpiSearch

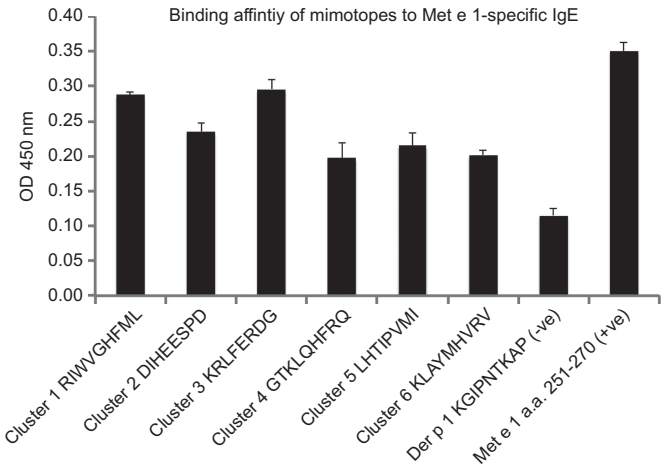

Figure 2 Peptide ELISA demonstrating the binding of Met e 1-specific IgE to the mimotopes. Mimotope KGIPNTKAP of Der p 1 and an IgE-binding epitope of Met e 1 (a.a. 251-270) were included as negative and positive controls, respectively. Met e 1-specific IgE could exclusively recognize the selected mimotopes from clusters 1–6, but not the irrelevant mimotope of Der p 1 ($P < 0.01$ except cluster 4 GTKLQHFRQ $P < 0.05$). Data are expressed as optical density at 450 nm (mean ± SEM) from three independent experiments.

program is an automated tool for predicting the possible location of conformational epitopes on the surface of an antigen, which are then ranked according to frequency distribution of similar residues in the patch of mimotope input. All EpiSearch predictions were based on default conditions (patch size = 12; area cutoff = 10; accuracy cutoff = 3). Linear or cyclic mimotopes from six irrelevant allergens previously identified were also mapped against Met e 1 as a negative control.

Statistical analysis

Met e 1-specific IgG_1 levels were compared between each group of mice receiving mimotope conjugates and the control group receiving KLH alone using *t*-test ($P < 0.05$) in SigmaStat 3.1.

RESULTS

Screening of the OBOC libraries

Five OBOC libraries with 8–12 amino acids were screened by pooled serum samples from adolescent or adult patients with shellfish allergy. Twenty-five mimotope sequences were obtained (Supplementary Table 2) in total. There is no significant difference in the pattern of epitope recognition between the two sera pools. No sequences were found on MimoDB2.0, confirming their relevance to this allergen but not to target-unrelated peptides. Twenty out of the 25 mimotope sequences could be grouped into seven clusters each with at least three amino acid residues in common at the same position, based on the Clustal Omega alignment (Figure 1). Non-clustering mimotopes were not included in subsequent analyses.

Binding reactivity of the mimotopes to Met e 1-specific IgE

Peptide ELISA was conducted to confirm the binding reactivity of the mimotopes to Met e 1-specific IgE (Figure 2). All mimotope peptides from clusters 1–6 had significantly higher reactivity to Met e 1 specific IgE than the irrelevant Der p 1 mimotope ($P < 0.01$; $P < 0.05$ for GTKLQHFRQ of cluster 4).

Induction of Met e 1-specific IgG_1 and IgG_{2a} in BALB/c mice after mimotope immunization

By ELISA, all six mimotope conjugates developed increased levels of Met e 1-specific IgG_1, compared to the control group receiving KLH alone or the irrelevant mimotope ($P < 0.05$) (Figure 3a). The capacity to induce Met e 1-specific antibodies was comparable between the mimotopes and the Met e 1 epitope, except for mimotope GTKLQHFRQ from cluster 4 which exhibited lower capacity ($P = 0.03$). IgG_{2a} levels were not significantly different among groups. Met e 1-specific IgG_1 was detected even at dilution 1:800 in mice receiving mimotope immunization. Met e 1-specific IgG_1 was still detectable at 1:1600 dilution in sera samples of mice immunized with mimotope DIHEESPD of cluster 2 (Figure 3b).

Homology modeling and the mapping of epitopes on Met e 1 by EpiSearch

Only the top two predictions produced by EpiSearch with a ranking score >0.800 were included in the analysis (Table 1). Eleven predicted patches were suggested by EpiSearch with cluster 6 having only one prediction above the threshold and cluster 7 having none. After integrating the 11 predicted patches, six epitope regions were identified: Met e $1^{43\text{-}57}$, Met e $1^{83\text{-}101}$, Met e $1^{123\text{-}145}$, Met e $1^{186\text{-}201}$, Met e $1^{221\text{-}239}$, and Met e $1^{252\text{-}283}$ (Table 2, Figure 4a).

Comparison between mimotope sequences and predicted patch

The patch similarity was calculated by the percentage of identical residues found in the mimotope input relative to the predicted patch (Figure 5). The amino acid residues of the mimotope sequences are generally more than 50% alike in the predicted patches (0.579-1.00). The amino acid residues in predicted epitope of Met e $1^{186\text{-}201}$ and Met e $1^{221\text{-}239}$ were all found in the corresponding mimotope input (Table 2).

Comparison between predicted and genuine epitopes

To validate the specificity of the mimotopes, the predicted patches were compared to the previously reported epitopes of Met e 1 and Pen a 1 by determining epitope coverage and overlapping ratio (Table 2). Five of the six predicted epitope regions, Met e $1^{43\text{-}57}$, Met e $1^{83\text{-}101}$, Met e $1^{123\text{-}145}$, Met e $1^{186\text{-}201}$, and Met e $1^{252\text{-}283}$, overlapped with Pen a 1 epitopes (Figure 4b)[29] with high epitope coverage (0.809–1.00). Met e $1^{43\text{-}57}$ exactly mapped to epitope region 1 from Val43 to Leu57 (epitope coverage = 1.00). Two predicted patches Met e $1^{43\text{-}57}$ and Met e $1^{252\text{-}283}$ were completely within the genuine epitope (overlap ratio = 1). Remarkably, the predicted patches covered all five major IgE-binding regions of Pen a 1. Met e 1 epitopes (Figure 4c)[22] were found to overlap with five of the six predicted regions (Table 2, epitope coverage = 0.285–0.882). The predicted epitope Met e $1^{221\text{-}239}$, which did not overlap with

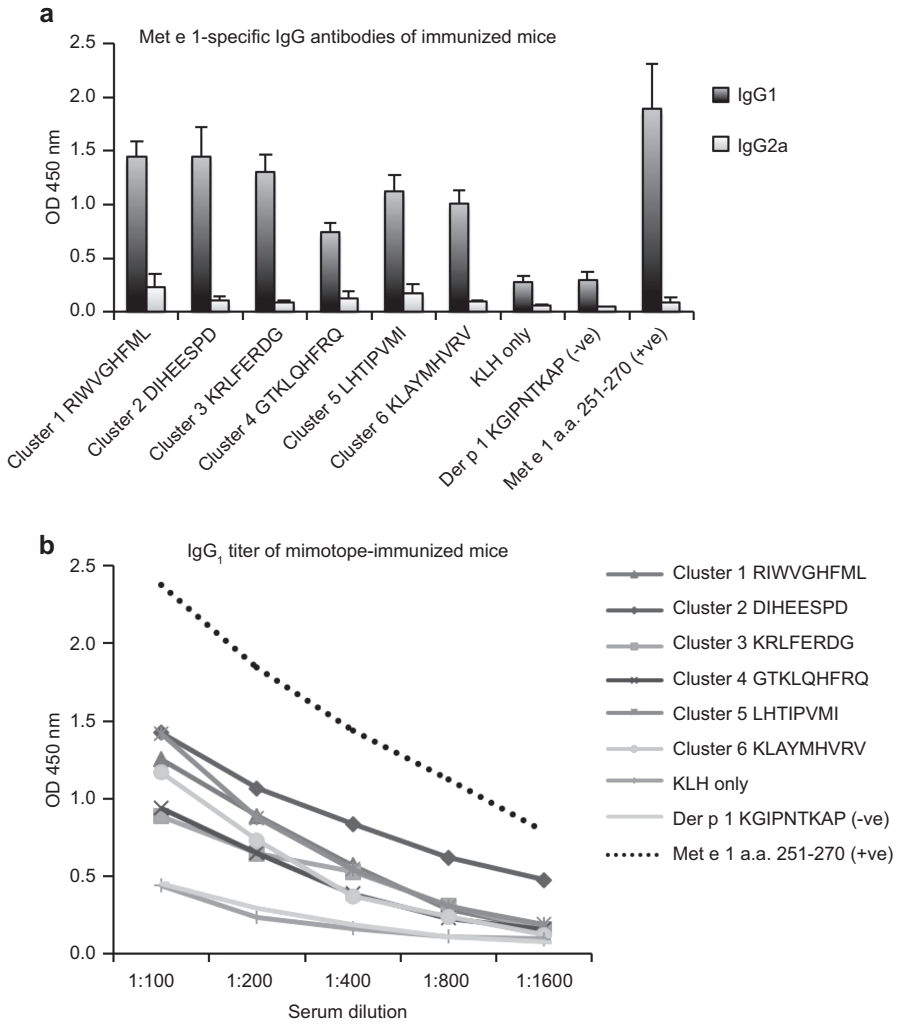

Figure 3 Serum profiles of BALB/c mice ($n = 4$) immunized with mimotopes conjugated to the carrier protein KLH. Results are expressed as mean optical density at 450 nm. (**a**) IgG_1 and IgG_{2a} levels of immunized mice (mean ± SEM). All six groups of mice immunized with mimotope conjugates have a significantly higher IgG_1 level compared to the control groups immunized with KLH alone or Der p 1 mimotope ($P < 0.05$). No significant increase in IgG_{2a} levels was observed. (**b**) IgG_1 titer of mimotope-immunized mice from serum dilution of 1:100 to 1:1600. Met e 1-specific IgG_1 was detected up to 1:1600 for the mimotope DIHEESPD.

any of the Pen a 1 epitopes, overlapped with epitope 7 (a.a. 236-241) of Met e 1. Linear or cyclic mimotopes from six irrelevant allergens (Supplementary Table 3) were also mapped to Met e 1 using EpiSearch (Table 2, Figure 4d). Only half of the irrelevant inputs matched to Met e 1, and only a prediction based on Pru p 3 overlapped with a short epitope at Met e $1^{25–30}$; none overlapped with Pen a 1 epitopes.

DISCUSSION

In this study, we have demonstrated the application of OBOC combinatorial library technology to obtain IgE mimotopes of shrimp tropomyosin using whole serum from patients with shellfish allergy. The mimicry potential and specificity of Met e 1 mimotopes were validated using *in vitro*, *in vivo*, and *in silico* analysis. Importantly, the specificity of the mimotopes to Met e 1-specific IgE were confirmed by peptide ELISA. Additionally, mice immunized with mimotope KLH conjugates exhibited elevated Met e 1-specific IgG_1, but mice administered with either an irrelevant mimotope or KLH alone did not. These data suggest that mimotope-specific antibodies are able to recognize at least one or more epitopes on tropomyosin. Met e 1-specific IgG_1 levels were detectable at as low of a dilution as 1:800 in mimotope immunized mice, which was comparable to the positive control groups. This indicates a strong affinity of the mimotope-induced antibodies to the native epitopes, even when the antibodies were raised against a peptide instead of against the whole antigen. From a clinical perspective, the data demonstrate the capacity of mimotopes to induce antibodies to their corresponding epitopes, which could be useful in generating blocking antibodies in SIT. Although the antibodies induced by mimotopes in this study are primarily of the IgG_1 isotype in this study, their potential to induce IgG_{2a} isotypes with Th1-adjuvant such as monophosphoryl lipid A[32] or chitosan[33] could be further investigated.

Table 2 Analysis of EpiSearch data and comparison between predicted epitopes and reported epitopes

Clusters/negative control	Predicted patch	Patch size	Epitope coverage[a] (Pen a 1/Met e 1)	Overlap ratio[b] (Pen a 1/Met e 1)	Patch similarity[c]
1a	43-57	15	1.00/0.833	1.00/1.00	0.667
1b, 5b	83-101	19	0.809/0.882	0.894/0.789	0.579
2b, 6	123-145	23	0.813/0.00	0.565/0.00	0.739
3b	186-201	16	0.938/0.285	0.938/0.375	1.00
4a, 4b, 5a	221-239	19	0.00/0.667	0.00/0.211	1.00
2a, 3a	252-283	32	0.868/0.773	1.00/0.531	0.75
Bet v 1	/	/	/	/	/
Der p 1	/	/	/	/	/
Der p 2	221-236	16	0.00/0.167	0.00/0.063	0.938
Bla g 2	114-128	15	0.00/0.00	0.00/0.00	0.933
Parvalbumin	/	/	/	/	/
Pru p 3	21-36	16	0.00/1.00	0.00/0.375	0.938

[a] Overlapping residues/total no. of residues in reported epitope.
[b] Overlapping residues/total no. of residues in predicted patch.
[c] Identical residues in mimotopes/identical residues in predicted patch.

By mapping the clustered mimotopes onto tropomyosin, we confirmed the validity of the mimotopes by comparing them with previously identified epitopes of Met e 1[22] and Pen a 1.[29] All epitopes predicted by EpiSearch were found to overlap completely or partially with at least one of the epitopes of Met e 1 or Pen a 1. The overlap ratios of the predicted clusters and genuine epitopes are higher in Pen a 1 than Met e 1 (Table 2), which might be due to the different mapping methods used for the two allergens. The Pen a 1 epitopes were identified by microarray of overlapping linear peptides spanning the entire length of Pen a 1 while the Met e 1 epitopes were mapped by both *in vitro* and *in silico* methods including ELISA,

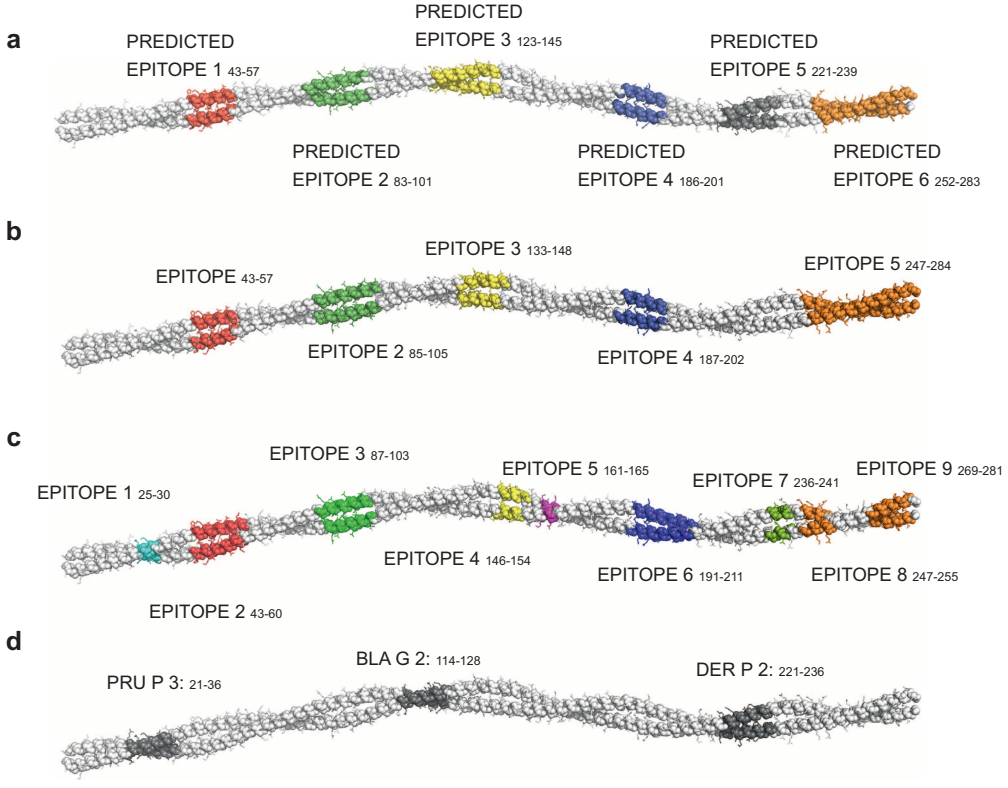

Figure 4 Homology model of Met e 1 based on *Sus scrofa* tropomyosin sequence for illustrating the location of predicted and identified epitopes. (**a**) Predicted epitopes based on Met e 1 mimotopes using the EpiSearch program. (**b**) Five major epitopes of Pen a 1 identified by Ayuso *et al.*[29] (**c**) Nine epitopes of Met e 1 identified by Wai *et al.*[22] (**d**) Predicted epitopes based on six sets of mimotopes from irrelevant allergens. The predicted epitopes using mimotopes as input overlap with 83% of both Pen a 1 and Met e 1 epitopes, while predicted epitopes using irrelevant allergens overlap with a single epitope (16%) from Met e 1 (Met e 1[25–30]) and none from Pen a 1.

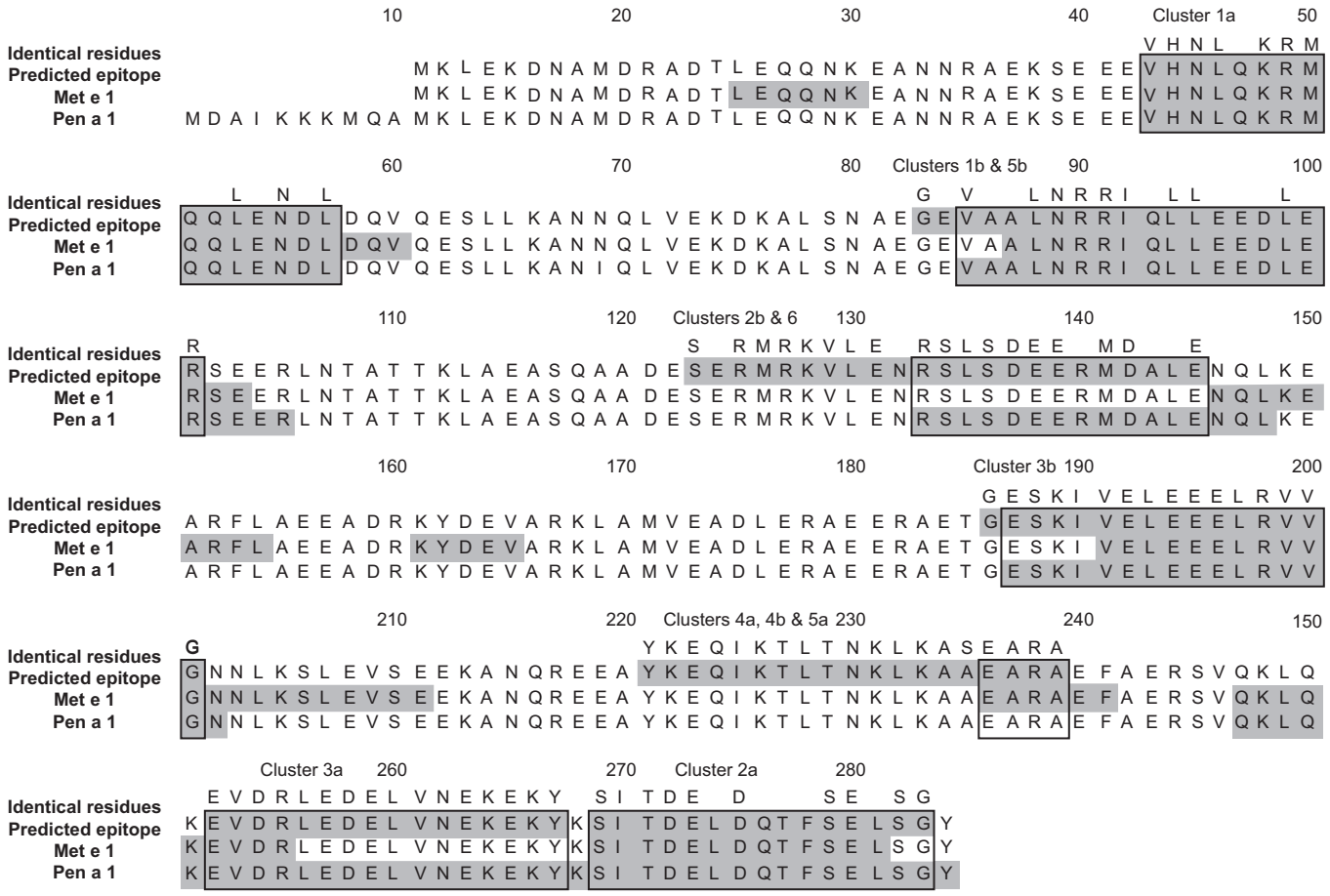

Figure 5 Predicted epitopes by EpiSearch using mimotopes as sequence inputs compared to the previously identified epitopes. Met e 1 and Pen a 1 differ by one amino acid at position 69. Identical residues refer to the consensus residues between the mimotope input and the predicted epitopes. Shaded amino acids represent predicted epitopes or identified epitopes of Met e 1 and Pen a 1. Boxed sequences represent overlapping regions between predicted and identified epitopes.

dot immunoblotting and epitope prediction models.[22] The Met e 1 epitopes would therefore be more distinct and refined, thus requiring a larger number of mimotopes to define the epitopes at a higher resolution. Nevertheless, only half of the six irrelevant mimotopes were mapped to Met e 1, with only a single predicted epitope found to overlap with a short epitope of Met e 1 and none with Pen a 1. These results highlight the specificity of the identified mimotopes and the power of EpiSearch as a tool for *in silico* validation. In fact, compared to a previous study using EpiSearch as an epitope mapping tool with subsequent verification by X-ray co-crystallography of mAbs and the predicted epitopes,[14] the EpiSearch predictions in our study have comparable or even higher epitope coverage, overlap ratio and patch similarity, affirming the credibility and relevance of mimotopes selected from an OBOC library. With advances in epitope mapping strategies, mimotopes can be used to deduce epitopes more precisely and serve as the basis for constructing hypoallergenic derivatives or predicting allergenicity of novel proteins in transgenic foods.[22,29]

Although mimotopes may not share sequence homology with their corresponding epitopes, the amino acid composition is quite similar between the mimotopes (Figure 5) and epitopes in our study as indicated by patch similarity (>0.500). This could be due to the relatively simple structure of tropomyosin and a lack of spatial organization of the epitopes. The mimicry of mimotopes is therefore mainly attributed to the similarity in physiochemical properties, i.e., the amino acid composition. For mapping conformational epitopes on the surface of globular proteins with more complex structures, a cyclic peptide library could be applied.[23]

To our knowledge, this is the first study that has utilized whole serum for identifying allergen-specific mimotopes. It is important to acknowledge that the mimotope sequences should not be analyzed and mapped to Met e 1 as a whole as they essentially bind to different epitopes of Met e 1, unlike the case when mAbs are used and all mimotopes bind to the same target. Multiple sequence alignment of the mimotopes revealed a high degree of homology within clusters (Figure 1), even compared to mimotopes that were identified by mAbs or affinity-purified specific IgE (Supplementary Table 3) in previous studies. Thus, we believe this simplest algorithm based on sequence identity is sufficient to resolve the mimotopes

into clusters without the need to account for gaps in the alignment or allow for substitutions of physiochemically similar amino acids within the aligned peptides, and these stringent conditions in clustering the mimotopes could largely contribute to the accuracy and relevance of the EpiSearch predictions.

Interestingly, there are two mimotopes from cluster 7 that could not be mapped to Met e 1 by EpiSearch. We speculate that these mimotopes might correspond to other shellfish allergens such as arginine kinase or myosin light chain. However, due to the lack of structural information about the other shellfish allergens, we could not possibly identify the origin of these two peptides at this stage. There are also five non-clustering mimotopes identified in our study, which could possibly be the result of insufficient sample size. Nevertheless, based on the fact that 18 out of 25 mimotopes could be mapped to Met e 1 and that all major epitopes were covered, we believe the use of whole serum in screening OBOC libraries is promising and the outcomes are comparable if not better than the outcomes from the conventional phage-displayed approach using monoclonal or purified antibodies.

In this study, peptide libraries of 8-mer to 12-mer in length were used for comprehensive library permutations and negligible redundancy. In general, if the peptides are too short, they do not possess the recognition specificity to their protein targets and could be easily lost during the washing steps. On the other hand, if the peptides are too long, the qualities of the OBOC libraries can be compromised and lead to high non-specificities. To increase the diversity of the libraries, all 19 eukaryotic amino acids were used except cysteine to avoid intra-molecular cross-linking. No unnatural amino acids were incorporated to minimize ambiguity during the *in silico* analysis.

Results from epitope mapping highlight the key advantage of the OBOC library: screening using pooled serum samples with polyclonal antibodies can simultaneously identify six epitopes with different specificities. The biopanning method for phage-displayed libraries usually utilizes the antibody as the capturing agent, and a homogenous population of antibodies is required to obtain a well-defined consensus sequence. Using polyclonal antibodies for biopanning is not feasible, as the sequences identified may correspond to different epitopes. It is also difficult to discern the positive hits against a background of non-binding sequences, as this process is non-quantitative.[34] Hence, most studies involving biopanning for mimotope screening of allergens requires mAb[13,14] or affinity-purified antibodies,[10,11] imposing a major technical limitation.

In contrast to the phage-displayed libraries which are non-quantitative, the OBOC combinatorial library offers a direct assessment of the affinity of individual peptides by the color intensity of beads. Moreover, unlike phage-displayed libraries where only a few peptides are displayed by each phage, there are up to 100 pmol or 10^{13} copies of the same peptide on a single bead in an OBOC library.[23] The high number of copies ensures higher sensitivity so that a more diluted antibody concentration (10- to 100-fold lower) can be used to reduce background signals. As such, a negative screening with non-allergic serum to deplete non-specific binding to irrelevant serum IgE is not needed. It is also unnecessary to deplete IgG in the serum samples, as the use of a specific anti-IgE secondary antibody would ensure the isotype specificity. By using pooled serum from multiple patients, the tropomyosin-specific IgE level would well exceed the level of irrelevant serum IgE. Moreover, the two-stage screening approach and cross-checking with sequences in MimoDB could further ensure the mimotope sequences are specific to the target allergen.

Screening of the OBOC library is much faster than biopanning with phage-displayed libraries. The whole screening process can be completed in two days for an OBOC library with 10 million beads compared to six days in biopanning using a standard protocol. Additionally, only standard laboratory equipment is required for the synthesis and screening of OBOC library. The rate-limiting step for library screening is usually peptide sequencing by Edman Degradation, but various methods are available to shorten the time required for peptide sequencing on beads.[35–38] Moreover, handling and maintenance of chemical libraries such as OBOC is much easier and does not require complex procedures such as phage amplification, phage titering, and strain maintenance. Lastly, the major disadvantages of biological libraries, such as phage contamination or inadequate peptide diversity due to biological pressure are avoided.

As demonstrated by this pioneering study, the identification of multiple IgE mimotopes using whole serum and OBOC combinatorial peptide libraries is an attractive alternative to the conventional biopanning of phage-displayed libraries. The OBOC library approach could shed light on the enormous potential of mimotopes for epitope mapping and the development of mimotope-based immunotherapy for allergy. Our group has previously developed a BALB/c mouse model of shrimp hypersensitivity[39,40] which could be a useful tool to investigate the therapeutic potential of tropomyosin-specific mimotopes. The therapeutic effects of mimotopes have been reported in an allergic asthma mouse model,[9] where a single Phl p 5-specific mimotope could alleviate asthmatic symptoms such as eosinophilic inflammation and mucus hypersecretion without eliciting any adverse side effects. However, it should be acknowledged that food allergens are likely to contain multiple linear IgE epitopes,[41] which are possibly responsible for the frequent anaphylactic side effects in clinical trials[42] and this increases the difficulty in designing safe hypoallergenic derivatives of food allergens. In this regard, a cocktail of mimotopes specific to different epitopes to the same allergen could be conjugated to a carrier such as KLH, tetanus toxoid, or multiple antigenic peptide system to increase the immunogenicity of mimotope peptides and generate a vast spectrum of blocking antibodies. Apart from therapeutic applications, the mimotopes could also be clinically validated and further developed for diagnostic applications on a peptide-based microarray platform to determine the sensitization profile of a patient. Such patient-specific mimotope screening would provide useful information about specific IgE epitopes, such

as the relevance and binding frequency of each IgE epitope. Furthermore, it could lead to a revolutionary approach for SIT by tailoring the most relevant blocking antibodies for each patient based on their own sensitization profile. This could reduce neo-sensitization and maximize the blocking capacity of antibodies induced, further improving the therapeutic safety and efficacy of SIT.

In conclusion, we have identified and validated mimotopes of tropomyosin obtained by screening OBOC combinatorial peptide libraries. This novel approach could overcome the limitations imposed by biopanning of phage-displayed libraries and paves the way for more prominent use of mimotopes to study allergy.

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