ABSTRACT

Cow milk is an important source of food protein for children; however, it could lead to allergy, especially for infants. α-Lactalbumin (α-LA) and β-lactoglobulin (β-LG) from whey protein make up a relatively high proportion of milk proteins and have received widespread attention as major allergens in milk. However, few studies have identified the epitopes of both proteins simultaneously. In this study, ImmunoCAP and indirect ELISA were first used for detection of sIgE to screen sera from allergic patients with high binding capacity for α-LA and β-LG. Subsequently, the mimotopes was biopanned by phage display technology and bioinformatics and 17 mimic peptide sequences were obtained. Aligned with the sequences of α-LA or β-LG, we identified one linear epitope on α-LA at AA 11–26 and 5 linear epitopes on β-LG at AA 9–29, AA 45–57, AA 77–80, AA 98–101, and AA 121–135, respectively. Meanwhile, the 8 conformational epitopes and their distributions of α-LA and β-LG were located using the Pepitope Server. Finally, glutamine and lysine were determined as common AA residues for the conformational epitopes both on α-LA and β-LG. Moreover, we found the addition of mouse anti-human IgE during the biopanning process did not significantly affect the identification of the epitopes.

Key words: cow milk allergy, α-lactalbumin, β-lactoglobulin, epitopes, phage display technology

INTRODUCTION

Of all the allergic diseases, a food allergy (FA) is the leading cause of allergic reactions in children and is a major public health problem (Mori et al., 2021). An FA is an abnormal immune response to a foreign antigen which is usually a protein. As a serious health problem, food allergic reactions can be life-threatening. Three hundred ninety kinds of proteins have been identified as allergens and more than 50% of food protein allergens are derived from eggs, milk, nuts, wheat, crustaceans, pulses, fish, and peanuts (Zhou et al., 2022). Cow milk allergy (CMA) is the most common FA in infants and children (Skripak et al., 2007), with a prevalence of approximately 2.5% globally and 2.69% in the Chinese pediatric population (Yang et al., 2019; Briceno Noriega et al., 2021).

A CMA may be mediated by IgE, non-IgE, or mixed, and symptoms from a CMA mediated by IgE can involve the skin, respiratory, and digestive tracts (Vandenplas et al., 2021). Cow milk protein is rich in nutrients, contains 9 essential AA for infants, and plays a crucial role in the growth and development of children. However, compared with human milk, cow milk contains more protein and mineral, thus making infants and children more prone to be allergic (Vincenzetti et al., 2021). The allergens involved in allergic reactions to CMA are very diverse, with milk containing over 30 different proteins at a concentration of approximately 30 to 35 g/L. The major allergens in cow milk are casein, α-LA, and β-LG in whey (Ogata et al., 2021).

B-cell epitopes are divided into linear and conformational epitopes, where linear epitopes consist of a contiguous sequence of AA and can be detected by screening patient sera in overlapping peptide libraries or allergen fragments. In contrast, conformational epitopes consist of folded, spatially closed AA and are identified in part by phage display technology or MS in combination with B-cell epitope prediction web servers. Other methods commonly used to locate B-cell epitopes include X-ray crystallography, nuclear magnetic resonance, hydrogen-deuterium exchange MS, and AA-targeted mutagenesis. However, these methods are...
sometimes difficult to perform due to difficulties in obtaining sufficient quantities of correctly folded, suitably processed allergens, and are laborious, time-consuming, and expensive. In summary, phage display technology combined with bioinformatics is an efficient and accurate method for allergen epitope location to be widely used. For foods, the phage display technology was once used by Chen et al. (2016), who identified 41 natural Ara h 2 and Ara h 6 mimic epitopes with affinity-purified IgE from 4 peanut allergic patients. In addition, with this technology, Zhang et al. (2023) identified the epitopes of the major allergenic antigenic peptide Ana o 2 in cashew nuts on AA 108–111, 113–119, 181–186, and 218–224; meanwhile, the epitopes of Ana o 3 were located at AA 10–24, 13–27, 39–49, 66–70, 101–106, 107–114, and 115–122. Currently, Li et al. (2015, 2016) have used phage display technology to identify the epitopes of α-LA and β-LG, respectively, but there remain few studies on the commonality of the 2 whey proteins, which are the major allergens in cow milk. In this study, to further produce hypoallergenic dairy products, sera from cow milk–allergic children were used to biopan the phage library, and the IgE epitopes of α-LA and β-LG were identified simultaneously, to find the common epitopes. In addition, we also explored whether the addition of mouse anti-human IgE during biopanning would affect epitope location.

**MATERIALS AND METHODS**

No animal experiments were done for this article, and only sera provided by the Wuhan Union Hospital from children allergic to cow milk were used.

**Materials**

Avidin-HRP and TMB were purchased from NeoBioscience. Biotinylated goat anti-human IgE and mouse anti-human IgE were from Sigma-Aldrich. HiTrap protein G HP was bought from GE. The phage display peptide library kit (Ph.D.-12) and *Escherichia coli* ER 2,738 were bought from New England Biolabs. The HRP-conjugated anti-M13 monoclonal antibody was from Amersham Biosciences. Other chemical reagents were from Sangon Biotech.

**Preparation of the Serum Pool**

The sera were provided by the Wuhan Union Hospital from children allergic to cow milk. Ethical approval from the Second Affiliated Hospital of Nanchang University was approved for the study. The sera were first detected using ImmunoCAP, and if sIgE ≥0.35 kUa/L, the sera were initially judged to be allergy positive, and further determination was performed by indirect ELISA as follows. The plates were dried and then washed 3 times for 5 min with 20 mM PBS (pH 7.4) between each of the following 2 steps. First, α-LA or β-LG was diluted to 1 μg/mL in 0.05 M carbonate buffer (pH 9.6) and added to 96-well plates (100 μL/well) at 4°C overnight. Afterward, PBS containing 1% BSA was added to block the plate (250 μL/well) and held at 3°C for 1 h. Then sera from cow milk–allergic patients diluted 200-fold in PBS was added (100 μL/well) and held at 37°C for 1 h (all the dilutions below were made using PBS). Afterward, biotinylated goat anti-human IgE was added at a dilution of 1:5,000 (100 μL/well) and held at 37°C for 1 h. Avidin-HRP was added at a dilution of 1:60, and held at 37°C for 1 h. Tetramethylbenzidine (TMB) substrate solution was then added (100 μL/well) and held at 37°C for 15 min. Finally, 2 M H2SO4 was added to terminate the reaction (50 μL/well) and the absorbance values at 450 nm were measured. Sera were judged positive at P/N > 2 and P > 0.2, where P and N represent the absorbance at 450 nm of samples and negative sera, respectively. Based on the results, the sera pool was obtained by mixing serum from patients with positive and significant levels of IgE specific to α-lactalbumin (α-LA-sIgE) or IgE specific to β-lactoglobulin (β-LG-sIgE).

**Separation of IgE**

Referring to the thesis of Li et al. (2016), protein G column was used for the separation of IgE in sera from cow milk–allergic children with appropriate modifications, sera were filtered through a 0.45-μm aqueous membrane after dilution with 20 mM PBS (pH 7.4) at a ratio of 1:1 (vol/vol) before loading and the flow rate was controlled to 1 mL/min during elution. The nonspecific elution collection tubes were concentrated by ultrafiltration at 4°C and 2,203 × g centrifugation for 10 min to obtain a mixture of IgE with little heteroprotein and then the buffer was replaced with 0.01 M PBS.

**Biopanning Specific Clones from Phage Display**

**The Selecting Process.** This assay was divided into 2 groups, A and B, from which the amplification process was slightly different. In group A, the nonspecific eluate was further enriched by adding mouse anti-human IgE, which was diluted to 30 μg/mL with 0.1 M NaHCO3 (pH 8.6), and blocked before adding anti-cow milk IgE. The IgE was diluted to 1,000 μg/mL (100 μL/well) with 0.1 M NaHCO3 (pH 8.6), and coated on the plate overnight at 4°C. Subsequently, the well was washed 3 times with TBST (TBS containing...
0.1% Tween-20) for 3 min each time (300 μL/well), and then was blocked with 0.5% BSA-PBS (300 μL/well) for 2 h at 37°C. The plate was next washed 6 times with TBST for 1 min each time (300 μL/well) and the original phage library (Ph.D.-12, 2.0 × 10^11 pfu) was added (100 μL/well) and incubated for 1 h at 37°C in a shaker. After that, the bound phages were eluted by 0.2 M glycine-HCl buffer (pH 2.2) for 6 min, followed by neutralizing with 1 M Tris-HCl buffer (pH 9.1). Ten microliters of the solution above was taken for titer determination and the rest was used for the amplification for the next round, referring to the Ph.D.-12 protocol for detailed steps.

After the third round of selecting, 20 blue plaques were stabbed with sterilized toothpicks from the culture plates in group A and B, respectively, and sent to Genewiz (Su zhou, China) for sequencing. The sequences using Reverse Complement Server and DNA-Star software. The AA sequences were then aligned with the negative control were identified as positive.

greater than 0.2 which also had a ratio greater than 2 to the original phage library (Ph.D.-12, 2.0 × 10^11 pfu) was added (100 μL/well) and incubated for 1 h at 37°C in a shaker. After that, the bound phages were eluted by 0.2 M glycine-HCl buffer (pH 2.2) for 6 min, followed by neutralizing with 1 M Tris-HCl buffer (pH 9.1). Ten microliters of the solution above was taken for titer determination and the rest was used for the amplification for the next round, referring to the Ph.D.-12 protocol for detailed steps.

After the third round of selecting, 20 blue plaques were stabbed with sterilized toothpicks from the culture plates in group A and B, respectively, and sent to Genewiz (Su zhou, China) for sequencing. The concentration gradient of mouse anti-human IgE was set at 30, 22.5, and 15 μg/mL depending on the antibody titer. The concentration gradient of mouse anti-human IgE was set at 30, 22.5, and 15 μg/mL according to the manufacturer’s protocol, whereas the concentration gradient of anti-cow milk IgE was set at 1,000, 750, and 500 μg/mL depending on the antibody titer. The concentration of the washing solution was 0.1, 0.25, and 0.5%; the phage binding time was 60, 45, and 30 min; the elution of the washing solution was 0.1, 0.25, and 0.5%; the phage binding time was 60, 45, and 30 min; the elution time was 6, 8, and 10 min in 3 rounds, respectively. Moreover, the number of phages added in each round was controlled to be 2 × 10^11 pfu.

**Phage ELISA.** Twenty-three phages potentially containing epitopes were initially selected and whether they were positive phage clones was identified by indirect ELISA. The phages above were transferred into ER2738 cell culture in log phase at 0.537 × g with a shaker for 5 h at 37°C, and then dissolved in 50 μL of TBS (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) after 2 precipitations by centrifugation and PEG solution. The final phage solution was assessed by titer test to determine the required dilution in ELISA. The subsequent steps were performed according to the thesis by Li et al. (2016) with some modifications, the anti-cow IgE was diluted to a concentration of 5 μg/mL by carbonate buffer (pH 9.6). The coating solution containing 3% BSA was used as the negative control. The phage solution was diluted 5-fold with PBS before addition. The substrate solution is substituted with TMB. Each clone was compared with 2 negative controls, the absorbance value was measured at 450 nm and clones with OD_{450} greater than 0.2 which also had a ratio greater than 2 to the negative control were identified as positive.

**Identification of Epitopes on α-LA and β-LG.** The DNA sequences were first translated into AA sequences using Reverse Complement Server and DNA-Star software. The AA sequences were then aligned with α-LA and β-LG through DNAMAN software, 3 or more consecutive overlaps and 4 or more nonconsecutive overlaps were considered as potential linear epitopes.

The Pepitope Server was used to locate the conformational mimic epitopes. Chain A of the X-ray structure of α-LA (Bos d 4; PDB code 2G4N) and β-LG (Bos d 5; PDB code 5K06) were submitted to the server, by which the mimic peptides screened from phage library were compared. The top 5 sequences in each of the 2 groups were identified as conformational mimic epitopes, considering the higher PepSurf algorithm, and lower P-value. In addition, the distribution in the spatial structure of α-LA and β-LG were described respectively by using Pymol software.

**Statistical Analysis.**

The data were expressed as mean ± SEM. All the data were analyzed using Instant version 5.0 (GraphPad, San Diego, CA; http://www.graphpad.com). Differences between values were compared by using unpaired t-test. A value of P < 0.05 was considered to be statistically significant.

**RESULTS**

**The Serum Screening and Separation of Specific Antibody**

**Screening Sera from Children Allergic to Cow Milk.** The detailed information of the sera involved in this experiment is shown in Table 1, including 2 males and 5 females, and their average age was 5.4 yr. The specific symptoms of allergic patients were urticaria, eczema, allergic rhinitis, allergic asthma, and wheezing, and so on. This is consistent with the previous study by Strinnholm et al. (2014) that CMA is common in children, with symptoms may affect single or multiple organ systems and are most frequently found in skin, such as urticaria, angioedema, or eczema, respiratory, or gastrointestinal tract, and oral mucous membranes. Moreover, for infants and children, IgE-mediated CMA usually manifests as eczema and diarrhea, while recent studies suggest that allergic symptoms may involve the respiratory system, whereas non-IgE-mediated CMA usually manifests as diarrhea only, so the CMA of patients in the trial was mainly IgE-mediated (Skripak et al., 2007; Sackesen et al., 2019).

ImmuNOCAP has become the “gold standard method” for sIgE detection, due to its good repeatability and accuracy (Park et al., 2017), and the results of ImmunoCAP are classified into 7 levels ranging from class 0 to class 6 (<0.35 kUa/L, class 0; 0.35–0.7 kUa/L, class 1; 0.7–3.5 kUa/L, class 2; 3.5–17.5 kUa/L, class...
In this experiment, ImmunoCAP was used to detect cow milk sIgE levels in the patients and sera with levels greater than or equal to 0.35 kUa/L were considered positive. All 7 patients had positive sera for further testing and patient 1 had the highest serum sIgE level at 7.57 kUa/L (Table 1). It has been suggested that CMA gradually subsides after the age of 6 yr, but children with IgE-mediated allergy may not acquire tolerance to cow milk until an older age (Bernard et al., 2012; Ogata et al., 2021; Edwards and Younus, 2022). The 3 children over 6 yr in this study still showed high sIgE levels, so it is further assumed that the cow milk–allergic reaction in these children was mainly mediated by IgE.

This study was followed by screening specific mimic epitopes with phage display technology. To improve its accuracy, the specific capacity of serum IgE was screened by indirect ELISA. The results showed that 85% (out of 6) of the patients had sIgE response to β-LG and 71% (out of 5) had sIgE response to α-LA, both of the recognition were higher than 50%. Thus, it was concluded that both α-LA and β-LG were the main allergens in CMA induced by whey proteins as reported (Geiselhart et al., 2021; Ogata et al., 2021). In addition, the concentration of purified IgE was measured using the BCA kit and was 10 mg/mL.

### Selecting Mimic Epitopes of Major Allergens in Whey Using Phage Display Technology

**Affinity Amplification of Phage Libraries.** After 3 rounds of screening, the phage output of group A increased from $2.0 \times 10^4$ at the beginning to $7.1 \times 10^5$, whereas that of group B increased from $1.7 \times 10^4$ to $6.8 \times 10^5$ at last, thus no obvious difference was found in the enrichment effect between group A and B, indicating that the addition of secondary antibody did not significantly affect the specificity of the screening process.

**Identification of Positive Phage Clones.** DNA sequences of 20 random clones were obtained from each group, and they were translated to AA sequences as shown in Table 2. Among these 40 12-mer peptides, the sequence ATMRGDQSVRIF appeared 4 times, the sequence VVGRAMAYSTIP appeared 3 times, and the remaining sequences all appeared only once.
With the sequences, we finally selected 23 phage clones as either potential linear or conformational epitopes, and they were further identified by indirect ELISA. As presented in Figure 3, 17 clones were judged to be positive clones for subsequent epitope analysis, for the absorbance values of A3, A4, A13, B12, B13, and B17 were less than 2 times that of the corresponding negative controls and were thus excluded.

**Figure 1.** The IgE level of patient sera specific to cow milk. (A) α-LA-sIgE detected by indirect enzyme-linked immunosorbent assay is showed as the column corresponding to the left y-axis, while the curve is the data from ImmunoCAP (Phadia AB) corresponding to the right y-axis. (B) β-LG-sIgE detected by indirect enzyme-linked immunosorbent assay is showed as the column corresponding to the left y-axis, while the curve is the data from ImmunoCAP corresponding to the right y-axis. Each value represents the mean of 2 independent experiments ± SD. OD = optical density. α-LA-sIgE = IgE specific to α-lactalbumin; β-LG-sIgE = IgE specific to β-lactoglobulin. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 2.** The SDS-PAGE patterns of the serum purified by protein G with appropriate dilute. M: markers; lane 1: nonpurified serum; lane 2: nonspecific eluent; lane 3: specific eluent. The electrophoretic stripe of IgE is marked with the red box.

**Epitope Location of Major Allergens in Whey**

**Identification of Linear Epitopes.** Figure 4 exhibits the linear epitopes of α-LA and β-LG after positive phage identification, from which we identified one linear epitope on α-LA at AA 11–26 and 5 linear epitopes on β-LG at AA 9–29, AA 45–57, AA 77–80, AA 98–101, and AA 121–135, respectively. In addition to these 5 epitopes, 2 other shorter epitopes, AA 36–38 and AA 146–148 were too short to be regarded
as epitopes. Moreover, we found that the sequence VVGRAMAYSTIP, which was repeated 3 times, was defined as a linear epitope on β-LG at AA 15–29, suggesting that the sequence with a high frequency in the binding peptide is more likely to be an epitope.

It is worth noting that the location results of the linear epitopes shared a large number of regions between group A and group B. It is tentatively assumed that the addition of mouse anti-human IgE did no change on the epitope results.

**Mapping of Conformational Epitopes.** Conformational mimic epitopes on α-LA and β-LG were identified based on the higher server algorithm and lower P-value. Tables 3, 4, 5 and 6 were obtained by excluding the negative clones A-IgE3, B-IgE13, B-IgE17.

Four conformational mimotopes of α-LA were identified in each of group A and B. From Table 3, it can be found that the 5 AA residues L105, Q2, L3, T48, and Q65 appeared twice in the epitopes identified in group A. As shown in Table 4, the AA residues C120, C6, T1, L3, E11, K13, F6, K5, Y36, and G35 were found to have higher frequencies in the epitopes identified in group B, with the exception of L3, which appeared 3 times, all the others appeared twice. Combining the results of both group A and B, it is concluded that L3 is the key AA in the conformational epitope of α-LA. Among the 2 groups of AA from the conformational epitopes, threonine, glutamine, lysine, and alanine occurred more than 7 times in total, 14, 8, 8, and 7 times, respectively.

Meanwhile, 4 conformational mimotopes of β-LG were identified in group A and B. From Table 5, it can be found that among the epitopes identified in group A, T5, V4, A143, P145, S28, M146, D12, Q14, and K15 had a higher appearance frequency, with twice for each AA; from Table 6, the AA residues T7, K9, Q14, R125, E159, L11, D12, and G10 appeared more frequently in group B, except for D12 which appeared 3 times and the rest appeared twice. Compared with the results of group A and B, it is assumed that D12 and Q14 are the key AA in the conformational epitope of β-LG. Among the 2 groups of AA from the conformational epitopes, threonine, glutamine, lysine, and alanine occurred more than 7 times in total, 14, 8, 8, and 7 times, respectively.

**DISCUSSION**

**Preparation of Serum Pool from Cow Milk–Allergic Children**

Allergic diseases are mainly diagnosed by vivo allergen skin testing or vitro specific IgE testing, while the latter is not interfered by the patient’s skin, medication and other factors, effectively avoiding inaccurate results and patient adverse reactions caused by individual differences, and has become the dominant tool for the diagnosis of allergic diseases (Wang et al., 2008; Lee et al., 2015). However, Novembre et al. (2012) found in their study of the peach allergen Pru p3 that sIgE levels are not correlated exactly with the severity of allergic reactions in pediatric patients, which explains the fact in this experiment that both low sIgE and high sIgE patients presented allergic rhinitis.

Table 1 shows that only one of the patients identified with CMA had eczema, whereas the majority of...
patients had allergic rhinitis or asthma, probably because about 50% of patients with eczema later develop allergic rhinitis or asthma (Fu et al., 2014). At the same time, some studies have observed that s-IgE levels in milk allergy are not related to the severity of eczema (Ong, 2014). In addition, 2 other patients presented

Table 3. The composition of conformational epitopes on α-LA from group A

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Peptide sequence</th>
<th>Mimotope</th>
<th>Score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-IgE5</td>
<td>VIAHGGLELTWH</td>
<td>A_{30}A_{31}A_{32}T_{33}G_{34}Q_{35}E_{36}T_{37}</td>
<td>17.870</td>
<td>0.00026</td>
</tr>
<tr>
<td>A-IgE15</td>
<td>SKVPAPDYTKQM</td>
<td>S_{58}K_{59}H_{60}Y_{61}T_{62}Q_{63}E_{64}</td>
<td>17.862</td>
<td>0.00005</td>
</tr>
<tr>
<td>A-IgE20</td>
<td>LPMLPLTATGW</td>
<td>E_{93}N_{94}Y_{95}N_{96}I_{97}K_{98}H_{99}L_{100}I_{101}</td>
<td>16.683</td>
<td>0.00040</td>
</tr>
<tr>
<td>A-IgE7</td>
<td>DRGSGVPADILW</td>
<td>D_{114}Q_{115}N_{116}T_{117}I_{118}A_{119}D_{120}Q_{121}</td>
<td>16.661</td>
<td>0.00139</td>
</tr>
</tbody>
</table>

Figure 3. The screen of positive clones by indirect enzyme-linked immunosorbent assay in group A (A) and B (B). * is used to mark the negative phage clones that should be excluded. Each value represents the mean of 2 independent experiments ± SD. Con = control; O.D. = optical density.
with urticaria, indicating imminent vasodilation and bronchoconstriction, which is often the initial manifestation of allergy, and later, similar to eczema, may develop into other atopic diseases such as asthma or rhinitis, and 75% of fatal allergic reactions are closely related to asthma, so further identification and treatment of patients should be carried out immediately after the onset of urticaria (Sarinho and Lins, 2017; Aydoğan et al., 2021). The size of serum samples in this study was small and the above conclusions need to be further confirmed by expanding the study sample. The study group only included children aged 3 to 12 yr and further information on CMA is needed supplementing in infants and adults.

Figure 4. The IgE binding linear epitopes identified from α-LA (A) and β-LG (B) by aligning with sequences of positive clones.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Peptide sequence</th>
<th>Mimotope</th>
<th>Score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-IgE6</td>
<td>KCCFALEKRGHR</td>
<td>K12C12G1T1L3E2K11F9R10</td>
<td>21.760</td>
<td>0.000005</td>
</tr>
<tr>
<td>B-IgE10</td>
<td>AQQCFCASDYDA</td>
<td>Q12K6C12F6K11E11E3K11Y3G10</td>
<td>19.277</td>
<td>0.00001</td>
</tr>
<tr>
<td>B-IgE8</td>
<td>NTLELPNVVLLNG</td>
<td>D12K11E20S2F2K12K12Y4K12Q12G15</td>
<td>18.611</td>
<td>0.00085</td>
</tr>
<tr>
<td>B-IgE18</td>
<td>SVYNALYLAASE</td>
<td>T1L3Y3G12F13H3L3A10K10S10E13</td>
<td>16.375</td>
<td>0.00062</td>
</tr>
</tbody>
</table>

Table 4. The composition of conformational epitopes on α-LA from group B

Affinity Amplification of Major Allergen Mimic Epitopes in Whey

The NEB phage random 12-mer peptide library used in the experiment is a fusion of a random 12-mer peptide to the M13 phage minor coat protein (pIII), a combinatorial library in which the 12-mer peptide is expressed at the N terminus of pIII (i.e., the first AA of the mature protein is the first AA of the random polypeptide) followed by a short spacer polypeptide consisting of Gly-Gly-Gly-Ser, and then the wild-type pIII protein.

During the 3 rounds of elution, antibody concentration, wash concentration, binding time and elution time
all affect the enrichment efficiency. With lower antibody concentration, the phage random peptides which can specifically bind IgE will preferentially bind IgE due to increased competition, resulting in more specific phages. The wash with a higher concentration effectively reduce the nonspecific binding of phage random peptides between blocking solutions and target molecules. With shorter binding time or longer elution time, the binding capacity of the peptide to the target molecule could be stronger (i.e., more specific clones were obtained). After each round of elution, and by keeping the same number of phages added in each round, the specific peptide sequences that bind to the target molecule were increased in the phage library gradually, thus achieving amplification.

Identification of Linear Epitopes

In this study, we attempted to locate both linear and conformational epitopes of α-LA and β-LG simultaneously using the same batch of serum. Finally, the linear IgE epitope on α-LA for children was identified at AA 11–26, which is highly overlapped with the IgE epitope AA 15–34 for infant identified by Hochwallner et al. (2010) using overlapping peptide, suggesting the sharing of α-LA IgE linear epitopes between children and infants. While the IgE epitopes on β-LG for children were identified at AA 9–29, AA 77–80, AA 98–101, and AA 121–135, respectively, are in good agreement with the IgE epitopes AA 17–31, AA 72–86, and AA 92–106 identified with infant by Cong and Li (2012) using synthetic peptides and the IgE epitope AA 121–135 identified by Cerecedo et al. (2008) with the cow milk–allergic children using overlapping peptides, indicating that children and infants also share common β-LG IgE linear epitopes.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Peptide sequence</th>
<th>Mimotope Score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-IgE10</td>
<td>ATDIAPKPLRIT</td>
<td>19.897</td>
<td>0.00029</td>
</tr>
<tr>
<td>A-IgE1</td>
<td>VSVPGHTGTLR</td>
<td>19.472</td>
<td>0.00015</td>
</tr>
<tr>
<td>A-IgE19</td>
<td>TLAPRTEKDKTAT</td>
<td>18.695</td>
<td>0.00033</td>
</tr>
<tr>
<td>A-IgE15</td>
<td>SKVPAPDYTKQM</td>
<td>17.560</td>
<td>0.00023</td>
</tr>
</tbody>
</table>

The pediatric IgE epitopes of β-LG identified in this study also are substantially similar to the epitopes AA 77–82 and AA 126–131 identified by Li et al. (2015) using rabbit serum, offering the prospect of using immunized rabbit serum as a substitute for the serum of allergic patients. In addition, according to the work of Järvinen et al. (2001), the IgE epitope AA 5–18 on α-LA and AA 124–134 on β-LG are homologous sequences, which is consistent with the results of the present study in which the IgE epitope on α-LA was located at AA 11–26 and the IgE epitope on β-LG was located at AA 121–135, indicating that this homologous sequence is an important IgE epitope in both α-LA and β-LG, and the AA of the epitope are potential key AA in CMA, which provides a new approach for the subsequent desensitization of milk proteins.

Linear epitope location also showed that there was a large amount of overlap between the 2 experimental groups. For instance, the linear epitope on α-LA was located at AA 18–26 in group A, whereas was located at AA 11–22 in group B; and the linear epitope on β-LG was located at AA 48–54 in group A, whereas was located at AA 45–57 in group B, indicating that the addition of secondary antibody did not affect the epitope location.

Location of Conformational Epitopes

For conformational epitopes, 8 pediatric IgE epitopes were located on α-LA, from which mimotopes D110K114L115E25 S22P24L119K5Y36L3Q2G35 and S70K58S69N66P67 D63Y50T48Q65Q43 were highly overlapped with results of Li et al. (2016) respectively, reflected in common AA residues K114, L119, and P67, T48, Q65, Q43. Combined with the previous results, it is further concluded that leucine, glutamine, and lysine are common residues in both CMA and milk allergy patients.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Peptide sequence</th>
<th>Mimotope Score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-IgE15</td>
<td>ATMRGDQSVRIF</td>
<td>18.899</td>
<td>0.00002</td>
</tr>
<tr>
<td>B-IgE8</td>
<td>NTLLELPVKNLG</td>
<td>18.847</td>
<td>0.00125</td>
</tr>
<tr>
<td>B-IgE10</td>
<td>AQKCCFASVDY</td>
<td>17.725</td>
<td>0.00002</td>
</tr>
<tr>
<td>B-IgE7</td>
<td>SNGNLDGNRT</td>
<td>17.484</td>
<td>0.00022</td>
</tr>
</tbody>
</table>
tamine, and lysine are key AA in the conformational epitopes of α-LA.

In addition, 2 mimotope T77K78I79P80L11D12Y100K15Q14 and E159Q160Q160C161C67W62Q36S37A35 located on β-LG in this study were highly overlapped with those located by Li et al. (2016) using rabbit sera, respectively, namely T7M8K9G10L11D12I13Q14K15A17G18T19Y21E45L58T77K78I79P80V82Y100R125 and A35Q36S37P39L40R41V42K61W62C67A68Q69F152N153Q156E159Q160H162I163. The high coincidence was reflected in common AA residues as follows: T77, K78, I79, P80, L11, Y100, K15, Q14, and Q160, C67, W62, S37, A35. Compared with the previous results, it is indicated that glutamine and lysine play an essential role in the conformational epitopes of β-LG, and further demonstrates the feasibility of using immunized rabbit sera as a substitute for allergic human sera.

Madsen et al. (2014) used Brown Norway rats in animal experiments to find that conformational epitopes were significant for α-LA, β-LG, and β-CN, respectively.
and that the importance of α-LA was greater than β-LG than casein, which could reflect their differences in structure, where both β-LG and α-LA are small structurally stable and compact proteins possessing 2 and 4 disulfide bonds, in contrast to β-CN which is regarded an unstructured and flexible protein. In addition to study the effect of polyphenols on the structure of β-LG, Xu et al. (2019) found β-LG has 14 lysine residues, of which 12 are located on the major IgE epitopes. In this study, lysine and glutamine have a high frequency of occurrence in the conformational epitopes of both α-LA and β-LG according to the previous results, thus we considered they are common AA residues identified in conformational epitopes on α-LA and β-LG. The analysis of the AA composition of conformational epitopes in this study provides a direction for reducing the allergenicity of cow milk by AA mutation in future.

From Figures 5 and 6, we can see the conformational epitopes of both groups were mainly located in the α-helical structure of the protein which is consistent with the previous study of Yang et al. (2022). Meanwhile, the conformational epitopes of group A also existed in the β-folded and randomly coiled regions compared with group B, suggesting that the β-folded and randomly coiled regions cannot be ignored in the

Figure 6. The mimic conformational epitopes on α-LA in group B. (A) The mimic peptides on α-LA surface. (B) The distribution of mimic peptides on the secondary structure of α-LA. (C) The AA composition of mimotopes on the surface.
As presented in Figures 7 and 8, the conformational epitopes of β-LG in group A and B were mostly in the random coiled region, with a few in the β-folded region and almost none in the α-helical structure. This indicates that although the main secondary structure of β-LG is the β-fold, the random coiled part may play a more dominant role in the conformational epitopes and the difference in the region of conformational epitope recognition between group A and B may be due to the fact that different cow milk–allergic patients recognize different conformational epitopes.

In the conformational epitope location, especially for β-LG, it existed a greater tendency of overlap between the constituent AA in group A and B as follows: T₅, K₁₅, A₁₇, T₁₂₆, R₁₂₅, Y₁₀₀, L₁₁, D₁₁₂, G₁₁₀, T₇₇, T₅₀, Q₁₄, K₉, and whether the addition of secondary antibodies had an effect on the conformational epitopes identification needs to be further discussed.

**CONCLUSIONS**

No significant effect of the addition of secondary antibodies on the identification of epitopes with phage display technology was found in this study. When the epitopes of α-LA and β-LG were located simultaneously, the AA residues comprising the homologous sequences of α-LA and β-LG were identified as common linear epitopes. Glutamine and lysine were determined as common AA residues for the conformational epitopes both on α-LA and β-LG. However, the amount of phage plaques selected in this experiment is limited, further experimental exploration should be performed with more patient sera for verity.
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Figure 8. The mimic conformational epitopes on β-LG in group B. (A) The mimic peptides on β-LG surface. (B) The distribution of mimic peptides on the secondary structure of β-LG. (C) The AA composition of mimotopes on the surface.


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