Research Article

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Identification and characterization of the antigenic site (epitope) on bovine β-lactoglobulin: common residues in linear and conformational epitopes

Xin Li,[a,b] Shuilin Yuan,[a,b] Shengfa He,[a,b] Jinyan Gao[a,b] and Hongbing Chen[a,c*]

Abstract

BACKGROUND: β-Lactoglobulin is recognised as one of major allergens in milk and its epitopes include linear and conformational epitopes contributed to milk allergy.

RESULTS: In our work, two types of epitopes have been identified. Linear epitopes identified by using SPOT™ peptide arrays approach and three common peptide sequences AA77–82 (KIPAVF), AA126–131 (PEVDNE) and AA142–147 (ALPMHI) were obtained by reacting with specific sera from two rabbits. At the same time, mimotopes were screened by the panning of a phage display peptide library and the corresponding conformational epitopes were calculated by the web tool of Peptiope server with Mapitope algorithm. Three conformational epitopes against two specific sera were identified, in which there were 15 common residues as well and located in the different position and appeared mainly as an α-helix.

CONCLUSION: Common residues on the linear and conformational epitopes were identified in the first time, respectively, which could be regarded as informative epitopes for detection of allergen in dairy products.

Keywords: bovine β-lactoglobulin; allergen; common residues; epitope

INTRODUCTION

Cow's milk allergy (CMA) is reported to be the most prevalent for infants or young children with an incidence of 2–3%.[1,2] CMA can present with a wide spectrum of clinical reactions, including cutaneous, gastro-intestinal and respiratory symptoms as well as systemic anaphylactic symptoms. Clinical symptoms involve immediate or delayed reactions, operating separately or together.[3] The symptoms occur within days or weeks after commencing feeding with a cow's milk-based formula or already at their first exposure. Milk proteins are the first exogenous proteins consumed in large quantities by children. All cow's milk proteins may be potential allergens, of which the main allergens were found to be caseins, α-lactalbumin and β-lactoglobulin (β-Lg),[4] and they can cause immunologically mediated adverse reactions.

Immunoglobulin (Ig)-binding epitopes on proteins are typically divided into two categories, linear (continuous) and conformational (discontinuous), although some epitopes appear to be a blend of the two forms. Linear epitopes typically derive from short contiguous eight to ten amino acid (AA) segments whereas conformational epitopes, as the name implies, require a defined three-dimensional peptide conformation dependent upon the scaffolding of the native protein which often (though not necessarily) is comprised of discontinuous segments brought abbreviations into close proximity as a consequence of peptide/protein folding. The technology for identifying linear epitopes is straightforward and is typically performed by reacting the antibodies of interest with enzymatically derived or cloned peptide fragments or sets of short (AA12–20) overlapping peptides encompassing the sequences of interest (i.e. peptide scanning).[5]

Ladics *et al.* have referred those allergens in complex with fragments of IgG antibodies recognising epitopes that overlap with IgE antibody-binding sites Api m2, Bet v1, Bla v1 and so on.[6] The use of IgG antibodies may prove an ideal method for the prevention of the development of allergic diseases. Facilitated antigen presentation and its inhibition by blocking IgG antibodies depend on IgE repertoire complexity, which might indicate that IgG and IgE epitopes recognised the same epitope. Although some linear and conformational epitopes have been determined, there is no comparison between them. Therefore it is still a hypothesis

* *Correspondence to: Chen Hongbing, State Key Laboratory of Food Science and Technology, Nanchang University, No. 235 Nanjing Donglu, Nanchang 330047, P.R. China. E-mail: chbgjy@hotmail.com*

a *State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, 330047, P.R. China*

b *School of Life Sciences and Food Engineering, Nanchang University, Nanchang, 330031, P.R. China*

c *Jiangxi-OAI Joint Research Institute, Nanchang University, Nanchang, 330047, P.R. China*

that some of linear epitopes were the component of conformational epitopes.[7] In our study, linear and conformational epitopes of bovine β-Lg were defined by the sera from two rabbit by using SPOT™ peptide arrays and phage display technique, respectively. In addition, the epitopes of bovine β-Lg have been explored to find some common residues.

MATERIALS AND METHODS

Materials

ECL western blotting detection reagents and CNBr-activated Sepharose 4B were from GE (Schenectady, New York, NY, USA). The heptapeptide library (PhD-C7C) was purchased from New England Biolabs (Beverly, MA, USA). Sheep anti-rabbit Ig/horseradish peroxidase and membrane with overlapping peptides were bought from Sigma (St. Louis, MO, USA). All the other chemicals were purchased from Sangon Co. (Shanghai, China).

Preparation of polyclonal antibodies against bovine β-lactoglobulin

Four 8-week-old Japanese white male rabbits were immunised subcutaneously according to a routine procedure as given in a previous study.[6] Purified bovine β-Lg (2 mg) dissolved in 0.5 mL 0.05 mol L^{-1} phosphate-buffered saline was emulsified with complete Freund's adjuvant (Sigma, St Louis, MO, USA) in a 1:1 ratio for the first injection. Then three booster injections were performed at 14-day intervals with incomplete Freund's adjuvant. Before each injection point, the titres of polyclonal antibodies from sera were checked by indirect ELISA. One week later, after the last boosting, the sera were collected by centrifugation, and then stored at −80 °C until used.

Purification of polyclonal antibodies against bovine β-lactoglobulin

The specific IgG was isolated from rabbit sera by affinity chromatography. Firstly, the prepared medium of Sepharose 4B was resolved, degassed and washed by 0.01 mmol L^{-1} HCl. Five milligrams of purified bovine β-Lg was coupled to 2 mL of CNBr-activated Sepharose 4B followed by packing it into a 5 mL syringe. The packed syringe was then pre-equilibrated with 10 mL of PBS at room temperature. Then, 2 mL of rabbit sera was loaded onto the column, and incubated for 20 min at room temperature. After washing with 10 columns of PBS, the bound antibodies were eluted with 3 mol L^{-1} $MgCl_2$ (pH 7.4, adjusted with 1 mol L^{-1} Tris). Finally, the purity and specificity of collected antibody were carried out by SDS-PAGE and indirect ELISA, respectively.

SPOT™ peptide arrays assay

'SPOT synthesis' is an easy and very flexible technique for simultaneous parallel chemical synthesis on membrane supports. The peptides are covalently bound to a Whatman 50 cellulose support by the C-terminus and usually have acetylated N-terminus and those solid phase-bound peptides are used for binding studies directly on the membrane. Peptides spanning six mimic epitope regions in AA14–20, AA26–32, AA36–42, AA70–80, AA82–86 and AA149–156 on the basis of previous study[8] were synthesised as a series of decapeptides overlapping by four amino acids on derivatised cellulose membranes. Single glycine was also synthesised on the membrane as a negative control.

To prevent precipitation of hydrophobic peptides during the following TBS washing procedure, the membrane containing the synthesised peptides was rinsed with a small volume of ethanol for 5 min. Then the membrane was washed three times in TBS for 10 min and blocked with 3% porcine skin gelatin with TBS for 1 h at 37 °C. After washing with TBS-T (TBS with 0.15% Tween-20) three times, the membrane was incubated for 3 h at 37 °C with 4 μg mL^{-1} of individual rabbit serum. The incubation was followed by a 10 min wash in TBS-T for three times. After that, the membrane was incubated with sheep anti-rabbit IgG conjugate with horseradish peroxidase diluted to 1:5000 in a mixture of TBS for 1 h at 37 °C. The bound antibody was subsequently detected with a chemiluminescent detection system.

Additionally, the membrane could be regenerated according to the manufacturer's instructions. It was washed with regeneration buffer (62.5 mmol L^{-1} Tris, 2% sodium dodecyl sulfate, pH 6.7 with 0.7 mL β-mercaptoethanol per 10 mL sodium dodecyl sulfate) for an incubation of 30 min at 50 °C, followed by washing with TBS and TBST, and then it could be dried for storing at −20 °C.

Epitope mapping by phage display

Panning procedures

A solution of 100 μg mL^{-1} of purified antibody was dissolved into 0.1 mol L^{-1} $NaHCO_3$ (pH 8.6) and coated in one well of a microplate overnight at 4 °C, followed by blocking with 3% bovine serum albumin in 0.1 mol L^{-1} TBS for 1 h at 37 °C. After that, the coated well was incubated for 1 h at 37 °C either with the original phage library (PhD-C7C) or the amplified one from the previous round diluted in 100 μL of TBST. Then the bound phages were eluted with the glycine-HCl buffer (pH 2.2) for 10 min, followed by neutralisation with Tris–HCl (pH 9.1) immediately. Five microlitres of eluted phages were serially diluted in LB media, followed by mixing with top agar, and then it was poured into Luria broth plates. The plates were incubated at 37 °C overnight, followed by counting the number of phage plaques for calculating the initial phage titre, while others were amplified in the solution with the mid-log phase of *Escherichia coli* ER2738 which is used for the next round of biopanning.

Phage ELISA

Random individual bacterial colonies (no more than 100) from the third round of panning were selected for inoculating into the medium containing the ER2738 in the log phase for 4.5 h at 37 °C. Then the medium was centrifuged, and the supernatant containing the phages was collected for screening the positive clones. The protocol of ELISA is similar to inhibition ELISA. It is should be noted that the aliquot with random picked clones should be incubated in another microplate for 15 min at 37 °C in order to remove the unspecific absorption. The optical density was detected at 490 nm using a Bio-Rad Microplate Reader (Bio-Rad model 600; Bio-Rad, Berkeley, CA, USA).

DNA sequencing

Phage DNA was extracted by a simple step with the protocol from Heptapeptide library (PhD-C7C). The solution of PEG/NaCl was used for precipitating the phage and iodide buffer/ethanol was to obtain phage DNA. DNA sequencing was carried out by Sangon Co. Shanghai, China.

Identification of conformational epitopes on bovine β-lactoglobulin by the Mapitope algorithm

The Pepitope server (http://pepitope.tau.ac.il/) can be used to computationally predict epitopes based on mimotopes derived

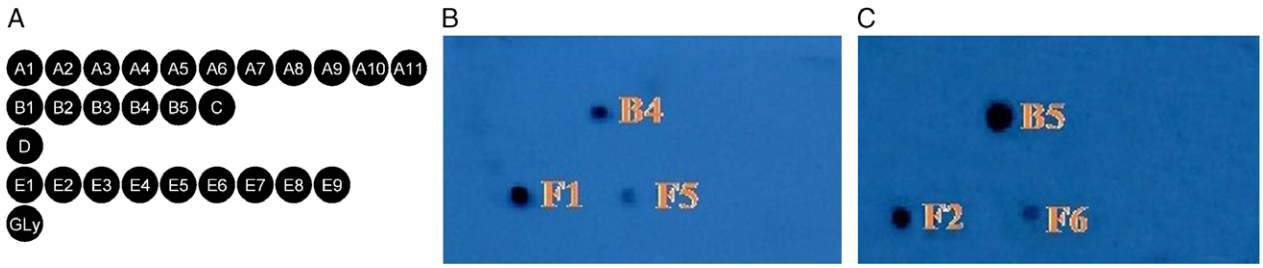

Figure 1. Spots for IgG binding epitopes on bovine β-lactoglobulin. (A) Arrangement of the peptides in Table 1; (B) spots binding with specific sera from rabbit 1; (C) spots binding with specific sera from rabbit 2.

from a phage display library.[9] Pepitope assumes that the peptides mimic surface residues (i.e. solvent-exposed residues). Thus, buried residues are eliminated from the search. The exposed residues of the given structure are extracted using the Surface Racer program.[10]

The server implements three epitope mapping algorithms and Mapitope were chosen in our work. The Mapitope algorithm is based on the underlying assumption that the entire set of peptides is enriched with amino acid pairs which mimic the epitope. Thus, Mapitope first identifies pairs of residues that are significantly over-represented in the panel of peptides, compared to their expected frequencies. Then it searches for patches on the surface that are enriched with these pairs. The resulting patches are presented on the three-dimensional protein structure. The user can specify a number of optional parameters: a distance threshold defining whether two surface residues are neighbours on the three-dimensional structure, a statistical threshold defining to which extent a pair of residue has to be enriched in the panel in order to be considered a significant pair, and a maximum gap 'fill-in' value, which is needed in the last step of the clustering to define which resides are considered part of the predicted patch.

RESULTS

Linear epitope of bovine β-lactoglobulin

Figure 1 shows a dot blotting profile and binding capacity of specific antibody from two rabbit sera. Negative sera did not bind to any peptides (data not shown) and no binding appeared in the negative control spot of a single Gly in SPOT™ peptide arrays assay. There are three peptides bound by the specific sera from two rabbits. The positions and sequences of reactive peptides are listed in Table 1. The positions of linear epitopes on the structure of β-Lg are shown in Fig. 2 and Fig. 3, respectively. From Table 1, we could find that the sequence of three peptides overlapped, which was recognised by specific sera from two rabbits, and the common peptide sequences were KIPAVF (AA77–82), PEVDNE (AA126–131) and ALPMHI (AA142– 147).

Table 1. Reactive peptides binding with sera from rabbits*

Name of peptide	Specific for	Position of peptide	Amino acid sequence
B4	R1	73–82	AEKTKIPAVF
B5	R2	77–86	KIPAVFKIDA
F1	R1	122–131	LVRTPEVDNE
F2	R2	126–135	PEVDNEALEK
F5	R1	138–147	KALKALPMHI
F6	R2	142–151	ALPMHIRLAF

*R1, rabbit 1; R2, rabbit 2.
Highlighted amino acids of were common residues for linear epitopes between two rabbits.

Conformational epitope of bovine β-lactoglobulin

Mimotope biopanning of the phage random library

After three rounds of biopanning, specific phages were enriched by eluting from phage random library and the phages with mimotopes for bovine β-Lg were obtained. A total of 60 random clones were identified by phage ELISA, and then 19 and 17 of phage clones were positive, respectively, which is specific for sera from rabbit 1 and 2 (R1 and R2). The deduced amino acid sequences of the mimotopes are displayed in Table 2 and Table 3, respectively. Moreover, the amino acid sequences of seven positive clones derived from the biopanning against serum of R1 were identical, while no similarities were found in the clones of R2.

Epitope match of conformational epitope by the Pepitope server

Mimotope sequences from Table 2 and Table 3 with the PDB file named as 2BLG (GI: 4388947) were put into the Pepitope server, respectively. Thirty-eight items of structure for bovine β-Lg were obtained from NCBI and 11 items of them are single protein by X-ray diffraction and 2BLG with high resolution of 2.56 Å was chosen as the model of molecular structure.

The position and composition of conformational epitopes specific for each rabbit were identified with Mapitope algorithm, as shown in Table 4. We found that the length of conformational epitopes was different from 7 to 22 and the common residues between two sera existed as well. Seven common residues in C-Epi1-1 and C-Epi2-2 were in the N-terminal, being T6, M7, K8, G9, L10, D11 and I12, which were continuous in the sequence. While another eight residues (C66–A67–Q68–F151–N152–Q159–H161–I162) identified in C-Epi1-2 also appeared in C-Epi2-1, in which two residues Q155–E158 has inserted between C66–A67–Q68–F151–N152 and Q159–H161–I162. Both the C-Epi1-3 and C-Epi2-3 were derived directly from positive clone sequences, being ARLHTSS and MTSVMMC. Therefore their length is only seven and both of them might be a part of the conformational epitopes. In addition, there were no common residues for them.

Molecular graphics of conformational epitope on bovine β-lactoglobulin

Conformational epitopes were modelled by PyMOL as shown in Fig. 4 and Fig. 5. There were three conformational epitopes

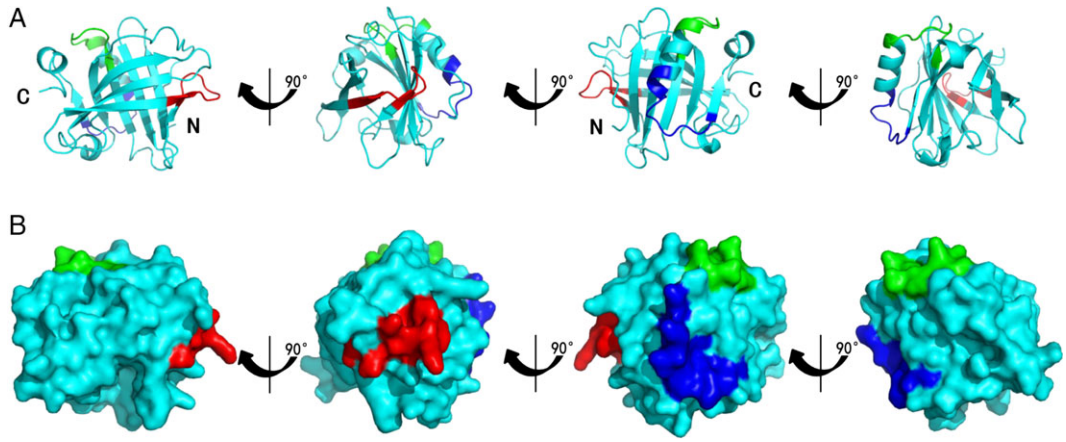

Figure 2. Location of linear epitopes specific for sera from rabbit 1 on the structure of BLG. (A) Ribbon diagrams; (B) surface. B4 marked in red; F1 marked in green; F5 marked in blue.

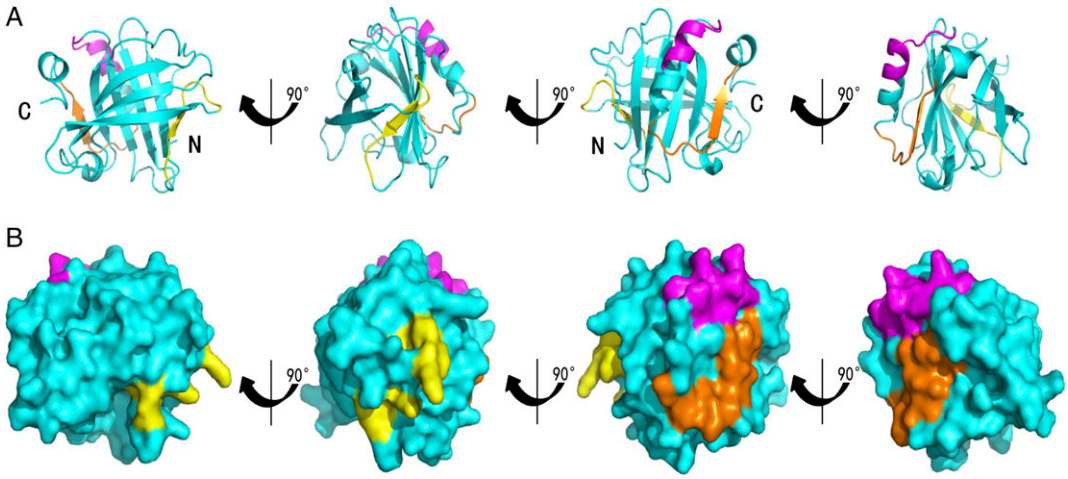

Figure 3. Location of linear epitopes specific for sera from rabbit 2 on the structure of BLG. (A) Ribbon diagrams; (B) surface. B5 marked in yellow; F2 marked in magenta; F6 marked in orange.

specific for each rabbit as well. From the ribbon diagram, we can find that most residues of conformational epitopes were located in the β-sheet and random coil, while only some residues of C-Epi1-2 and C-Epi2-1 appeared in the pattern of α-helix. The secondary structure of common residues was located in the region of the N-terminal (T6–M7–K8–G9–L10–D11–I12), C-terminal (F151–N152–Q159–H161–I162) and the middle (C66–A67–Q68) and most of them were located in the region of α-helix and some were a β-sheet.

DISCUSSION

Principally, allergenic epitopes are divided in two main classes, namely (1) conformational epitopes, which are expected to be

Table 2. Sequences of positive clones obtained by screening phage library (PhD-C7C) with sera from rabbit 1

Original nucleotide sequence	Complementary nucleotide sequence	Amino acid sequence	No. of clones
GCACTTCCCAAAAGGACCATT	AATGGTCCTTTTGGGAAGTGC	NGPFGKC	1
GCAATAATGCTTATAATCCAA	TTGGATTATAAGCATTATTGC	LDYKHYC	1
GCAAGGCTTCAAATGATAATC	GATTATCATTTGAAGCCTTGC	DYHLKPC	1
GCACGTCTGCACACAAGCAGC	GCTGCTTGTGTGCAGACGTGC	AACVQTC	1
GCAAAACACCCTCGGAATCTT	AAGATTCCGAGGGTGTTTTGC	KIPRVFC	1
GCACGTCTGCACACAAGCAGC	GCTGCTTGTGTGCAGACGTGC	ARLHTSS	7
GCACATAATCTTAGTCATAGA	TCTATGACTAAGATTATGTGC	SMTKIMC	5
GCAAGGCTTACTCACACTCAT	ATGAGTGTGAGTAAGCCTTGC	MSVSKPC	1
GCACAACACCGCACGCACCTT	AAGGTGCGTGCGGTGTTGTGC	AQHRTHL	1

Table 3. Sequences of positive clones obtained by screening phage library (PhD-C7C) with sera from rabbit 2

Original nucleotide sequence	Complementary nucleotide sequence	Amino acid sequence	No. of clones
GAAAGGTACCACTAAAGGAAT	ATTCCTTTAGTGGTACCTTTC	IPLVVPF	2
GCAATTCCGCCCATGCTCACT	AGTGAGCATGGGCGGAATTGC	SEHGRNC	1
GCAACCATTCTGCTCCATCCA	TGGATGGAGCAGAATGGTTGC	WMEQNGC	1
GCACCGCACAGGCTTAACACT	AGTGTTAAGCCTGTGCGGTGC	SVKPVRC	1
GCAATACAGCCAATCAATCCC	GGGATTGATTGGCTGTATTGC	GIDWLYC	1
GCAATACAGAGGATGCGAAAA	TTTTCGCATCCTCTGTATTGC	FSHPLYC	1
GCACGCCGGACCCCTCTGCGT	ACGCAGAGGGGTCCGGCGTGC	TQRGPAC	1
GCACATCATCACCGACGTCAT	ATGACGTCGGTGATGATGTGC	MTSVMMC	1
GCACGTCTGCACACAAGCAGC	GCTGCTTGTGTGCAGACGTGC	AACVQTC	2
GCAAAAAACAGCCGGAATCTT	AAGATTCCGGCTGTTTTTTGC	KIPAVFC	2
GCACATAATCTTAGTCATAGA	TCTATGACTAAGATTATGTGC	SMTKIMC	3
GCAAGGCTTCAAATGATAATC	GATTATCATTTGAAGCCTTGC	DYHLKPC	1

Table 4. Position and residues of conformational epitopes on bovine β-lactoglobulin calculated by Pepitope server*

Epitope	Corresponding mimotopes	Positions and residues of conformational epitopes	No. of amino acids
Rabbit 1			
C-Epi1-1	LDYKHYC KIPRVFC SMTKIMC MSVSKPC AQHRTHL SMTKIMC	T6-M7-K8-G9-L10-D11-I12-Q13-K14-A16-G17-T18-Y20-E44-L57-T76-**K77-I78-P79-V81**-Y99-R124	22
C-Epi1-2	NGPFGKC DYHLKPC AACVQTC	S30-D33-P38-K60-C66-A67-Q68-F151-N152-Q159-H161-I162	12
C-Epi1-3	ARLHTSS	L31-A34-R40-N109-S110-Q115-S116	7
Rabbit 2			
C-Epi2-1	SEHGRNC WMEQNGC SVKPVRC FSHPLYC TQRGPAC AACVQTC KIPAVFC DYHLKPC	A34-Q35-S36-P38-L39-R40-V41-K60-W61-C66-A67-Q68-F151-N152-Q155-E158-Q159-H161-I162	19
C-Epi2-2	IPLVVPF GIDWLYC SMTKIMC	T6-M7-K8-G9-L10-D11-I12-P48-**I78-P79**-Y99	11
C-Epi2-3	MTSVMMC	D28-I29-S30-L31-**M145-H146-I147**	7

*Distance threshold value is 9.0; statistical threshold value is 3.0; maximum gap 'fill-in' is 3.
Highlighted amino acids of C-Epi1-1/C-Epi2-2 and C-Epi2-1/C-Epi1-2 were common residues.
Underlined amino acids were common residues for linear and conformational epitopes.

more susceptible to processing-induced changes; and (2) linear epitopes, which are likely to be more resistant to physical treatments and to be deactivated only by hydrolysis.[11,12] The allergic reaction has two stages: sensitisation and challenge. In the first stage, food allergens are digested into small peptides which make a person sensitised. After that, if a person takes the same protein, whether it is the small peptide or whole protein, an allergic reaction may be triggered. This procedure is named the 'challenge'. In general, linear epitopes mainly contribute to the first stage during digestion in the gastro-intestinal tract, and both the conformational and linear epitopes play an important role in the second stage. Clinically, insight into the IgE-binding epitopes of allergens, linear or conformational, is extremely useful.[13] In the respect to the theory that the linear epitopes are part of conformational epitopes, we identified three linear and conformational epitopes, respectively. More interestingly, common residues have been found between the two specific sera.

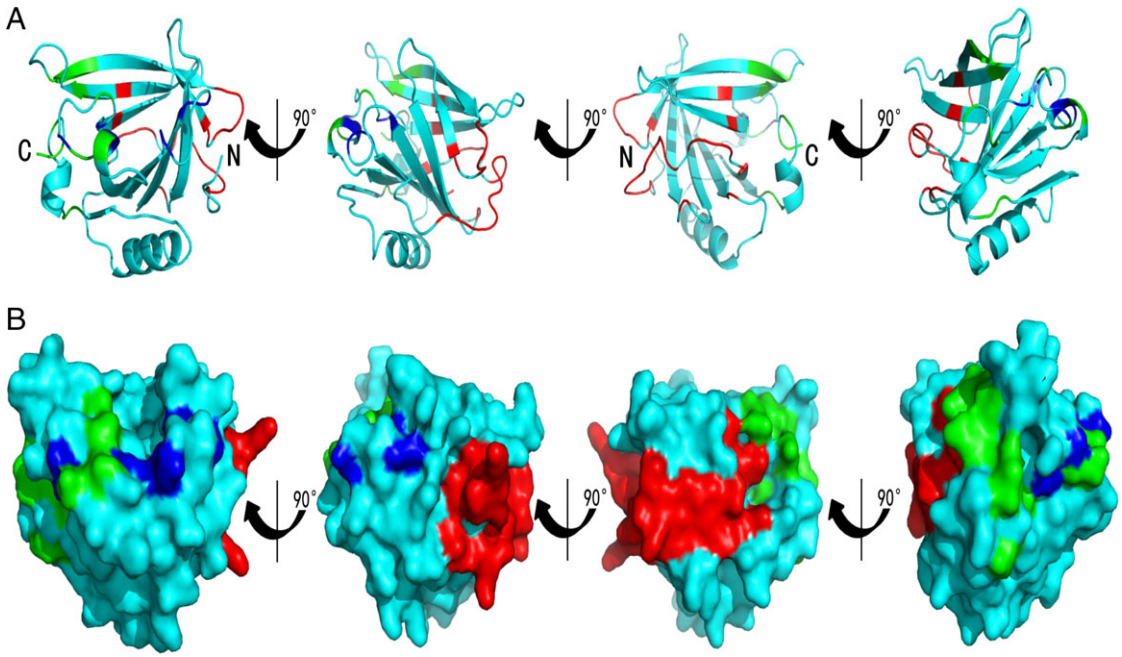

Figure 4. Conformational epitopes specific for sera from rabbit 1. (A) Ribbon diagrams; (B) surface. Epi1-1 marked in red; Epi1-2 marked in green; Epi1-3 marked in blue.

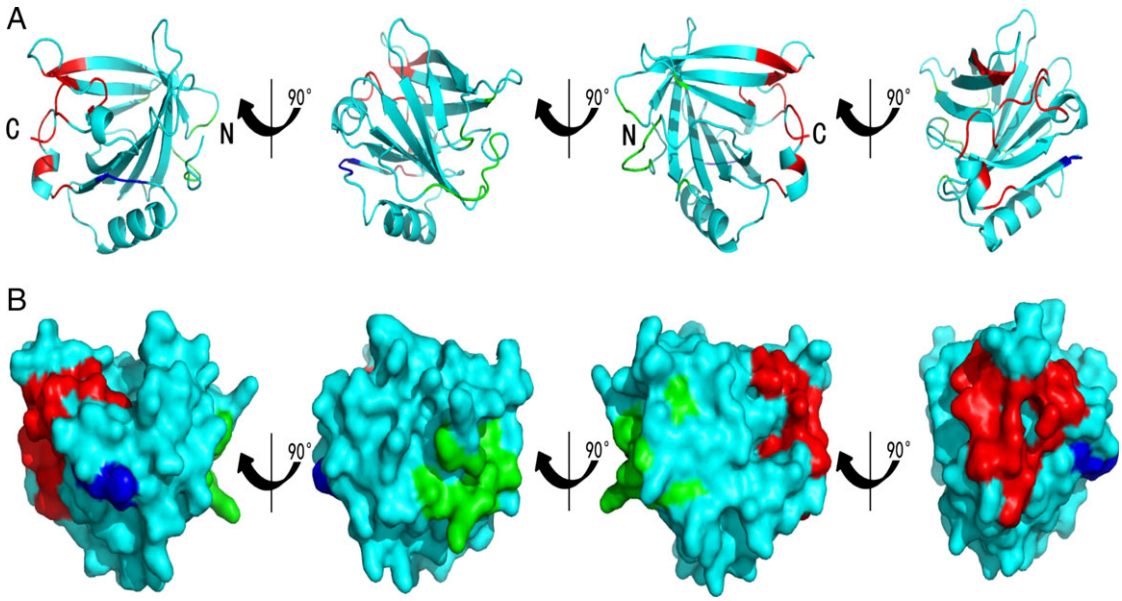

Figure 5. Conformational epitopes specific for sera from rabbit 2. (A) Ribbon diagrams; (B) surface. Epi2-1 marked in red; Epi2-2 marked in green; Epi3-3 marked in blue.

For linear epitopes, it has recently been reported that the length of epitopes was between five and ten.[14] The length of synthetic peptides in our work was decapeptide and that of the common sequence is hexapeptide. Therefore, both items of data were consistent with the theoretical number of linear epitopes. Three common sequences on linear epitopes were recognised by both of specific sera, which might prove that these sequences are dominant epitopes. Moreover, the composition of these amino acids were similar, especially for AA77–82 (KIPAVF) and AA142–147 (ALPMHI), both of which were comprised of six hydrophobic and one charged amino acids, while the one remaining amino acid was flexible. Based on analysis of the common residues of linear epitopes, we further found that hydrophobic amino acids account for 67% of linear epitope, which occupied a dominant percentage of composition on linear epitopes.

In our previous study, eight linear epitopes and 18 critical amino acids of buffalo β-Lg were determined by SPOT™ assay.[8,15] We found two of linear epitopes (B4 and B5) and four residues of critical amino acids (Val81, Phe82, Ala142 and Leu143) on linear epitopes in the previous study and these are displayed in Table 1.[15] Moreover, one epitope region derived from phage display is similar to the epitope AA77–82.[8] In Cong's study, one of four IgE-binding epitopes (AA72–86) was identified, which overlapped with the

same epitope (AA77–82) in the present study, which demonstrated IgG and IgE binding have the similar epitope sequences.[14] These studies also indicated that the epitope AA77–82 was a dominant one. Compared with the previous study, linear epitopes on buffalo and bovine β-Lg are overlapped slightly, although there are just two amino acids different in the N- and C-terminal, which should be explored in further.

As we know, conformational epitopes play a major role in food allergy because they account for 90%[7] but it is difficult to explore with flexible conformation of allergen. In our work, we found that three conformational epitopes have been mapped for each serum, respectively. Consistent with the theory, some continuous epitopes were component of conformational epitopes, as shown in Table 4. Seven continuous residues, T6–M7–K8–G9–L10–D11–I12, were located in two conformational epitopes of C-Epi1-1 and C-Epi2-2; these residues mainly appeared in the form of an α-helix, while some two or three sequential amino acids such as H161–I162 and C66–A67–Q68 were common residues in C-Epi2-1 and C-Epi1-2. Compared with linear and conformational epitopes, seven common residues of linear epitopes appeared in only three conformational epitiopes, C-Epi1-2 C-Epi1-1 C-Epi2-2 and C-Epi2-3 and only two residues of three common peptides on linear epitopes were found in conformational epitopes as shown in Table 4.

Conformational epitopes should be induced by bioinformatics tools. In our previous study, we introduced the web tool of MIMOX, which was designed for linear and conformational epitopes based on the data of mimotope from the phage random library.[16,17] However, MIMOX uses one peptide sequence (or a consensus peptide sequence) at a time, as reported in the study by Negi and Braun.[18] The Peptiope server with the Mapitope algorithm is in terms of physico-chemical properties and spatial organisation.

There are many studies on the role of IgG in blocking antibody, although IgG is not the specific antibody in most allergic reactions. As early as 1998, IgG antibodies from allergic patients binding to BLG was found to be a different form to that of control sera.[19] Recently, IgG Abs induced with PreS-2XP4P5 which was a non-allergenic peptide from the C-terminal IgE epitope-containing part of Der p 23 inhibited Der p 23-induced basophil activation.[20] The mimotope-induced IgG antibodies are then directed not only against the mimotopes, but co-recognise the three-dimensional allergen epitope via molecular mimicry. Therefore, they are able to prevent the high-affinity interaction between allergen and specific IgE.[21] The high level of IgG sub-class was tested in the early childhood although it decreased at the age of 8 years.[22] Rabbit IgG antibodies induced by Dpg-Pol effectively inhibit human IgE binding to allergens which may be helpful for understanding the mechanism of action of specific allergen immunotherapy.[23] However, many studies on the relationship between IgG and irritable bowel syndrome have been investigated in recent years. Therefore, the role of IgG in food allergy and food intolerance should receive more attention and need further investigation.

CONCLUSION

Linear and conformational epitopes were two important forms which react with antibody when food allergies occur. In our study, it is the first time that common residues have been discovered in the linear and conformational epitopes among the different sera, which could be regarded as informative residues for detection of allergen in dairy products and production of hypoallergenic dairy foods.

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