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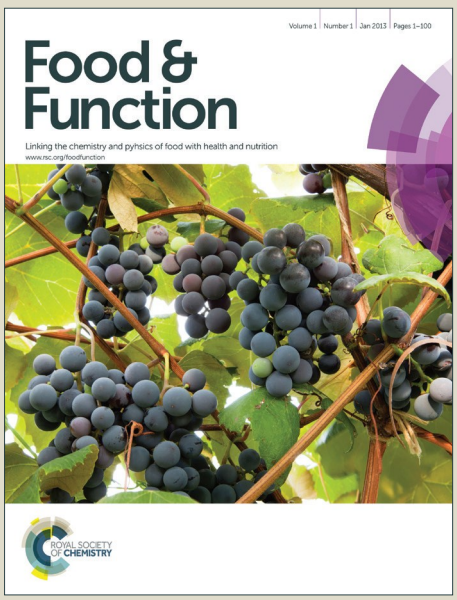

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Identification of IgE and IgG epitopes on native Bos d 4 allergen specific to allergic children

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ABSTRACT:

Alpha-lactalbumin (ALA) is one of the major allergens in cow's milk. However, research on its conformational epitopes have been relatively limited. In our study, specific antibodies against cow's milk ALA were purified from eight children by two-step affinity chromatography. Subsequently, mimotopes against IgG and IgE were biopanned from Ph.D.-12 and Ph.D.-C7C, respectively. Based on the mimotopes, linear epitopes were defined with UniProt alignment tool. Conformational epitopes were computed using the Pepitope Server. Six IgE and seven IgG linear epitopes were identified. Meanwhile, five IgE and three IgG conformational epitopes were revealed with PyMOL. The results showed that common residues were identified in both IgE and IgG epitopes and some residues of conformational epitopes were composed of linear epitopes on bovine α-lactalbumin. The results indicated that the data could be used for developing of hypoallergenic dairy products on the basis of epitopes and providing a diagnostic tool for the assessment of patients who are allergic to cow's milk.

INTRODUCTION

Food allergy is a type of adverse reaction and it has been reported that approximately 3% of adults and up to 8% of children suffer from food allergies in develop countries.[1] There are about 160 foods causing allergy and 90% of allergies are caused by eight kinds including milk, peanuts, egg, wheat, soybeans, crustacean, fish and tree nuts.[2-4] Among them, cow's milk is the leading cause of food allergy for infant and children. Recently, the prevalence of cow's milk allergies in preschoolers, older children and adults were reported to be 0-2.5%, 0.3% and less than 0.5%, respectively.[5]

There are more than 25 different types of proteins in milk, and the most important allergens are casein, α-lactalbumin (ALA, Bos d 4) and β-lactoglobulin.[6, 7] ALA belongs to the lysozyme family and its molecular weight is 14.2 kDa with 123 amino acid residues. It is a monomeric, calcium-binding, globular protein with four disulfide bonds.[8, 9] Cow's milk ALA is secreted by mammary epithelial cells, and one of its important functions is to regulate the synthesis of lactose by the galactosyltransferase system. The amino acid sequences of bovine and human ALA share 74% identity and 6% similarity.[9] In the food industry, ALA is an important additive in infant formula due to the fact that it contains many essential amino

acids and performs various physiological functions,[10] such as inhibiting colon carcinogenesis and anti-inflammatory activity and so on.[11, 12]

An epitope is a specific region on the surface of an antigen that is part of a macromolecule (usually a protein) recognized by antibody of the immune system. [13] There are two types of epitopes: linear (sequential and continuous) and conformational (discontinuous).[13-16] Until now, linear epitopes have been detected on cow's milk allergens.[9, 17-20] However, relatively limited research has been performed to explore conformational epitopes of cow's milk allergy, despite the finding that 90% of the epitopes on protein antigens are conformational, with antibody-binding abilities that are dependent on their structures.[20]

Random peptide libraries displayed on filamentous phages is a unification of the gene and its corresponding protein in vitro and it has been widely used in many fields such as life sciences, medication and so on. [21, 22] It is a robust method for epitope mapping since this technique is based on the interaction of antibody and mimic peptides. The aim of work is to explore the information on epitopes of ALA and characterization of linear and conformational epitopes, which is expected that the results of epitopes can be used for developing of

hypoallergenic dairy products on the basis of epitopes. In our work, linear and conformational mimotopes on cow's milk ALA were biopanned from phage display peptide libraries(Ph.D.-12 and Ph.D.-C7C) and then IgG and IgE epitopes were identified by bioinformatics tools.

MATERIALS AND METHODS

Materials

Purified cow's milk ALA were purchased from Sigma Co. (St. Louis, MO) with purity of higher than 85%. Phage display libraries (Ph.D.-C7C and Ph.D.-12) were purchased from New England Biolabs (Beverly, MA, USA). CNBr-activated Sepharose 4B and HiTrap Protein G columns were obtained from GE (Fairfield city, State of Connecticut-CT, USA). All other chemicals were purchased from Sangon Co. (Shanghai, China).

Sera collection for diagnosis was performed by staff members of the First Affiliated Hospital of Guangxi Medical University and the Children's Hospital of Zhejiang University. None of the authors were involved in the sample collection. Verbal informed consent was obtained from the children's parents, who agreed that their children's sera could be used for research on epitope mapping as long as their private information was protected.

Pooling of sera from patients with allergy to cow's milk

Serum samples from 19 children with allergy to cow's milk were tested in this study. Among them, 14 are boys and 5 are girls, which accounts for about 75% and 25%, respectively. Serum-specific IgE (S-IgE) antibodies specific to ALA were analyzed using an ImmunoCAP 100E (Phadia AB, Uppsala, Sweden). All selected serum samples with S-IgE values greater than or equal to 0.35 kU_A/L were pooled.

Purification of IgG and IgE antibodies to cow's milk ALA

According to the instruction of 4B CNBr-Sepharose, 0.6g CNBr-activated Sepharose 4B powder has been washed with 1 mM HCl and transferred to 2 mL column. After that, 15mg ALA was added to the column for coupling and the concentration of protein in the supernatant was monitored for every half hour.

According to the instructions of CNBr-activated Sepharose 4B，10 mL of PBS was used for equilibration firstly. Then 1ml of sera samples were diluted to 2ml with PBS and then were loaded onto the CNBr-activated Sepharose 4B column and the column medium was washed with a 10-fold volume of 20 mM PBS (pH 7.4) in order to remove the unbound material. Finally, specific antibody consisting of

both IgG and IgE were eluted with 10 medium volumes of 3 mol/L $MgCl_2$ and excess salts in solution were removed by centrifugation with cut-off molecular weight of 5KDa Millipore tube.

In a second step, 1ml of concentrated solution from the previous step were separated using the HiTrap Protein G column. 2 ml specific antibodies obtained from the previous step were loaded slowly onto the activated HiTrap Protein G and IgE antibodies have been flowed through by 10 column volumes of binding buffer washing. After that, specific IgG antibodies were eluted by 5 volumes of 0.1 M glycine-HCl (pH 2.7) and neutralized with 1M Tris-Cl (pH9.0). The purities of IgG and IgE were calculated by software Quantity One from Bio-Rad which based on bands in SDS-PAGE gel.

Epitope mapping by phage display

ALA mimotope selection from random peptide libraries

Ph.D.-12™ and Ph.D.-C7C™ phage display peptide libraries were subjected to three cycles of panning to select sequential and conformational epitopes of ALA, respectively. The biopanning protocol was conducted as described by Li et al with minor modification.[20] Briefly, microplate wells were coated with purified IgE and IgG antibody in 0.1 mol/L $NaHCO_3$ (pH 8.6) overnight at 4°C. Subsequently, 200 μL of 3% (w/v) BSA (or OVA) in TBS (50

mmol/L Tris-HCl [pH 7.5] and 150 mmol/L NaCl) was dispensed into the wells to block nonspecific binding, and the plate was incubated at 37°C for 2 h. Then, the wells were washed three times with TBST (TBS containing 0.1% Tween 20), and 100 μL of phage solution (2.0×10^{11} pfu) from the random peptide library was added to each well and incubated at 37°C for 1 h. After washing with TBST to remove unbound phages, 100 μL of elution buffer (0.2 mol/L glycine-HCl, pH 2.2) was added and immediately neutralized with 1 mol/L Tris-HCl (pH 9.1). The eluted phages were used to infect *Escherichia coli* ER 2738 for amplification purposes, which was followed by a further round of biopanning. After the third round of biopanning, individual colonies were selected randomly from the titre culture plates (LB medium) and amplified for identification by phage ELISA.

Phage ELISA

We firstly used a sterile toothpick to stab one of the blue plaques (< 100 plaques) resulting from the third round of random panning. The phage was then transferred to an *ER2738* cell culture in log phase, and the cells were incubated at 37°C with shaking for 4.5-5 h. The cultures were then centrifuged at 10,000 rpm for 1 min, and the supernatants were assessed by a titre test.

ELISA plates were coated with 100 μg/mL IgE or IgG against cow's milk ALA suspended in 3% BSA in 0.1 mol/L TBS (100 μL /well) overnight at 4°C. Two non-allergic sera were used for negative control. On the following day, the ELISA plates were washed three times with 0.05% (v/v) Tween-20 in PBS (300μL /well), blocked with 1% gelatin in PBS (250μL /well). After that, the above supernatants were added to the plates and incubated for 1 h at 37°C. An HRP-conjugated anti-M13 monoclonal antibody was added (100μL /well; diluted 1/5,000 in TBST), and the plates were again incubated at 37°C for 1 h. The plates were washed three times, and o-phenylene diamine (OPD, 4 mg/mL) in citrate buffer (100μL /well) was dispensed into the wells and incubated at 37°C for 15 min in the dark. A stop solution of 2M H_2SO_4 (50μL /well) was added to terminate the reaction. The optical density of 490 nm was immediately recorded using a Bio-Rad Microplate Reader (Bio-Rad model 600).

DNA purification and peptide alignment

Single-stranded DNA was purified from the bacteria infected with specific phage by precipitation with 20% PEG/2.5 M NaCl, resuspended in iodide buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA and 4M NaI), precipitated again with absolute ethyl alcohol and

resuspended in TE buffer. Ten microliters of the resuspended template was sent for DNA sequencing, which was carried out by Jinsirui (Nanjing, China) using the primer 5'-CCC TCA TAG TTA GCG TAA CG-3'. Sequence of the mimetic peptide and cow's milk ALA were aligned using the UniProt alignment tool.

Location of mimotopes on ALA

The Pepitope Server is a web-based tool for predicting discontinuous epitopes based on a set of peptides that have been affinity-selected against an antibody of interest.[20] Chain A of the X-ray structure of ALA (Bos d4; PDB code 2G4N) was submitted to the Pepitope Server. This server implements the following three algorithms for epitope mapping: PepSurf, Mapitope, and a combined algorithm incorporating the first and second algorithms, which were used to identify conformational epitopes with mimotope sequences.[23] The parameters included a substitution matrix, gap penalty and the probability of obtaining the best path. Finally, conformational epitopes on ALA were modelled using PyMOL, which is an open-source, user-sponsored, molecular visualization system created by Warren Lyford DeLano.[24]

RESULTS

Preparation of pooled patient serum samples

S-IgE levels in serum samples collected from 19 patients who were allergic to cow's milk were measured by ImmunoCAP, as shown in Table 1. Equal volumes of eight samples (sample patient nos. 2, 4, 6, 7, 8, 9, 10, and 15) were pooled since their S-IgE levels were greater than 0.35 kU_A/L, which is regarded as the cut-off value for food allergy.[25]

Affinity purification of IgG and IgE antibodies to cow's milk ALA

Specific antibodies against ALA binding onto the column medium were eluted with 3 M $MgCl_2$. As shown in Figure 1A, the fractions with a high concentration of specific antibody against ALA (from the sixth milliliter to the tenth milliliter) were collected, centrifuged, concentrated and desalinated through ultrafiltration tubes with a cut-off of 5 kDa (Millipore). The highest concentration of IgE reached in the ninth millilitre with 0.580 mg/mL. The specificity and purity of antibody are shown in Figure 1B, and these values indicated that the antibody can be used in the next step.

During non-specific elution for IgG antibodies using Protein G (Figure 2A), we found that most of the protein had been eluted in the first three milliliters. Most of this protein was IgE as shown in

SDS-PAGE pattern (Figure 2C) according to the molecular weight of IgE (190 kDa, including two heavy chains and two light chains) and similar studies about affinity purification of IgG and IgE antibodies. [26-30] The protein concentration of eluent was sharply reduced until the ninth millilitre, in which the protein concentration was as low as 0.010 mg/mL. Purified IgG was collected from the first to the sixth milliliter (Figure 2B) during the specific elution. In total, 630 μl of 0.153 mg/mL ALA S-IgE and 840 μl of 0.158 mg/ml ALA S-IgG were obtained and the purities of ALA S-IgE and S-IgG were 93.8% and 85.3%.

IgE- and IgG-binding linear epitopes of cow's milk ALA

After three rounds of biopanning, 48 clones against IgG and IgE respectively were obtained from the phage random library Ph.D.-12. Among them, included 39 positive clones against anti-ALA IgE and 43 positive clones positive clones against anti-ALA IgE have been identified by ELISA, respectively (Table 2 A and B). Six amino acid sequences (RKQTRQKRIQSR, IKTMIRMNTIKL, NIRRNLTIRSRI, HNRKRS SSIRIT, LNNRIRSSINSL and RTRLRRKRRSLI) were identical with different frequencies among the IgE-binding mimotopes. While for IgG mimotopes, four amino acid sequences (HHQNLTQRSRRR, RRLPPLPKIPMH, HRSKQITHTRRH and

243 KQNTKRIIKRRS) have been identified more than once from
244 positive clones, which were regarded as candidate epitope sequences.
245 The UniProt alignment tool was used to align the sequences of
246 mimotopes with the cow's milk ALA amino acid sequence (Locus
247 AAF63624.1) to identify linear epitope candidates with three or more
248 similar or identical amino acids. As shown in Figure 3, linear
249 IgE-binding epitopes were found at AA 41-46, AA 55-60, AA 62-72,
250 AA 74-76, AA 85-90 and AA 92-99, and linear IgG-binding epitopes
251 were detected at AA 37-46, AA 52-54, AA 56-59, AA 63-72, AA
252 74-76, AA 81-90 and AA 92-99.
253 **Locations of conformational IgE- and IgG-binding epitopes of**
254 **cow's milk ALA**
255 After three rounds of biopanning, 96 clones were obtained from the
256 phage random library Ph.D.-C7C, 73 of which were determined to be
257 positive by phage indirect ELISA (Table 3), including 39 IgE-binding
258 (Table 3 A) and 34 IgG-binding mimotopes (Table 3 B). Moreover,
259 six amino acid sequences appeared to be repetitive among the 39 IgE
260 mimotopes. Specifically, the sequences RIRSRRN, LRSLKRP and
261 LRKLKRP appeared 13, 8 and 3 times, respectively. In addition, the
262 peptides LRHLKRP, LSSLQRP and NTTKHIK appeared twice.

263 While among the 34 IgG-binding mimotopes, the sequences of
264 IPMRRIR, SHRRTR, KPSLPNL and LTNSSIQ appeared twice.
265 Six mimic peptides recognized by IgE and four mimic peptides
266 recognized by IgG and a PDB file of bovine ALA were put into the
267 Pepitope Server. High algorithm scores and lower P values were
268 judged as the standard of optimal epitopes (Table 4). Thus, we
269 determined that the most probable bovine α-lactalbumin
270 conformational IgE-binding epitopes were ME 1 (K62-N71-I75
271 -S76-K79 and K62-I75-S76-K79-L81), ME 2 (P24-E25-K114- L115-
272 Q117-L119), ME 3 (L105-H107-K108-L110), ME 4 (V42-Q43-S47-
273 T48-Q65-P67), and ME5(N44-S47-T48-E49-Y50-K79-L81) as
274 shown in Figure 4 and conformational IgG-binding epitopes on
275 bovine α-lactalbumin were MG 1 (F9-R10-K13-K16-L23-P24-
276 L119), MG 2 (I59-Q65-P67-S69-N71-I75), and MG 3
277 (Q39-I41-N44-S47- T48) in Figure 5.
278

279 ## DISCUSSION

280 We isolated specific anti-ALA IgE and IgG antibodies from the
281 sera of patients with an allergy to cow's milk by two-step affinity
282 chromatography using CNBr-activated Sepharose 4B and HiTrap
283 Protein G columns[31,32] and high-purity IgG (purity = 93.8%) and IgE

(purity = 85.3%) were obtained for epitope mapping. A small number of additional miscellaneous bands were observed which might be IgA and IgM since they existed in the form of dimmer and pentamer and could not totally reduced by β-mercaptoethanol.

As we know, X-ray is an accurate method for epitope mapping.[33] However, the main limitation of X-ray based approach for B cell epitope mapping is the lack of natural IgE mAb in milligram amount required for X-ray crystallography studies, resulting in limiting the broad use of X-ray for epitope mapping.[13] Therefore, it was concluded that the mimotope identification strategy can be an alternative way for epitope mapping of different food allergen，and until now several conformational epitopes were identified by biopanning phage libraries. In 2006, one conformational IgE epitope of Bet v1 has been identified which was also the cross-reacting basis with other three allergens homologues Gly m 4 (soybean), Ara h 8 (peanut) and Pru av 1 (cherry) [34]. In the same year, conformational epitopes of parvalbumin recognized by both IgE and IgG antibody were obtained by screening of a constrained decamer phage library[35]. Following this approach, two relevant conformational IgE-binding epitopes of peach Pru p 3 have been identified.[36] Krisztina et al mapped a conformational epitopes

of house dust mite allergens Der p 1 and Der p 2 with human monoclonal antibody[37]. The phage display technique is used to assess large sets of random peptides to select those with high binding affinity to an antibody of interest, and it has been used in many fields as well, including drug discovery, enzymatic substrate and inhibitor discovery, vaccine design, analysis of protein-protein interactions, identification and engineering of polypeptides and mapping of allergen epitopes. [21, 22, 38-40] In 2012, Bøgh et al. conducted competitive immunoscreening of a phage-displayed random peptide library to map IgE epitopes on intact and digested Ara h 1 with both human and rat sera. The identified epitopes were similar for intact and digested proteins; therefore, the IgE epitopes can be considered to be informative epitopes.[41] Untersmayr et al. have determined three major specific IgE epitope regions on the parvalbumin molecule and have confirmed these epitope regions using two independent matching algorithms.[42] A recent study has demonstrated the utility of phage-display technology for distinguishing between the epitope patterns of IgE and IgG4, providing detailed information on fine specificity and affinity.[43]

Knowledge about antigen epitopes can reveal the nature of antigen-antibody reactions and contributes to improved

326 understanding of the role of food allergens in allergic reactions. Prior
327 to this study, some studies investigated IgE-binding linear epitopes
328 on ALA. Jarvinen et al. described four IgE epitopes at AA 1-16, AA
329 13-26, AA 47-58, and AA 93-102 of native bovine ALA.[9] Maynard
330 et al. showed that AA 17-58 and one large tryptic peptide containing
331 this sequence were the most strongly and frequently recognized by
332 IgE.[18] Adams et al. used the RAST assay to show that a synthetic
333 peptide comprising AA 5-18 contains an IgE-binding epitope.[19]
334 Hochwallner et al. identified sequential epitopes on ALA using 8
335 synthetic overlapping peptides spanning the ALA sequence.[44]
336 In the current study, six linear IgE-binding epitopes on bovine
337 ALA were identified, including AA 41-46, AA 55-60, AA 62-72, AA
338 74-76, AA 85-90 and AA 92-99. The linear IgE-binding epitopes AA
339 41-46, AA 55-60, and AA 92-99 overlap with those reported in
340 Maynard's study,[18] which included AA 17-58, and with those
341 reported in Jarvinen's study,[9] which included AA 47-58 and AA
342 93-102. Epitope AA93-102 reported by Jarvinen contained an
343 additional epitope AA92-99.[9] Although exact comparisons are
344 difficult to perform, these findings may be related to patients' living
345 environments, including geographical factors, and concentrations of
346 specific IgE antibodies. More importantly, three new linear

IgE-binding epitopes on bovine ALA—AA 62-72, AA 74-76, and AA 85-90—were identified. However, the carboxy terminus (AA 109-123) was not found to be identical to that of the IgE from our cow's milk-allergic patients.[9, 18, 19]

Regarding linear IgG epitopes on ALA, only one study has identified three IgG-binding sequences—AA 7-18, AA 53-62, and AA 89-108—in human sera from CMA patients.[9] In the present study, seven linear IgG-binding epitopes were found: AA 37-46, AA 52-54, AA 56-59, AA 63-72, AA 74-76, AA 81-90 and AA 92-99. Among these, five epitopes—AA 37-46, AA 52-54, AA 56-59, AA 81-90 and AA92-99—overlapped with epitopes previously reported by Jarvinen et al. Moreover, epitopes AA 52-54 and AA 56-59 are included within AA 53-62, while AA 92-99 is included within AA 89-108, which was reported by Jarvinen et al.[9]

In addition, the current study revealed that the linear IgE-binding epitopes AA 74-76 and AA 92-99 were exactly the same as the linear IgG-binding epitopes AA 74-76 and AA 92-99 and that the linear IgE-binding epitopes AA 55-60 and AA 62-72 were exactly the same as the linear IgG-binding epitopes AA 56-59 and AA 63-72 on ALA. Other epitopes contained large numbers of shared amino acids. Thus, the sequences and position of linear IgE- and IgG-binding epitopes

368 on cow's milk ALA were very similar since both of them were
369 located in AA 37-99. Even some of linear epitopes were the same
370 such as AA 74-76 and 92-99. Based on their overlap position, it was
371 indicated that common residues could with different frequencies be
372 regarded as informative maker for detecting and diagnosis of milk
373 allergy in further.

374 With respect to linear IgE epitopes from figure 3A, the highest
375 frequency of residue is Asparagine in the position of 66 since there
376 were 12 positive clones containing this residue. The second highest
377 frequency was Lysine which appeared ten repetitions at the position
378 of 93, 94 and 98, respectively. Based on full sequence of bovine
379 ALA, we found that the highest frequency of amino acids identified
380 as the composition of epitopes were Asparagine (50 times), Lysine
381 (47 times), Isoleucine (45 times), Aspartic acid (28 times), Glutamine
382 (22 times), successively. Moreover, the characterization of IgG
383 epitopes showed the same results, which means the same residues
384 such as Aspartic acid, Lysine acid, Asparagine, Isoleucine and
385 Glutamine showed the highest frequencies as shown in figure 3B.

386 Related studies have indicated that more than 90% of epitopes
387 involved in allergic reactions were conformational epitopes and that
388 some linear epitopes were components of conformational epitopes.[20]

Maynard et al. concluded that conformational epitopes are 'more important' because the majority of patients (60%) respond to native ALA and larger peptides.[18] IgG antibodies from patients with cow's milk allergy also showed marked preferences for conformational epitopes.[45] These findings further confirm that the epitopes on the milk-based allergen ALA are conformational epitopes. However, ALA conformational epitopes have not been identified before our study. In our study, we identified five IgE-binding conformational epitopes (ME 1, ME 2, ME 3, ME 4, and ME 5) in addition to three IgG-binding conformational epitopes (MG 1, MG 2, and MG 3) on bovine ALA. We found that most of these residues of these epitopes also appeared in linear epitopes in Figure 3. A total of 61% of the amino acids in the IgE-binding conformational epitopes and 94% of the amino acids in the IgG-binding conformational epitopes identified in this study have been previously reported as linear epitope sequences. In particular, the amino acids of the two conformational epitopes MG 2 and MG 3 have been reported as IgG-binding linear epitopes. These findings also support those of a previous study by Aalberse, who found that some linear epitopes are components of conformational epitopes.[46]

In conclusion, B cell epitopes including linear and conformational epitopes have been identified successfully by phage display technique at the same time. Common residues were found in IgG and IgE epitopes. Some Asparagine (50 times), Lysine (47 times), Isoleucine (45 times), Aspartic acid (28 times), Glutamine (22 times) were more be the composition of residues. It is the first time to identify conformational epitopes of ALA which could provided a diagnostic tool for the assessment of patients who are allergic to cow's milk. These findings also indicate that some residues of conformational epitopes are composed of linear epitopes on bovine α-lactalbumin.

Acknowledgements

The work was supported by National High Technology Research and Development Program of China (863 Program, No. 2013AA102205), International Science & Technology Cooperation Program of China (No.2013DFG31380), National Natural Science Foundation of China (No. 31260204 and 31301522), Research Program of State Key Laboratory of Food Science and Technology (No. SKLF-ZZA-201612 and SKLF-ZZB-201510) and Special project of Technology Research from Guangdong Provience in 2013 (No. 2013B090600060).

The authors declare no competing financial interest.

REFERENCES

1 N. Prieto, E. Iniesto, C. Burbano, B. Cabanillas, M. M. Pedrosa, M. Rovira, J. Rodríguez, M. Muzquiz, J. F. Crespo, C. Cuadrado and R. Linacero, *J. Agr. Food Chem*., 2014, **62**, 5617-5624.

2 P. M. Christopher, A. D. Wendy, L. W. Richard, Y. Megumi, C. Brian and C. G. Casey, *Food Chem,* 2014, **62**, 6746-6755.

3 Z. Husain and R. A. Schwartz, *Int. J. Dermatol.,* 2013, **52**: 286-294.

4 E. C. McGowan and C. A. Keet, *J. Allergy Clin. Immun*., 2013, **132**: 1216-1219.

5 H. Hochwallner, U. Schulmeister, I. Swoboda, S. Spitzauer and R. Valenta, *Methods*, 2014, **66**, 22-33.

6 C. Beck, A. Castro, A. Gushken, L. Watanabe, A. Brandao, G. Yonamine, A. Pastorino and C. Jacob, *Allergy*, 2012, **67**, 366-366.

7 E. M. Savilahti, V. Rantanen, J. S. Lin, S. Karinen, K. M. Saarinen, M. Goldis, M. J. Makela, S. Hautaniemi, E. Savilanti and H. A. Sampson, *J. Allergy Clin. Immun.*, 2010, **125**, 1315-1321.

8 G. Bu, Y. Luo, Z. Zheng and H. Zheng, *Food Agr. Immunol*., 2009, **20**, 195-206.

9 K. M. Järvinen, P. Chatchatee, L. Bardina, K. Beyer and H. A. Sampson, *Int. Arch Allergy Immunol*., 2001, **126**, 111-118.

10 M. Zhang, J. Zheng, K. Ge, H. Zhang and B. Fang, *Int. Dairy. J.*, 2014, **34**, 220-228.

11 M. Yamaguchi, S. Takai, A. Hosono and T. Seki, *Biosci. Biotech. Bioch,* 2014, **78**, 672-679.

12 M. Yamaguchi, K. Yoshida and M. Uchida, *Biol. Pharm. Bull*, 2009, **32**, 366-371.

13 A. Pomés, *Int. Arch. Allergy Imm*., 2010, **152**, 1-11.

14 H. A. Sampson, *J. Allergy Clin. Immun.,* 2004, **113**, 805-819.

15 J. M. Gershoni, A. Roitburd-Berman, D. D. Siman-Tov, N. Tarnovitski Freund and Y. Weiss, *BioDrugs*, 2007, **21**, 145-156.

16 L. A. N. Willison, Q. Zhang, M. Su, S. S, Teuber, S. K. Sathe and K. H. Roux, *Mol. Immunol*., 2013, **55**, 253-263.

17 S. Benedé, I. López-Expósito, G. Giménez, G. Grishina, L. Bardina, H. A. Sapson, R. López-Fandino and E. Molina, *Food Res. Int*., 2014, **62**, 1127-1133.

18 F. Maynard, R. Jost and J. M. Wal, *Int. Arch. Allergy Immunol*., 1997, **113**. 478-488.

19 S. L. Adams, D. Barnett, B. J. Walsh, R. J. Pearce, D. J. Hill and M. E. H. Howden, *Immunol. Cell Biol*., 1991, **69**, 191-197.

20 X. Li, H. B. Chen, J. Y. Gao, S. F. He and Y. Y. Wu, *J. Food Sci*., 2014, **79**, 748-756.

21 T. Bratkovič, *Cell. Mol. Life Sci*., 2010, **67**, 749-767.

22 E. E. Guhsl, G. Hofstetter, C. Ebner, W. Hemmer, H. Breiteneder and C. Radauer, *Clinical and Translational Allergy*, 2014, **4**, 1-1.

23 I. Mayrose, O. Penn, E. Erez, N. D. Rubinstein, T. Shlomi, M. T. Freund, E. M. Bublil, E. Ruppin, R. Sharan, J.M. Gershoni, E. Martz and T. Pupko, *Bioinformatics*, 2007, **23**, 3244-3246.

24 P. Tosco and T. Balle, *J. Mol. Model*., 2011, **17**, 201-208.

25 Y. T. Lin, C. T. Wu, J. L. Huang, J. H. Cheng and K. W. Yeh, *J. Microbiol. Immunol.,* 2014. Mar 22. doi: 10.1016/j.jmii.2014.01.002.

26 C. Jin, B. Hantusch, W. Hemmer, J. Stadlmann and F. Altmann, *J. Allergy Clin. Immun.*, 2008, **121**(1): 185-190. e2.

27 H. Dai, H. L. Dong, F. Y. Gong, S. L. Sun, X. Y. Liu, Z. G. Li, S. D. Xiong and X. M. Gao, *J. Immunol.*, 2014, **192**(10): 4533-4 540.

28 A. Gieras, P. Cejka, K. Blatt, M. Focke-Tejkl, B. Linhart, S. Flicker, A. Stoecklinger, K. Marth, A. Drescher, J. Thalhamer, P.

493 Valent, O. Majdic and R. Valenta, *J. Immunol.*, 2011, **186**(9):
494 5333-5344.
495 29 P. Rupa, K. Hamilton, M. Cirinna, M. Cirinna and B. N. Wilkie,
496 *Immunol. Immunop.*, 2008, **125**(3): 303-314.
497 30 Y. Y. Shiung, C. Y. Chiang, J. B. Chen, P. C. Wu, A. F. H.
498 Hung, D.C.S.Lu, R. L. Pan and T. W. Chang, *Immunobiology*,
499 2012, **217**(7): 676-683.
500 31 C. G. Jakobsen, U. Bodtger, P. Kristensen, L. K. Poulsen and E.
501 L. Roggen, *Mol. Immunol*., 2004, **41**, 941-953.
502 32 B. Hantusch, I. Schol, C. Harwanegg, S. Krieger, W. M. Becke,
503 S. Spitzauer, G. Boltz-Nitulescu and E. Jensen-Jarolim, *Immunol.*
504 *Lett*., 2005, **97**, 81-89.
505 33 W. M. Abbott, M. M. Damschroder and D.C. Lowe,
506 *Immunology*, 2014, **142**, 526-535.
507 34 D. Mittag, V. Batori, P. Neudecker, R. Wiche , E. P. Friis,
508 B.K. Ballmer-Weber , S. Vieths, E.L. Roggen , *Mol.*
509 *Immunol*., 2006,**43**(3), 268–278.
510 35 E. Untersmayr, K Szalai, A. B. Riemer, W. Hemmer,
511 I. Swoboda, B Hantusch, I Schöll, S. Spitzauer, O. Scheiner,
512 R. Jarisch, G. Boltz-Nitulescu, E Jensen-Jarolim,
513 *Mol. Immunol*., 2006, **43** (9), 1454–1461.

514 36 L.F. Pacios, L. Tordesillas, J. Cuesta-Herranz, E. Compes
515 R. Sánchez-Monge, A. Palacín, G. Salcedo and A. Díaz-Perales,
516 *Mol. Immunol*., 2008, **45**, 2269-2276.
517 37 K. Szalai, J. Fuhrmann, T. Pavkov , M. Scheidl, J.Wallmann, K.
518 H. Brämswig, S.Vrtala, O. Scheiner, W. Keller, J. Saint-Remy, D.
519 Neumann, I. Pali-Schöll, E. Jensen-Jarolim, *Mol. Immunol.*
520 2008,**45** (5) 1308–1317.
521 38 J. Pande, M. M. Szewczyk and A. K. Grover, *Biotechnol. Adv*.,
522 2010, **28**, 849-858.
523 39 K. J. Lee, J. H. Lee, H. K. Chung, J. Choi, J. Park, S. S. Park, E.
524 J. Ju, J. Park and S. H. Shin, *Amino Acids*, 2014, **47**, 281-289.
525 40 S. S. Sidhu, W. J. Fairbrother and K. Deshayes, *Chembiochem*,
526 2003, **4**, 14-25.
527 41 K. L. Bøgh, H. Nielsen, C. B. Madsen, E. N. C. Mills, N. Rigby,
528 T. Eiwegger, Z. Szepfalusi and E. L. Roggen, *Mol. Immunol*.,
529 2012, **51**, 337-346.
530 42 E. Untersmayr, K. A. B. Szalai, Riemer, W. Hemmer, I.
531 Swoboda, B. Hantuschd, I. Schölla, S. Spitzauerc, O. Scheinera,
532 R. Jarischb, G. Boltz-Nitulescua and E. Jensen-Jarolima, *Mol.*
533 *Immunol*., 2006, **43**. 1454-1461.

43 K. L. Bøgh, H. Nielsen, T. Eiwegger, C. B. Madsen, E. N. C. Mills, N. M. Rigby, Z. Szepfalusi and E. L.Roggen, *Mol. Immunol*., 2014, **58**, 169-176.

44 H. Hochwallner, U. Schulmeister, I. Swoboda, M. Focke-Tejkl and V. Civaj, *J. Allergy Clin. Immun*., 2010, **125**, 1279-1285.

45 J. Duchateau, A. Michils, J. Lambert, B. Gossart and G. Casimir, *Clin. Exp. Allergy*, 1998**, 28**, 824-833.

46 R. C. Aalberse, *J. Allergy Clin. Immun*., 2000, **106**, 228-238.

Figure captions

Fig. 1. Affinity chromatography by CNBr-Sepharose 4B. (A) Specific elution curve with 3 mol/L $MgCl_2$. (B) SDS-PAGE patterns of solutions during CNBr-Sepharose 4B affinity chromatography (M, protein marker; 1, serum pool (diluted to 1:10); 2, solution from the first non-specific elution; 3, specific concentrated elution).

Fig. 2. Affinity chromatography by protein G. (A) The concentration change during non-specific elution. (B) The concentration change during specific elution. (C) SDS-PAGE patterns of different solutions (M, Marker; 1, IgE; 2, IgG).

Fig. 3. Positions of linear epitopes located in amino acid sequence of ALA. (A) IgE binding epitopes. (B) IgG binding epitopes.

Fig. 4. Ribbon (A) and globular (B) presentations of cow's milk α-lactalbumin mimic peptides to IgE binding composition epitopes. ME 1-5 from Table 4A are marked in green, blue, yellow, magenta and hotpink, respectively. Overlapping amino acids (K79, L81) in ME 1 and 5 are marked in orange, while overlapping amino acids (S47, T48) in ME 4 and 5 are marked in red.

Fig. 5. Ribbon (A) and globular (B) presentations of cow's milk α-lactalbumin mimic peptides to IgG binding composition epitopes. MG 1,2 and 3 in Table 4B are marked in green, blue and yellow, respectively.

Table 1. Demographic, clinical and serologic characterization of bovine milk allergic children

Patient no.	Age range	Milk-related symptoms	α-La S-IgE(kU_A/L)
1	1-5 years	asthma	0.11
2	1-5 years	asthma	4.14
3	less than 1 year	bronchial asthma	0.19
4	less than 1 year	bronchial asthma	0.45
5	1-5 years	eczema	ND
6	1-5 years	cough variant asthma	12
7	1-5 years	asthma	2.42
8	1-5 years	NK	0.75
9	less than 1 year	leukocytosis	1.09
10	NK	NK	25.3
11	less than 1 year	NK	0.15
12	less than 1 year	urticaria	0.22
13	less than 1 year	NK	0.11
14	1-5 years	urticaria	0.31
15	1-5 years	rhinoconjunctivitis	3.18
16	6-10 years	rhinoconjunctivitis	0.04
17	5-10 years	rhinoconjunctivitis	0.25
18	1-5 years	pneumonia	0.27
19	5-10 years	rhinoconjunctivitis	0.02

NK, not known; ND, not done.

Table 2. Mimotope sequences from the phage display random peptide libraries Ph.D.-12

A: Target molecule is anti-ALA IgE

Phage	Peptide sequence	Times	Phage	Peptide sequence	Times
Ph 5, Ph 6, Ph 29, Ph 43, Ph 45	RKQTRQKRIQSR	5	Ph 32, Ph 41, Ph 42	IKTMIRMNTIKL	3
Ph 11, Ph 37, Ph 39	NIRRNLTIRSRI	3	Ph 9, Ph 14	HNRKRSSSIRIT	2
Ph 17, Ph 31	LNNRIRSSINSL	2	Ph 1, Ph 46	RTRLRRKRRSLI	2
Ph 18	HKRNRRPPRLLN	1	Ph 19	NIPRITIRLHMP	1
Ph 15	HRIRSPSSLRKP	1	Ph 10	NRPKKRIKQIQL	1
Ph 36	HRRPRHRKRRRL	1	Ph 25	NTRIRRRTNRTI	1
Ph 23	IHKRQQKRIKPI	1	Ph 4	PRQLQRRNHRHH	1
Ph 34	ILHNPRRIKRHI	1	Ph 7	QKQRINLILTNR	1
Ph 24	IPRTIRTKRKLI	1	Ph 3	QQLTITRKLLPK	1
Ph 8	KSKQKRIKTRIT	1	Ph 47	QQQRMKKRIKRT	1
Ph 35	KSMRSSIKSINI	1	Ph 22	RHNTIRSRIMRI	1
Ph 16	LIIRRLLQKPMT	1	Ph 13	RHRNNSIRSSHI	1
Ph 21	LNNHRKRRRPRL	1	Ph 44	SPWSPKFPGDPT	1
Ph 30	MLPIIRNLIHTT	1	Ph 20	TNLRRTTTHRLN	1

B: Target molecule is anti-ALA IgG.

Phage	Peptide sequence	Times	Phage	Peptide sequence	Times
Ph 24, Ph 30, Ph 36	HHQNLTQRSRRR	3	Ph 20, Ph 33, Ph 47	RRLPPLPKIPMH	3
Ph 5, Ph 19	HRSKQITHTRRH	2	Ph 26, Ph 29	KQNTKRIIKRRS	2
Ph 22	IKTPSLMHQSNI	1	Ph 7	NIIKNRLRITPL	1
Ph 9	ITTLLIKRMTKI	1	Ph 42	NKSKPLIQRLIN	1
Ph 4	KKHRKQLIKRLI	1	Ph 10	NRRQQLRNSRRT	1
Ph 17	KMRMNHRRISNN	1	Ph 48	PIIHIILNMTHS	1
Ph 12	KQSPQLRKIQRI	1	Ph 43	PRNHNLLQKNRR	1
Ph 6	KRPLIHRRNRLR	1	Ph 14	PSKMLIQTRITI	1
Ph 39	LLKSTTKSSNIR	1	Ph 35	PTIRHSKRLHQN	1
Ph 46	LMKPNNLLKISI	1	Ph 31	QKQNIIIRNLLN	1
Ph 23	MNQRTKKIRSRR	1	Ph 45	QQHINSRRRIMK	1
Ph 16	MQTKMTKKKMPI	1	Ph 2	RIKIRLNRIKPH	1

Ph 34	MRRSSHQSLKKR	1	Ph 13	RIRTIITNTIMK	1
Ph 41	NHKHPIKNKIHI	1	Ph 21	RKHRTQSTQIIR	1
Ph 1	RMIRRINPTIII	1	Ph 44	STRVVVPDGNLP	1
Ph 25	RNKHLSHQRRMS	1	Ph 15	TLIHRHKKLNIN	1
Ph 8	RRKRIHRRNPLR	1	Ph 38	TNRNISKIRIRR	1
Ph 37	SRKRSHMRRRNQ	1	Ph 28	TTSTIPPTLRMT	1
Ph 32	SSIMNSKSLHKH	1			

Table 3. Mimotope sequences from the phage display random peptide libraries Ph.D.-C7C

A: Target molecule is anti-ALA IgE.

Phage	Peptide sequence	Times	Phage	Peptide sequence	Times
Ph 7, Ph 10, Ph 12, Ph 14, Ph 18, Ph 20, Ph 21, Ph 24, Ph 30, Ph 32, Ph 37, Ph 39, Ph 44	RIRSRRN	13	Ph 6, Ph 15, Ph 16, Ph 29, Ph 36, Ph 38, Ph 40, Ph 42	LRSLKRP	8
Ph 19, Ph 22, Ph 45	LRKLKRP	3	Ph 31, Ph 35	LRHLKRP	2
Ph 25, Ph 26	LSSLQRP	2	Ph 5, Ph 9	NTTKHIK	2
Ph 41	ILKRRPI	1	Ph 3	LTRKLRS	1
Ph 23	IPRKLPN	1	Ph 13	RRLTRIQ	1
Ph 48	LIRRTSI	1	Ph 27	RRTQLHL	1
Ph 2	LPRKRHS	1	Ph 33	TLKRRPN	1
Ph 43	LRPLKRP	1			

B: Target molecule is anti-ALA IgG.

Phage	Peptide sequence	Times	Phage	Peptide sequence	Times
Ph 45, Ph 46	IPMRRIR	2	Ph 47, Ph 48	ISHRRTR	2
Ph 33, Ph 34	KPSLPNL	2	Ph 17, Ph 18	LTNSSIQ	2
Ph 25	HIQRTTP	1	Ph 13	LRHTIMN	1
Ph 14	HIRIMIP	1	Ph 2	MSHNTRR	1
Ph 24	HLRRRHT	1	Ph 39	NPLRKRR	1
Ph 11	ILLKRPR	1	Ph 26	PLRLRRP	1
Ph 38	ILQRRPS	1	Ph 29	QQITRRP	1
Ph 9	KIRMLRR	1	Ph 35	RHSLMPM	1
Ph 7	LHPPLTL	1	Ph 32	RIRMRRL	1
Ph 4	LITQMMP	1	Ph 15	RLHRRIH	1

Ph 36	LLHKLRQ	1	Ph 21	RMSRHLN	1
Ph 41	LLKRRPT	1	Ph 30	RRPLRIR	1
Ph 42	LLLLRNL	1	Ph 20	RTKLRKL	1
Ph 12	LLLRPMT	1	Ph 16	RTTMQQI	1
Ph 37	LLRRLRL	1	Ph 23	TLRKRRP	1

Table 4. Composition and position of conformational epitopes on ALA by Pepitope Server

A：Mimic peptide binding to IgE

Phage	Mimic peptide	Amino acid sequence	Algorithm scores	P values	Name
Ph 5, Ph 9	NTTKHIK	N44-S47-T48-E49-Y50-K79-L81	12.6053	0.0024	ME5
Ph19, Ph22, Ph 45	LRKLKRP	P24-E25-K114-L115-Q117-L119	10.5609	0.0027	ME2
Ph31, Ph35	LRHLKRP	L105-H107-K108-L110	10.1631	0.0010	ME3
Ph7, Ph10, Ph12, Ph14, Ph18, Ph20, Ph21, Ph24, Ph 30, Ph 32, Ph 37, Ph 39, Ph 44	RIRSRRN	K62-N71-I75-S76-K79	7.5717	0.0013	ME1
Ph 6，Ph15，Ph 16，Ph 29, Ph 36, Ph 38，Ph 40, Ph 42	LRSLKRP	K62-I75-S76-K79-L81	7.4178	0.0056	ME1
Ph 25, Ph 26	LSSLQRP	V42-Q43-S47-T48-Q65-P67	6.1000	0.0040	ME4

B：Mimic peptide binding to IgG

Phage	Mimic peptide	Amino acid sequence	Algorithm scores	P values	Name
Ph33, Ph 34	KPSLPNL	I59-Q65-P67-S69-N71-I75	10.2792	0.0009	MG2
Ph17, Ph 18	LTNSSIQ	Q39-I41-N44-S47-T48	9.4523	0.0013	MG3
Ph 45, Ph 46	IPMRRIR	F9-R10-K13-K16-L23-P24-L119	8.8165	0.0011	MG1

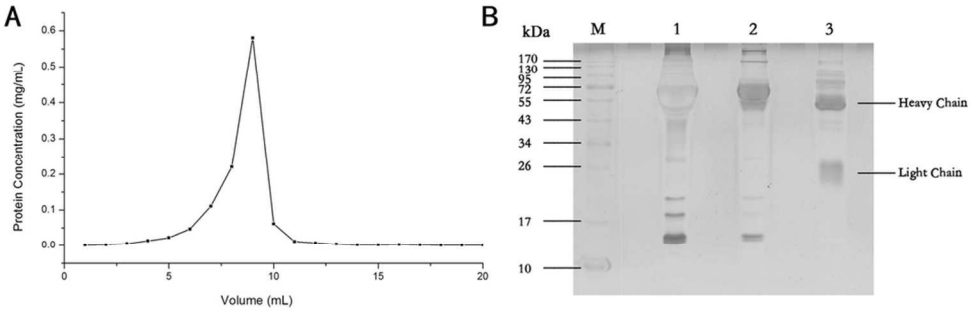


Fig. 1. Affinity chromatography by CNBr-Sepharose 4B.
92x32mm (300 x 300 DPI)

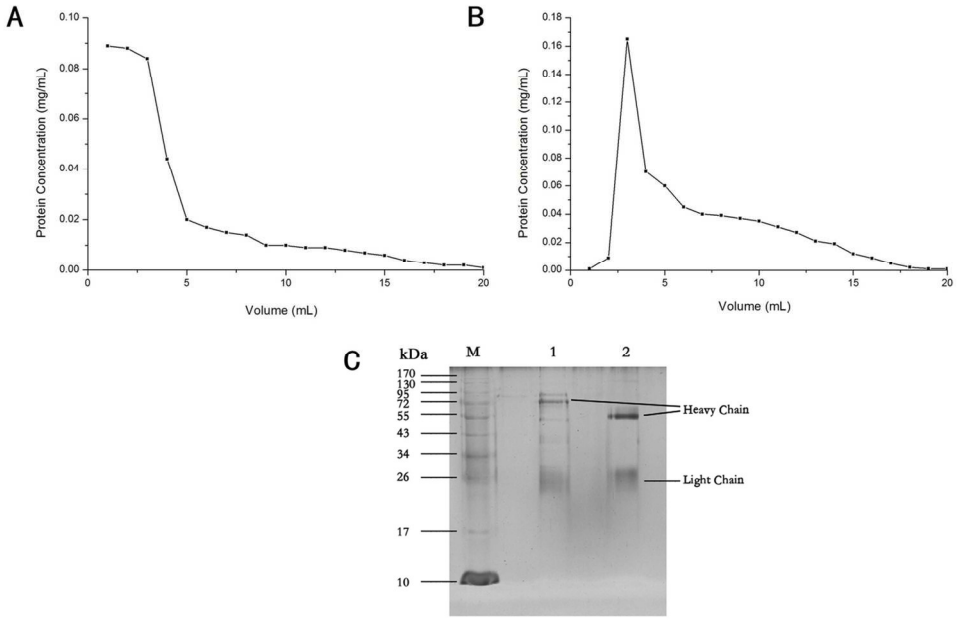


Fig. 2. Affinity chromatography by protein G.
125x81mm (300 x 300 DPI)

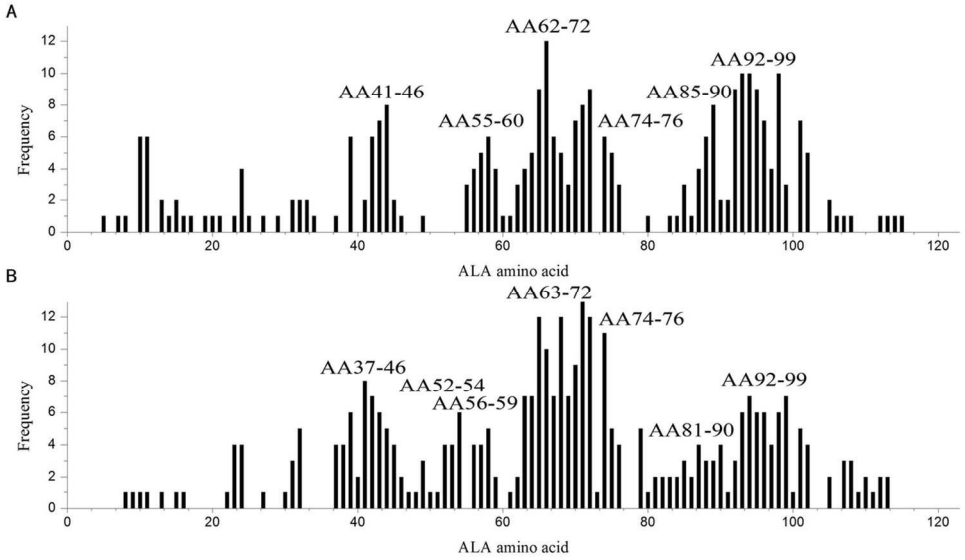


Fig. 3. Positions of linear epitopes located in amino acid sequence of ALA.
120x71mm (300 x 300 DPI)

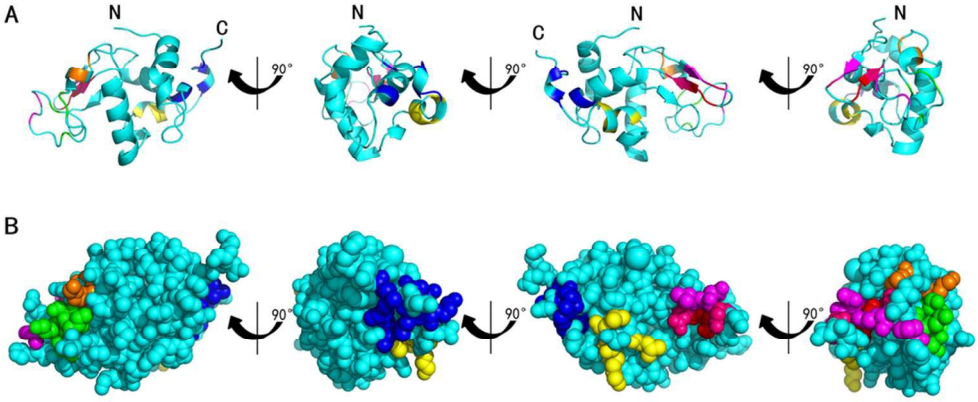


Fig. 4. Ribbon (A) and globular (B) presentations of cow's milk α-lactalbumin mimic peptide to IgE binding composition epitopes.
95x43mm (300 x 300 DPI)

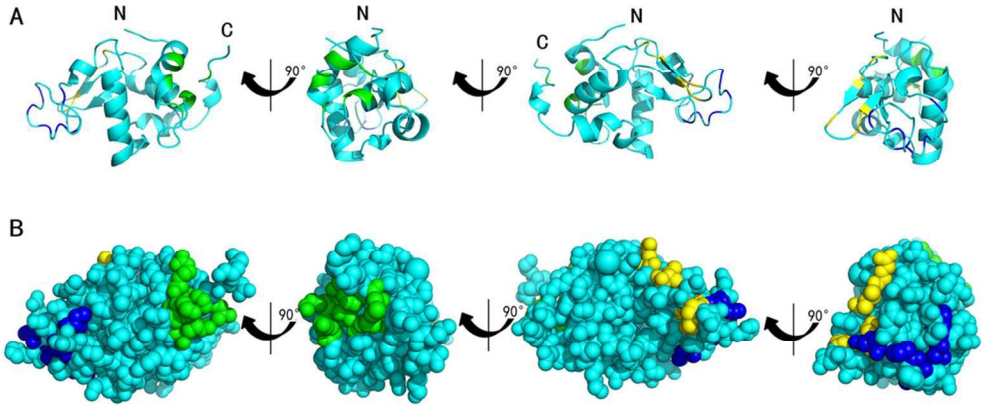
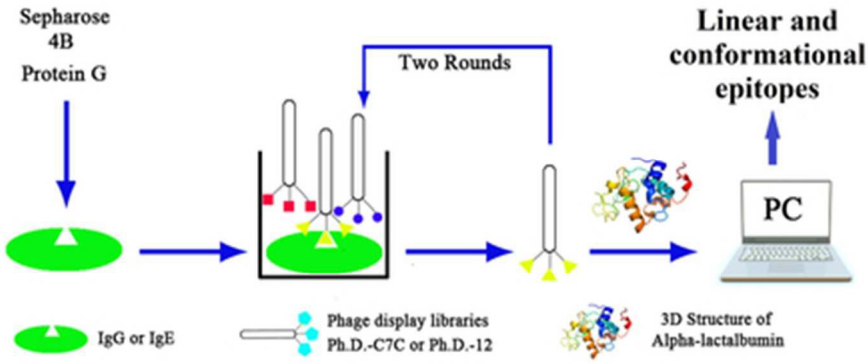


Fig. 5. Ribbon (A) and globular (B) presentations of cow's milk α-lactalbumin mimic peptide to IgG binding composition epitopes.
95x43mm (300 x 300 DPI)



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