

Identification of Conformational Antigenic Epitopes and Dominant Amino Acids of Buffalo β -Lactoglobulin

Li Xin, Gao Jinyan, He Shengfa, Wu Yuanyuan, and Chen Hongbing

Abstract: Major allergen β -lactoglobulin exists in many mammalian types of milk except human breast. Buffalo milk also contains this major allergen but the detailed information on its epitopes is not available. The aim of this work was to map and characterize its conformational antigenic epitopes. Sixty mimotopes of buffalo β -lactoglobulin were produced by biopanning of phage display peptide library and then 2 mimotopes, specific for sera from rabbit 1 and 2, respectively, were predicted to be conformational epitope candidates by the use of DNASTar and web tool of MIMOX. On the basis of bioinformation analysis, 5 conserved amino acid residues PL-ENK were identified in 2 conformational epitope sequences and 7 conformational epitopes were derived from 2 mimotopes by molecular modeling. The result showed that these conformational epitopes were located in the 2 regions on buffalo β -lactoglobulin and composed of 5 hydrophilic and 2 hydrophobic amino acids.

Keywords: antigenicity, buffalo β -lactoglobulin, conformational epitope, phage display technique, MIMOX

Practical Application: It is the first time to define conformational epitopes binding to buffalo β -lactoglobulin and a simple and operable method has been established for identification of conformational epitope on food allergens.

Introduction

Epitope is a specific region on the surface of an antigen and recognized by a specific antibody. In general, there are 2 types of epitopes categorized as linear or continuous, and conformational or discontinuous. So far, many sequences and linear epitopes of allergenic proteins have been identified and archived in databases. However, structural and physicochemical discriminators that define their specific properties are lacking. Regarding to conformational epitopes, it was first explored in 1986, in which 2 distinct epitopes on horse cytochrome c were characterized (Jemmerson and Paterson 1986). Recently, it has been reported that 90% of epitopes on antigen are conformational and their binding to antibody depends on the epitope structure (Van Regenmortel 2009).

β -Lactoglobulin, one of the major allergens in milk, exists in many mammalian types of milk. The globular protein is one main composition of whey fraction, comprising 10% of total milk proteins. It belongs to the lipocalin family, in which there are many members identified as allergens (Zeiler and others 1999; Mäntyjärvi and others 2000; Saarelainen and others 2008). It has been reported that cow's milk-protein allergy likely affects range from 2% to 7% (Turck 2013) and 82% of cow's milk-allergic patients are sensitive to β -lactoglobulin (Aoki and others 2006). β -Lactoglobulin is a polypeptide which usually exists as a dimer with

Cys-Cys linkages, resulting in being resistant to acid digestion, and enabling primary structure of some proteins to remain intact after passing through the stomach (Creamer and others 2004; Wada and others 2006). Moreover, the pioneered researches have suggested that both linear and conformational epitopes can play an important role in the allergenicity of bovine β -lactoglobulin (Kaminogawa and others 1989; Takahashi and others 1990). In previous work, linear B-cell epitopes on bovine β -lactoglobulin had been identified by many groups and some sequential epitopes had turned out to be located on the surface of the molecule (Ball and others 1994; Williams and others 1997; Williams and others 1998; Selo and others 1999; Järvinen and others 2001; Fritsché and others 2005). However, very limited information on conformational epitopes on β -lactoglobulin is available.

Buffalo milk is the second largest milk supply in the world after cow milk, representing more than 12% of total milk production (Li and others 2008; Bonfatti and others 2013). Buffalo β -lactoglobulin is homologous to variant B from bovine β -lactoglobulin but differs in 2 residues Leu1Ile and Ile162Val (Bolognesi and others 1979). In our previous study, cross-reactivity ratio with 69.7% has been evaluated between purified buffalo and standard bovine β -lactoglobulin. In this work, purified rabbits IgG specific for buffalo β -lactoglobulin was used to screen mimotopes, those represented mimic IgG epitopes on buffalo β -lactoglobulin, by the biopanning of the disulfide constrained heptapeptide phage display library. On the basis of mimotopes, conformational epitopes were defined using Web service of MIMOX.

Materials and Methods

Materials

Buffalo β -lactoglobulin was prepared according to the protocol in our previous work (Li and others 2008). Heptapeptide library (PhD-C7C) was purchased from New England

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Biolabs (Beverly, Mass., U.S.A.). HRP/Anti-M13 Monoclonal Conjugate and CNBr-activated Sepharose 4B were from GE (Sch-enectady, N.Y., U.S.A.). All the other chemicals were bought from Sangon Co., Shanghai, China.

Immunization of rabbits

Eight-week-old Japanese white male rabbits were from Institute of Occupational Medicine of Jiangxi (permission number SYXK (Gan) 2009-003). A 4 mL of 1:1 solution of purified buffalo β -lactoglobulin (2 mg/mL in PBS, pH 6.8) and Freund's complete adjuvant was used for the first injection. Then, each rabbit was injected for 3 times with a booster dose of 1 mL β -lactoglobulin (2 mg/mL) in suspension with 1 mL Freund's incomplete adjuvant on day 14, 28, and 42, respectively. One week after the last injection, rabbits were bled, and each rabbit's antiserum was collected by centrifugation at $5000 \times g$ for 10 min. The titers of antibodies were determined by indirect ELISA.

Purification of polyclonal antibodies against buffalo β -lactoglobulin

The purification was performed by affinity chromatography. Before packing Sepharose 4B, the prepared medium and solutions were degassed slurries. CNBr-activated Sepharose 4B was washed by 0.01 mmol/L HCl, and purified buffalo β -lactoglobulin was coupled to the CNBr-activated Sepharose 4B according to the manufacturer's instruction (GE). Then, 2 mL of the CNBr-activated Sepharose 4B coupled to 10 mg of purified buffalo β -

lactoglobulin was packed into a 5 mL syringe, which had been pre-equilibrated with 10 mL of PBS at room temperature. The anti- β -lactoglobulin rabbit serum (2 mL) was loaded onto the column, followed by incubation for 20 min at room temperature. The column medium was washed with 20 mL of 0.01 mol/L PBS (pH 7.4). The anti- β -lactoglobulin antibody was eluted with 3 mol/L MgCl_2 (pH 7.4, adjusted with 1 mol/L Tris), followed by assaying its purity and specificity by SDS-PAGE and ELISA, respectively.

A general regeneration protocol of Sepharose 4B is described as below. An affinity medium was washed with 2 to 3 column volumes of alternating buffers of high pH (0.1 mol/L Tris-HCl, 0.5 mol/L NaCl, pH 8.5) and low pH (0.1 mol/L NaAc, 0.5 mol/L NaCl, pH 4.5). The cycle was repeated for 3 times, followed by equilibration with the binding buffer.

Epitope mapping by phage display

Panning procedures. A solution of 100 $\mu\text{g/mL}$ of purified antibody was dissolved into 0.1 mol/L NaHCO_3 (pH 8.6) and coated in one well of a microplate overnight at 4 °C followed by blocking with 3% BSA in 0.1 mol/L TBS (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) for 1 h at 37 °C. After washing with TBS containing 0.1% Tween 20 (TBST) for 3 times, coated well was incubated for 1 h at 37 °C either with the original phage library or the amplified one from the previous round diluted in 100 μL of TBST (Figure 1). Then the unbound phages were removed by washing with TBST, and the bound phages were eluted with

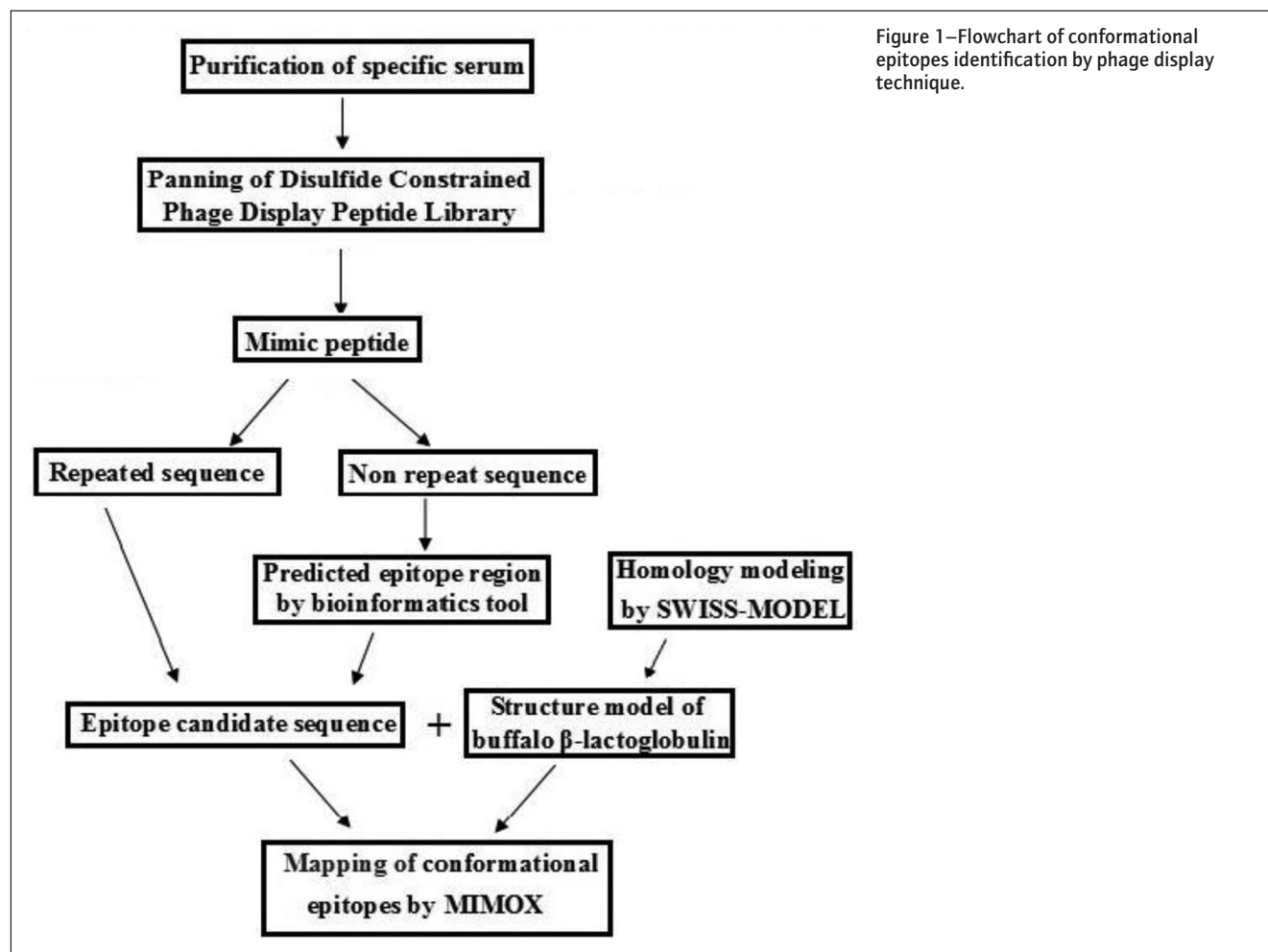


Figure 1—Flowchart of conformational epitopes identification by phage display technique.

100 μ L of 0.2 mol/L Glycine-HCl (pH 2.2) for 10 min with gentle agitation, followed by neutralization with 15 μ L of 1 mol/L Tris-HCl (pH 9.1) immediately. The eluted phages were kept at 4 °C until further use as described below.

Measurements of phage titer were carried out following plaque assay method, described briefly as follows. *Escherichia coli* ER2738 was grown to mid-log phase, and the aliquots inoculated with phage preparations were serially diluted in LB media, followed by mixing with top agar, and then it was poured into LB plates. The plates were incubated at 37 °C overnight, followed by counting the number of phage plaques for calculating the initial phage titer.

The remaining aliquot was used to infect *E. coli* ER2738 cells for phage amplification. *E. coli* ER2738 bacterial cells in log phase were infected with the bound phage, followed by incubation for 4.5 h, and the collected bacterial culture was centrifuged twice for 10 min at $6527 \times g$ at 4 °C. The phages were then precipitated from the supernatant using 25% PEG 8000 and 2.5 mol/L NaCl for 10 min at 4 °C followed by collecting by centrifugation for 15 min at $6527 \times g$, and the phages pellet was resolved into 1 mL TBS and precipitated again. The collected phage pellet was resuspended in 200 μ L of TBS for the further bio-panning.

Phage ELISA. The output from the third round of panning was plated out on LB/IPTG/Xgal agar, followed by incubation overnight at 37 °C, and random individual bacterial colonies (no more than 100) were selected for inoculating into the medium containing the ER2738 in log phase for 4.5 h at 37 °C. Then the medium was centrifuged, and the supernatant containing the phages was collected for titer test.

ELISA plates were coated with 100 μ L of 1 mg/mL purified antibody against buffalo β -lactoglobulin overnight at 4 °C, followed by washing 3 times with 0.05% (v/v) Tween-20 in PBS,

and then blocking with 3% nonfat milk powder in PBS for 1 h at 37 °C. At the same time, the aliquot with random picked clones in different dilutions with blocking buffer were incubated in another microplate for 15 min at 37 °C to remove the un-specific absorption, and it was then transferred to the coated microplate, incubated for 1 h at 37 °C. The microplate was washed with TBST again, and 100 μ L of HRP-conjugated anti-M13 monoclonal antibody diluted 1:5000 with TBST was added to each well, followed by incubation for 1 h at 37 °C. After a further washing with TBST for 3 times, 100 μ L of O-phenylene diamine (OPD, 4 mg/mL) in citrate buffer was then added to each well for color development at 37 °C for 15 min. The color reaction was stopped with 50 μ L H_2SO_4 and the optical density was detected at 490 nm using a Bio-Rad Microplate Reader (Bio-Rad model 680, Calif., U.S.A.).

DNA sequencing. Bacterial cultures (2 mL) infected with positive phage clones was amplified for 4.5 h, followed by centrifugation for 1 min at $6527 \times g$. A total of 500 μ L of the phage containing supernatant was transferred to another tube, followed by addition of 200 μ L PEG/NaCl to precipitate the phage. The incubation lasted for 10 min at room temperature, followed by collecting the phage through centrifugation for 10 min at $6527 \times g$. The phage pellet was dissolved in 100 μ L iodide buffer and 250 μ L ethanol, followed by incubation on ice for 10 min, and the pellet was collected by centrifugation and washed with 70% ethanol again. Finally, the phage pellet was suspended in 30 μ L of TE buffer for DNA sequencing. The sequencing primer (-96 gIII: 5'-HOCCC TCA TAG TTA GCG TAA CG-3') has been used for automatic sequencing, which is carried out by Sangon Co. Shanghai, China. The primers hybridize downstream of the insert and the sequence being read corresponds to the anticodon strand of the template. The complementary strand has

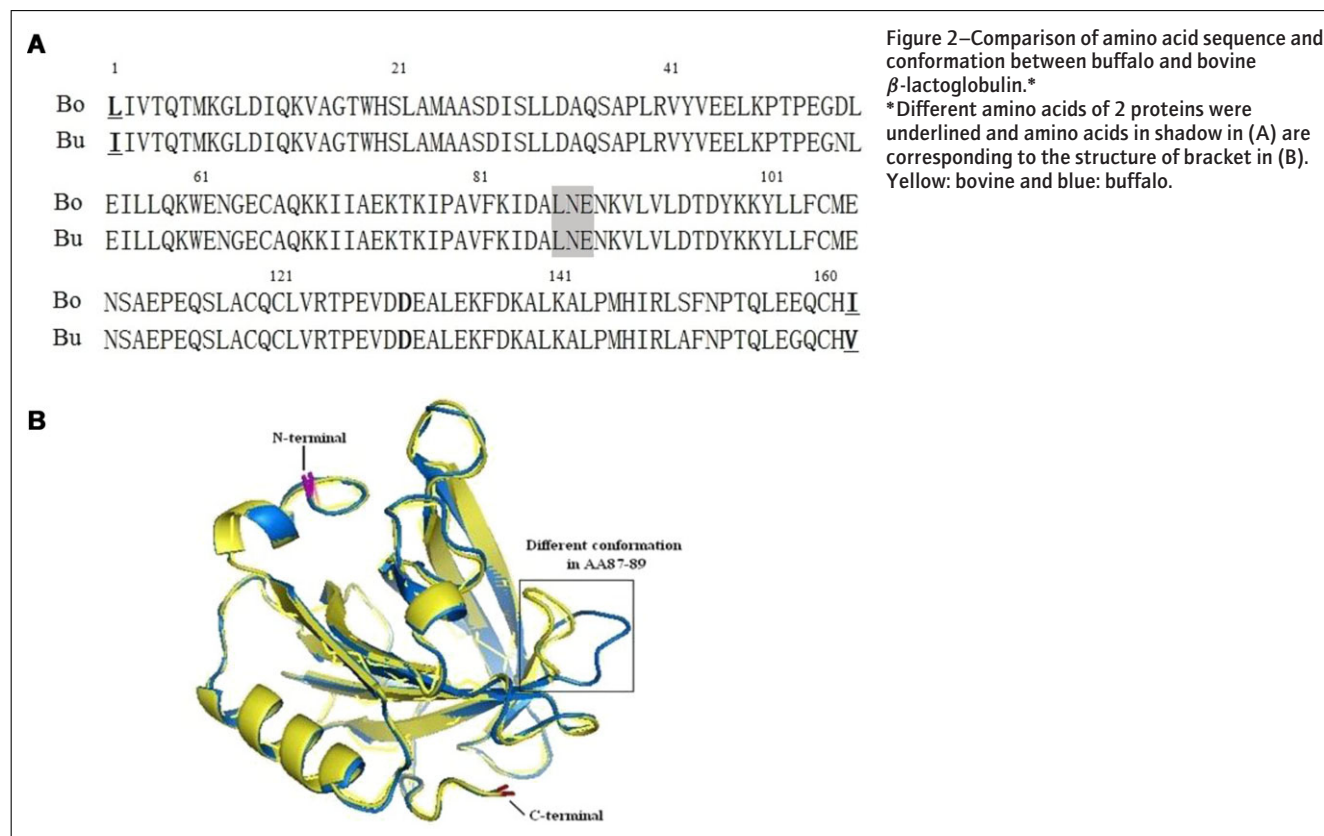


Table 1–DNA sequences of positive clones derived from biopanning against sera from rabbit 1.

Original nucleotide sequence	Complementary nucleotide sequence	Deduced amino acid sequence	Nr. of Clones
CTT ATT CTC ATT CAA CGC CGT	ACG GCG TTG AAT GAG AAT AAG	T A L N E N K	1
CTT ATT CTC CGT AAG CGG CGA	TCG CCG CTT ACG GAG AAT AAG	S P L T E N K	7
CAT CAG CGA CGA CTT CTG ACT	AGT CAG AAG TCG TCG CTG ATG	S Q K S S L M	1
CGT ACC CAC CGA CGA CGA AGA	TCT TCG TCG TCG GTG GGT ACG	S S S S V G T	1
CGC ATT CGG CTT CTT CGG AAA	TTT CCG AAG AAG CCG AAT GCG	F P K K P N A	1
CTG CGG CTG AGT CGA CTT CGG	CCG AAG TCG ACT CAG CCG CAG	P K S T Q P Q	1
CTG CGA ACG ATC CAT ATA CTG	CAG TAT ATG GAT CGT TCG CAG	Q Y M D R S Q	1
ATC CGT CAC AAA CGC CCG ATT	AAT CGG GCG TTT GTG ACG GAT	N R A F V T D	1
ATC CTC ATT CCC CCG ATG AGG	CCT CAT CGG GGG AAT GAG GAT	P H R G N E D	1

Table 2–DNA sequences of positive clones derived from biopanning against sera from rabbit 2.

Original nucleotide sequence	Complementary nucleotide sequence	Deduced amino acid sequence	Nr. of Clones
CTT ATT CTC CTC CAG CGC ACC	GGT GCG CTG GAG GAG AAT AAG	G A L E E N K	1
CCG ATT CTC CTG ATA CGG ACT	AGT CCG TAT CAG GAG AAT CGG	S P Y Q E N R	1
ATC CTT ATT CTC ATT CAG CGG	CCG CTG AAT GAG AAT AAG GAT	P L N E N K D	1
CTC CCG ATT CTC ATT CGT AGG	CCT ACG AAT GAG AAT CGG GAG	P T N E N R E	1
CTT AGG ACT CTG ACC AGC AAC	GTT GCT GGT CAG AGT CCT AAT	V A G Q S P K	1
ATT CGC AAG CGG CGC ATG CGT	ACG CAT GCG CCG CTT GCG AAT	T H A P L A N	1
CTT ATT CTC AGA AAG AGC ATT	AAT GCT CTT TCT GAG AAT AAG	N A L S E N K	1
CTT ATT CTC CGA CCC AGG ATT	AAT CCT GGG TCG GAG AAT AAG	N P G S E N K	1
CCT ATT CTC AAG CAA CGG ACT	AGT CCG TTG CTT GAG AAT AGG	S P L L E N R	1
CGC AGC AGA CCG AGG AAA AGG	CCT TTT CCT CGG TCT GCT GCG	P F P R S A A	1
ACT CTT ATT CTC ATG AAG AGG	CCT CTT CAT GAG AAT AAG AGT	P L H E N K S	1
CCT ATT CTC CGA CTG AGG CCC	GGG CCT CAG TCG GAG AAT AGG	G P Q S E N R	1
CGC ACG ATT CTC CGA ATA CGG	CCG TAT TCG GAG AAT CGT GCG	P Y S E N R A	1
CGT AAG CGG CGG AGT CTT AGA	TCT AAG ACT CCG CCG CTT ACG	S K T P P L T	1
CGA CTT ATT CTC ATC AAA AGG	CCT TTT GAT GAG AAT AAG TCG	P F D E N K S	1
CTT ATT CTC CGC CAG AGC AGA	TCT GCT CTG GCG GAG AAT AAG	S A L A E N K	1

been written out and check against the top strand of the insert sequence.

Molecular modeling of epitopes on buffalo β -lactoglobulin by MIMOX

MIMOX is a tool for epitope mapping based on phage display, coded in Perl using modules from the Bioperl project (Huang and others 2006). It has 2 sections. In the first section, MIMOX provides a simple interface for ClustalW to align a set of mimotopes. In the second section, MIMOX can map a single mimotope or a consensus sequence of a set of mimotopes, on to the corresponding antigen structure and search for all of the clusters of residues that could represent the native epitope.

Modeling the 3 dimensional structure of buffalo β -lactoglobulin, was employed by homology modeling server SWISS-MODEL using bovine β -lactoglobulin (PDB ID: 2BLG) as a reference which was derived from the Protein Data Bank. Moreover, the structure model of buffalo β -lactoglobulin should be resolved into only one chain according to the demand of MIMOX. Both of the amino acids sequences of positive clones from the biopanning and PDB format of buffalo β -lactoglobulin were input into the first section of MIMOX for identification of epitope candidates. The parameter of candidate residue pickup mode and distance calculating method were chosen as strict (exact) match mode and distance between C alpha atoms, respectively. Distance factor can vary between 0.4 nm and 1.2 nm with a default value 0.7 nm. Finally, the molecular graphics of conformational epitope candidates of buffalo β -lactoglobulin were rendered with PyMOL 0.99.

Results

Comparison of molecular models of buffalo and bovine β -lactoglobulin

The amino acid sequences of bovine and buffalo β -lactoglobulin were shown in Figure 2(A). The different amino acids (underlined in Figure 2A) existed in the N and C terminal of 2 variants of β -lactoglobulin (Accession No. 0601265A and P02754). The 3-D structure of buffalo β -lactoglobulin was modeled by the Web service of Swiss model with bovine β -lactoglobulin (<http://www.ncbi.nlm.nih.gov/pubmed/>, PDB: 2BLG). It was found that the 2 different amino acids did not result in a different conformation between 2 varieties. However, the local regions in AA87–89 formed distinct fold orientations, although they had identical amino acids (shadow in Figure 2A).

Phages derived from the biopanning against antibuffalo β -lactoglobulin antibody

After 3 rounds of biopanning, specific phages were enriched by eluting from phage random library, and peptide mimotopes for buffalo β -lactoglobulin were obtained. A total of 60 random clones were identified by phage ELISA, and it was found that phage clone 15 for serum from rabbit 1 and phage clone 16 for serum from rabbit 2 were positive, respectively. The deduced amino acid sequences of the mimotopes were listed in Table 1 and 2, respectively. Moreover, the amino acid sequences of 7 positive clones derived from the biopanning against serum of R1 were identical, whereas no similarities existed in the clones of R2.

Table 3–Position and combination of mimotope SPLTENK^a.

Nr.	Amino acid residues and locations of epitope candidates	Accessible areas (nm ²)
1	S(21)-P(126)- <u>L(156)</u> - <u>T(154)</u> -E(127)-N(130)-K(135)	4.7668
2	S(21)-P(126)- <u>L(156)</u> - <u>T(125)</u> -E(127)-N(130)-K(135)	4.3704
3	S(21)-P(126)- <u>L(122)</u> - <u>T(125)</u> -E(127)-N(130)-K(135)	4.2909
4	S(21)-P(126)- <u>L(22)</u> - <u>T(125)</u> -E(127)-N(130)-K(135)	4.2909

^aDistance threshold: 10 and underline is variable amino acids.

Location of conformational epitopes on buffalo β -lactoglobulin

Conformational epitopes recognized by the serum of rabbit 1. The sequence of the 7 identical mimotopes (SPLTENK, Table 1) was dominant in the positive clones and therefore could be considered as a conformational epitope candidate. With web tool of MIMOX, the identical sequence was folded and formed 4 probably conformational compositions as shown in Table 3. The molecular graphics of these conformational compositions of epitope on buffalo β -lactoglobulin were shown in Figure 3, which revealed that SPLTENK did not form a cluster but 2 parts, resulting in enough space for epitope binding to the specific antibody.

The essential amino acids in the conformational compositions of epitope were 5 amino acids, S (21)-P (126)-E(127)-N(130)-K(135). However, the other 2 residues, T and L were variable (underlined in Table 3). We can find that T was located in the position of 145 and 125 for conformational compositions of No. 1 and 2, in Table 3, respectively. Although L (22) of Nr. 4 was replaced and compared to L (122) in Nr. 3.

Conformational epitopes recognized by the serum of rabbit 2. Because there were no identical sequences of the positive clones against sera derived from R2 (Table 2), the mimotope sequences were connected continuously, forming a new peptide (named as P) shown in Figure 4(A). The antigenicity of peptide P was evaluated by DNASTar and Web service in Figure 4(B).

The characteristics of hydrophobicity, flexible region, antigenic index, and surface probability for peptide P were defined by DNASTar’s blocks of Kyte-Doolittle, Karplus-Schulz, Jamson-Wolf, and Emini, respectively (Figure 4B-1). Moreover, the secondary structure of α -helix, β -turn, and β -sheet was predicted by Chou and Fasman on the web service (Figure 4B-2-4). According to bioinformatics analysis, 4 of the 16 mimotopes were determined to be potential conformational epitope candidates, including mimotope-3(PLNENKD), mimotope-5(VAGQSPK), mimotope-14(SKTPPLT), and mimotope-15(PKDENKS; shown

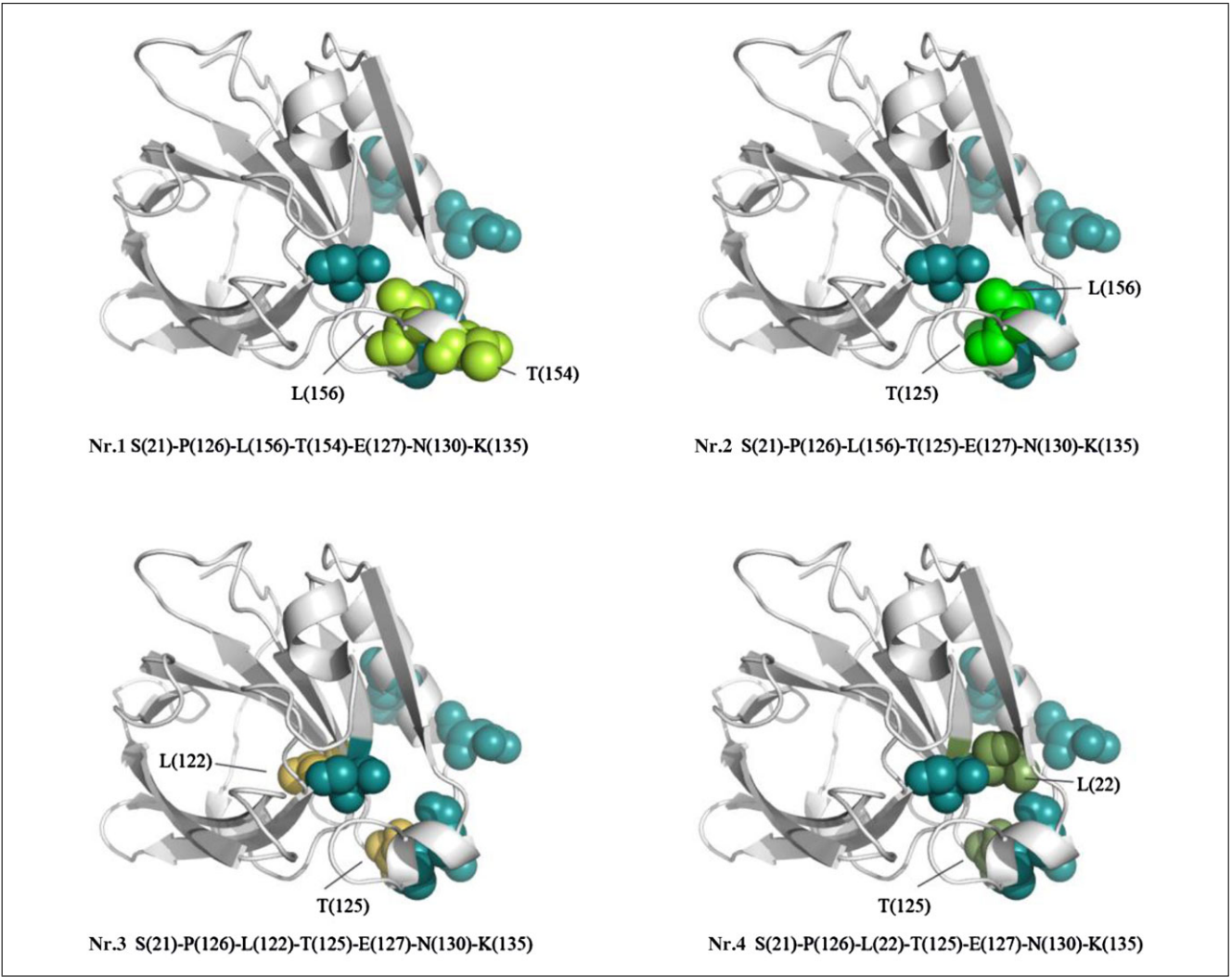


Figure 3–Molecular graphics of SPLTENK peptide recognized as conformational epitopes on Cyan-blue balls stand for conserved amino acid residues and other balls were variable.

in Table 2). At the same time, the consensus sequence “-PLXEN [KR]-” was calculated using the first section of MIMOX on the basis of the sequences of 16 biopanned mimotopes (Figure not shown).

Compared with consensus sequence by MIMOX and the 4 potential conformational epitope candidates derived from Figure 3, mimotope-3 and mimotope-15 were further confirmed as the conformational epitope ones. The 2 mimotope sequences were then mimicked into their conformation on buffalo β -lactoglobulin with web tool of MIMOX. Moreover, mimotope-15 was excluded because more than 20 conformation compositions were obtained through MIMOX, which is not realistic for conformational epitope. However, regarding to mimotope-3, only 3 conformational compositions were produced. It should be considered as a conformational epitope candidate for sera from R2.

Three conformational compositions of mimotope-3 (PLNENKD) were displayed in Table 4 and the molecular graphics of epitopes was depicted in Figure 5. It was found that there were 3 conserved residues including N (90), K (83), and D (85). More specifically, 4 continuous residues were located in the position of 87 to 90 shown in first conformational compositions

in Table 4, suggesting it might contribute to be a part of the linear epitope as well. Interestingly, P (38) is far away from other residues in the prime sequence, but it located near the position of 87 to 90 in the space shown in Figure 5. With respect to the other 2 conformational compositions of epitopes, the only one residue E located in different position of 89 and 108, respectively. It can be seen from Figure 5 that their spatial locations were similar.

Discussion

As early as in 1993, a random nonapeptide library was constructed, and it was used to mimic discontinuous epitopes on human H-subunit ferritin (Luzzago and others 1993). Actually, many conformational epitopes on allergens were defined by phage display technology, such as the plant panallergen profiling (Leitner and others 1998), the major birch pollen allergen Der p 1 (Jensen-Jarolim and others 1999; Furmonaviciene and others 1999). Therefore, it is considered as a robust method for the identification of conformational epitopes on allergens. Currently, epitope mimics by phage display technique are playing an important role in defining the conformational epitopes, and it has been used

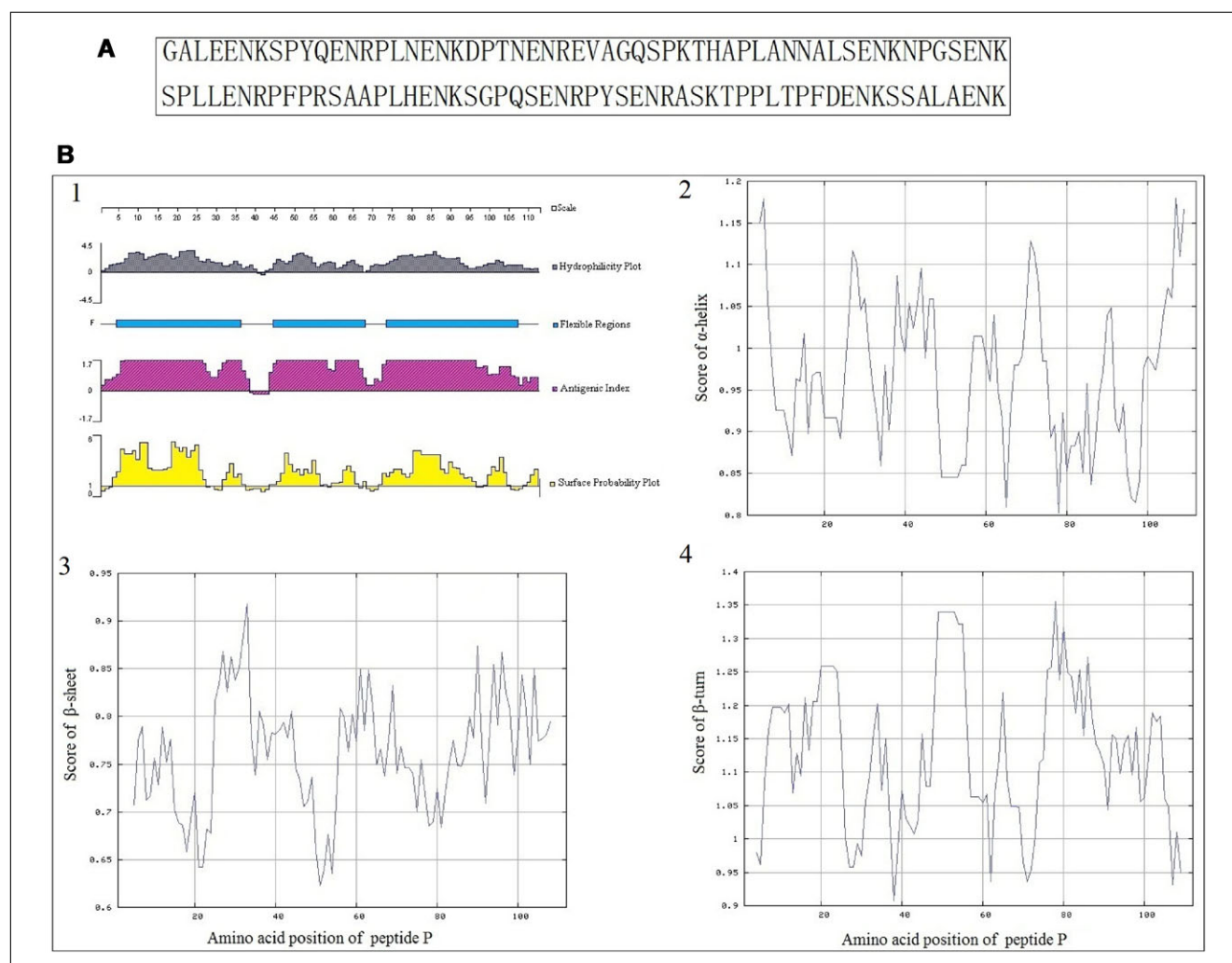


Figure 4—Amino acid sequence and antigenicity characterization of peptide P.

(A) Amino acid sequences of peptide P. (B) Antigenicity of peptide P characterized by DNASTar and Web service. 1, Antigenicity with DNASTar software; 2 to 4, Antigenicity by web service (<http://www.expasy.org/>), 2, α -helix by using Chou and Fasman; 3, β -sheet by using Chou and Fasman; 4, β -turn by using Chou and Fasman.

Table 4–Position and combination of mimotope PLNENKD^a.

Nr.	Residues and locations of candidate cluster	Accessible areas (nm ²)
1	<u>P</u> (38)- <u>L</u> (87)- <u>N</u> (88)- <u>E</u> (89)-N(90)-K(83)-D(85)	4.8244
2	<u>P</u> (113)- <u>L</u> (117)- <u>N</u> (109)- <u>E</u> (89)-N(90)-K(83)-D(85)	3.6503
3	<u>P</u> (113)- <u>L</u> (117)- <u>N</u> (109)- <u>E</u> (108)-N(90)-K(83)-D(85)	3.5913

^aDistance threshold: 8 and underline is variable amino acids.

widely, and developed quickly (Riemer and others 2004). For example, one research group reported that the mimotopes facilitated the localization of conformational IgE binding epitopes on Phl p 5, suggesting them to be suitable candidates for the development of an epitope specific immunotherapy (Hantusch and others 2004). In 2006, on the basis of biopanning of phage library, 5 positive phage clones were recognized by parvalbumin-specific IgE as well as IgG, suggesting these mimotopes can be candidates for an epitope-specific immunotherapy of fish-allergic patients (Untersmayr and others 2006). All these researches demonstrated that phage display technique is a robust method for the biopanning of mimotope.

Conformational epitope candidate sequences should be simulated on the structure of the molecule with information including mimotope and antigen sequence, or with mimotope sequence and antigen structure, or integrating the different approaches. For example, Pep-3-D-search approach is used to predict the epitope

area through mimotopes or a motif sequence derived from a set of mimotopes (Huang and others 2008). Given by the 3-D structure of an antigen and a set of mimotopes (or a motif sequence derived from the set of mimotopes), Pep-3-D-Search can be used in 2 modes: mimotope or motif. In respect to 3-D-Epitope-Explorer (3DEX) software, Schreiber and others has localized mimotopes from phage displayed peptide libraries with polyclonal antibodies of HIV-positive patient plasma within the 3-D structure of gp120, the exterior glycoprotein of HIV-1 (Schreiber and others 2005). MIMOX used in our work is designed for modeling the conformational epitope with mimotopes biopanned from phage display. This web tool is the first free web tool for structural mapping with mimotope information and implemented as the script jmol.pl, which wraps a Java applet version of Jmol and is used to calculate the surface accessibility of the mapping results. All mapping results are ranked based on their solvent accessible surface and each mapping result has detailed information of the accessibility of each candidate residue, which is parsed through the script parsapl and displayed as a table in a new window. Moreover, MIMOX was more simple and operable. Eventually, we have chosen this web tool to localize conformational epitopes on buffalo β -lactoglobulin.

Prime structure of β -lactoglobulin has been reported early in 1950s (Dawson 1951; Riley 1951), and that of water buffalo β -lactoglobulin was defined in 1979 (Braunitzer and others 1979).

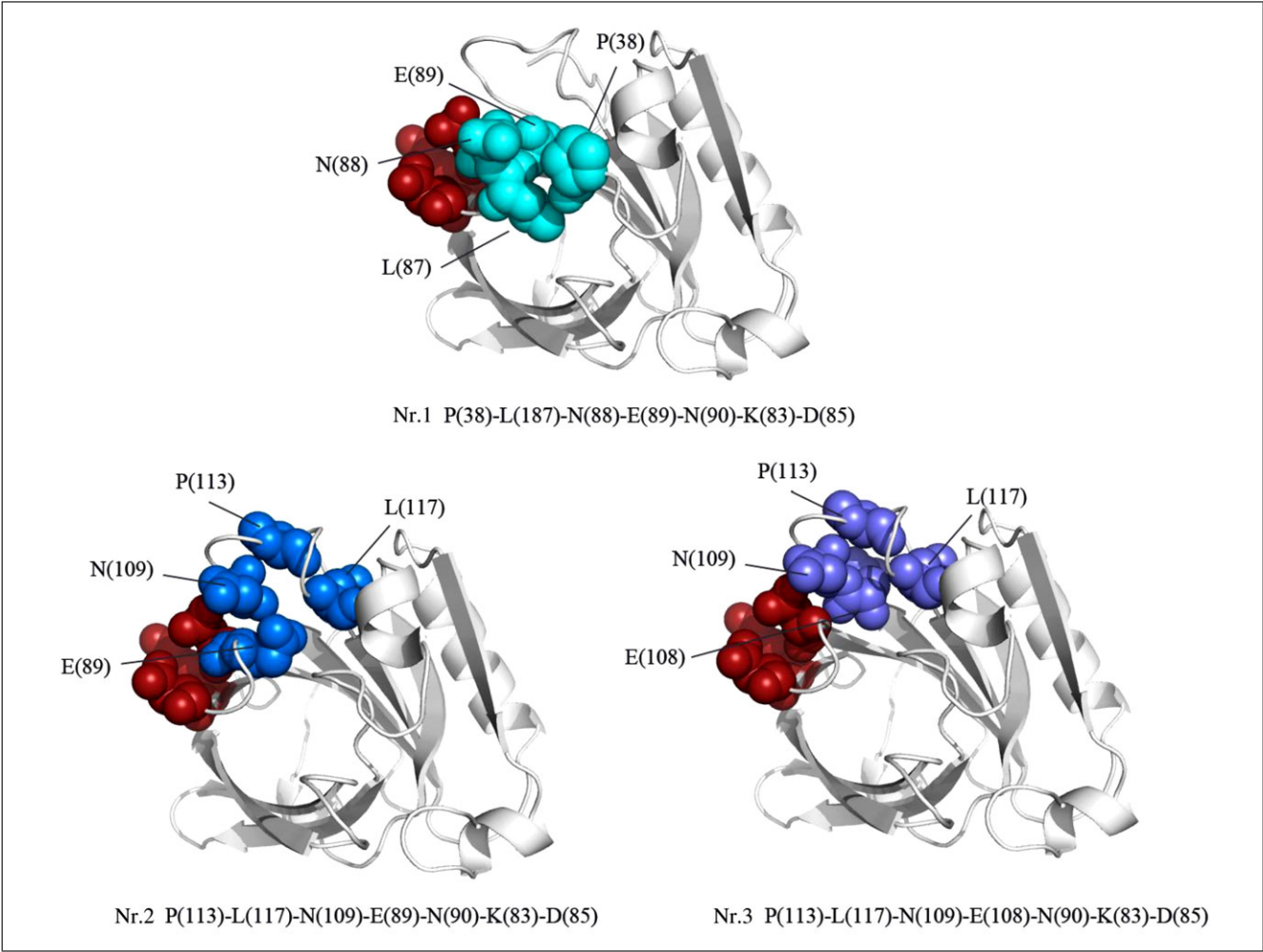


Figure 5–Conformational patterns of peptide PLNENKD. Red balls stand for conserved amino acid residues and the others were variable.

β -Lactoglobulin belongs to lipocalin family and its structure is a highly symmetrical β -structure dominated by a single 8-stranded antiparallel β -sheet closed back on itself to form a continuously hydrogen bonded β -barrel as shown in Figure 6 (Ragona and others 1999). Those parts from the 3 main structures and sequences, and conserved regions (SCRs) of the fold (SCR1, SCR2, and SCR3) are marked as heavy boxes. Compared with the sequences of conformational epitopes on buffalo β -lactoglobulin, we found that 4 conformational compositions of epitopes from R1 are mainly located in the region of SCR3 structure of lipocalin family protein (Flower and others 2000) and 4 conserved amino acids were located in the region of α -helix except for Ser (21), which indicating that the conformational epitopes are universal in lipocalin family, resulting in cross-reactivity among the family members. The conserved amino acids of conformational compositions of epitopes from R2 were in the position of E and F, whereas other amino acids were located dispersedly. Undoubtedly, our work revealed that the conformational epitopes on buffalo β -lactoglobulin are dominated in conserved structure of lipocalin. However, it was reported that most important IgE binding regions in β -lactoglobulin appear to be located in the carboxy-terminal portion of the molecules to form a continuous hydrogen-bonded β -barrel (Niemi and others 2007). This difference need for further investigation.

Although there is no data about the prevalence of buffalo milk allergy, cow's and buffalo's milk cross-reactivity showed a similar protein composition as early as in 1999 and presented a similar pattern in immunoblotting with 6 cow's milk-allergic children and anticasein monoclonal antibodies have a strong capacity with buffalo milk (Restani and others 1999). In 2002, the research group further proved that buffalo milk displayed a highly similar pattern in SDS-PAGE and immunoblotting with anti- β -lactoglobulin monoclonal antibody (Restani and others 2002). Katz has explored that all patients allergic to cow's milk were tested positive for cross-reactivity to buffalo by skin-prick test, which indicated a significant cross-sensitization to milk proteins derived from buffalo milk (Katz and others 2008). In further, the cross-reactivity

between purified buffalo and standard bovine β -lactoglobulin has been evaluated with 69.7% in our previous study (Li and others 2008). Therefore, there were strong evidences of cross-reactivity between buffalo and bovine β -lactoglobulin and it was a high risk because buffalo milk has been a common milk resource in many developing countries.

Compared with the sequence and structure between buffalo and bovine β -lactoglobulin (Figure 2), both proteins might have the same conformational epitopes because the epitopes were located in the conserved region. Although the prime sequence of AA87–89 of β -lactoglobulin from both bovine and buffalo was conserved, their conformations were slightly different in Figure 2. Therefore, the first conformational composition of epitopes from R2, P(38)–L(87)–N(88)–E(89)–N(90)–K(83)–D(85), might be unique to buffalo β -lactoglobulin. Moreover, the identified conformational epitopes proved the theory that conformational epitopes are located in 2 or more separate regions of the protein to bind an antibody tightly.

Although the structure of β -lactoglobulin has long been well-described, the conformational epitope has not been identified yet. In our work, 2 conformational epitope candidates specific for sera from 2 rabbits were identified to have several spatial structure combinations with conserved and variable amino acids, respectively. We also found that the defined conformational epitopes on buffalo β -lactoglobulin have 5 conserved amino acid residues “PL–ENK,” which located in the different positions. In addition, the sequences of 2 conformational epitope candidates were composed of five hydrophilic amino acids and 2 hydrophobic amino acids, corresponding to polar and nonpolar, respectively. It suggested that some amino acids were more prone to be the part of conformational epitope, such as proline, leucine, asparagine, glutamine, and lysine.

In addition, 8 linear epitopes and 18 critical amino acids were defined in our previous studies (Li and others 2012a, 2012b). Compared with the present data, 4 amino acids of the conformational epitope candidate from R1 were overlapped with the linear epitopes in previous study. Although all of amino acids in the conformational epitope candidates from R2 were also found in linear epitopes. Moreover, the sequence “ENK” from all the conformational epitopes were identified in our previous work of critical amino acids, which further proved that the conformational epitope played an important role in the linear epitope as well.

Conclusion

Seven conformational IgG-binding epitopes, specific for 4 and 3 from 2 rabbit sera, were defined by biopanned from phage random peptide library with the web tool of MIMOX. More importantly, five amino acids (PL–ENK) of identified epitopes were conserved. It is the first time to define conformational epitopes binding to buffalo β -lactoglobulin and a simple and operable method has been established for identification of conformation epitope on food allergens.

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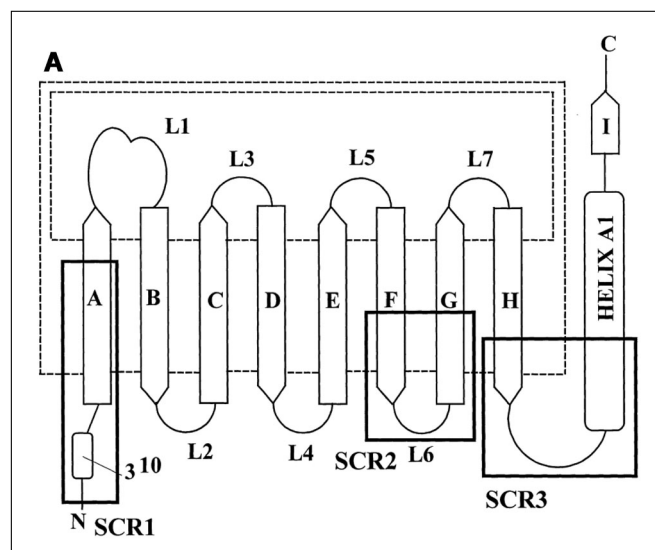


Figure 6—Structure of the lipocalin protein fold. An unwound view of the lipocalin protein fold orthogonal to the axis of the barrel. The 9 L-strands of the antiparallel β -sheet are shown as arrows and labeled A–I. The C-terminal α -helix A1 and N-terminal 310 like helix are also marked. Connecting loops are shown as solid lines and labeled L1–L7. A pair of dotted lines indicates the hydrogen-bonded connection of 2 strands.

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