

Identification of the Critical Amino Acid Residues of Immunoglobulin E and Immunoglobulin G Epitopes in α -Lactalbumin by Alanine Scanning Analysis

Cong Yanjun, Zhou Shengyun, and Li Linfeng

Abstract: α -Lactalbumin represents one of the major allergens causing cow milk allergy. Few studies have clearly evaluated immunological relationships between immunoglobulin E (IgE) and immunoglobulin G (IgG)-binding epitopes of α -lactalbumin. IgE- and IgG-binding epitopes were immunolabeled with individual sera from cow milk-allergic patients. Alanine scanning of immunodominant epitopes was used to identify the critical amino acid (aa). Our initial data revealed Val⁸, Phe⁹, Arg¹⁰, Tyr¹⁰³, Leu¹⁰⁵, and His¹⁰⁷ were the critical aa for IgE-binding epitope. The critical aa of IgG-binding epitopes were Phe⁹, Leu¹⁵, Pro²⁴, Trp²⁶, and His³². This study will provide necessary information to alter the cDNA to encode a protein capable of activating milk-specific T cells, but with reduced IgE- or IgG-binding capacity.

Keywords: cow milk allergy, critical amino acid, epitope, α -lactalbumin

Introduction

Cow's milk allergy (CMA) is a common and often transitory disease affecting approximately 2.5% of children less than 2 years of age (Osterballe and others 2005). The majority of children "outgrow" their cow's milk allergy by 3 to 4 years of age. However, 15% of infants with immunoglobulin E (IgE)-mediated CMA retain their sensitivity into the second decade (Sampson 2003).

Milk contains caseins and whey proteins, accounting for 80% and 20% of total protein, respectively. α_{s1} -, α_{s2} -, β -, and κ -caseins as well as the whey proteins, α -lactalbumin (ALA) and β -lactoglobulin (BLG) are the most important allergens. ALA is a monomeric globular protein of 123-amino acid (aa) residues with a molecular weight of 14.4 kD and 4 disulfide bridges (Wal 1998). Marked homology is found between bovine and human ALA, with 74% of the residues identical and another 6% showing chemical similarity. It possesses a high-affinity binding site for calcium, which stabilizes its secondary structure. Its hydrophilic profile suggests that the major antigenic site is located on peptide corresponding to aa 5 to 18, which corresponds to the homologous sequence 124 to 134 on the BLG molecule (Järvinen and others 2001).

An epitope is the part of an antigen that is recognized by B cell (IgE and immunoglobulin G [IgG] antibodies), and T cells. Information regarding the immunodominant epitopes may be useful in the development of more specific diagnostic methods of CMA and eventually lead to safe, effective therapeutic approaches for patients with CMA. In the current study, a region was defined as an epitope if it was statistically associated with reactive groups and

recognized by at least 65% of reactive patients. Significantly higher levels of IgG antibodies against certain milk proteins have been found in children with persistent CMA (Host and others 1992; James and Sampson 1992) and adult milk-intolerant patients (Little and others 1998). Few studies have clearly evaluated immunological relationships between IgE- and IgG-binding epitopes of cow milk allergens.

Previous reports on mutational analysis of other food allergens, such as the peanut allergens Ara h1, Ara h2, and Ara h3, showed that single aa substitutions within each IgE-binding epitope led to elimination or marked reduction of IgE-binding by antibodies within pooled sera from peanut-allergic patients (Burks and others 1997; Stanley and others 1997; Rabjohn and others 1999). Our initial study found the critical aa residues of IgE and IgG immunodominant epitopes for reducing the allergenicity of β -lactoglobulin and α_{s1} -casein (Cong and Li 2012; Cong and others 2013).

In this study, we aimed to map IgE and IgG epitopes using sera from infant patients allergic to cow milk and found the critical aa for reducing the allergenicity of α -lactalbumin by alanine scanning analysis.

Materials and Methods

Sera from patients with CMA

Sera from 8 patients with documented cow milk hypersensitivity (the median age at the time of collection of sera was 1 y; range: 6 mo to 3 y) were used to identify IgE- and IgG-binding epitopes and the critical aa for these epitopes. Cow milk specific IgE antibodies ranged from 25 to 100 kU/L as measured by CAP System FEIA (Phadia Diagnostics Uppsala, Sweden). All 8 patients presented atopic dermatitis as clinical symptom. Five non-milk-allergic children (aged 1–3 y) with atopic dermatitis and allergy to other foods (that is, tomato, shellfish, peanuts, and nuts) were used for control sera. Informed consent was obtained from the patients and their parents and the study was approved by Beijing Friendship Hospital (Beijing, China).

MS 20151915 Submitted 11/18/2015, Accepted 7/20/2016. Authors Cong and Zhou are with Beijing Key Laboratory of Food Flavor Chemistry & Beijing Higher Inst. Engineering Research Center of Food Additives and Ingredients, Beijing Technology and Business Univ., Haidian District, Beijing 100048, P. R. China. Author Li is with Dept. of Dermatology, Beijing Friendship Hospital, Capital Medical Univ., Xichen District, Beijing 100050, P. R. China. Direct inquiries to author Cong Yanjun (E-mail: congyj@th.btbu.edu.cn).

Table 1—The peptides synthesized based on the sequence of α -lactalbumin.

Code	Sequence	aa Position	Code	Sequence	aa p
P1	EQLTKCEVFRELKDL	1–15	P15	NICNISCDKFLDDDL	71–85
P2	CEVFRELKDLKGYGG	6–20	P16	SCDKFLDDDLTDDIM	76–90
P3	ELKDLKGYGGVSLPE	11–25	P17	LDDDLTDDIMCVKKI	81–95
P4	KGYGGVSLPEWVCTT	16–30	P18	TDDIMCVKKILDKVG	86–100
P5	VSLPEWVCTTFHTSG	21–35	P19	CVKKILDKVGINYWL	91–105
P6	WVCTTFHTSGYDTQA	26–40	P20	LDKVGINYWLAHKAL	96–110
P7	FHTSGYDTQAIVQNN	31–45	P21	INYWLAHKALCSEKL	101–115
P8	YDTQAIVQNNNSTEY	36–50	P22	AHKALCSEKLDQWLC	106–120
P9	IVQNNNSTEYGLFQI	41–55	P23	CSEKLDQWLC EKL	111–123
P10	DSTEYGLFQINNKIW	46–60			
P11	GLFQINNKIWCKDDQ	51–65			
P12	NNKIWCKDDQNPHSS	56–70			
P13	CKDDQNPHSSNICNI	61–75			
P14	NPHSSNICNISCDKF	66–80			

Synthesis of α -lactalbumin-derived peptides

For mapping and characterization of a dominant epitope and a critical aa, a lot of peptides (15 aa in length) based on the sequence of α -lactalbumin, were synthesized using the multiple method according to the Fmoc (9 fluorenylmethoxy carbonyl) strategy (Ruiter and others 2006). The alanine substitution scanning of a single aa residue of a dominant epitope was used to identify the critical aa.

Synthesis of the peptides was accomplished on a peptide synthesizer (Applied Biosystems 431) using an Fmoc-aa-Wang-resin containing free hydroxyl groups. Synthesis of each peptide was started by esterification of an Fmoc-aa to Wang-resin. After washing, all aa residues were blocked by acetylation to render them unreactive during the subsequent steps. Fmoc protective groups were then removed by addition of piperidine to render nascent peptides reactive. Each additional Fmoc-aa is esterified to the previous one by this same process. After addition of the last aa in the peptide, the aa side chains de-protected and the resin was cleaved using a mixture of 1:1:0.05 (by volume) dichloromethane/trifluoroacetic acid/triisobutylsilane, followed by washing with dichloromethane and methanol. After filtration, cleavage reagents were removed *in vacuo*, the crude peptides were precipitated with ice-cold tert-butylmethyl ether, dried *in vacuo*, dissolved in water, and lyophilized.

The crude peptides were purified by C₁₈ reverse-phase HPLC (Macherey-Nagel GmbH & Co. KG, Düren, Germany) at a flow rate of 12.0 mL/min and a gradient of 1% acetonitrile per min. Acetonitrile/water with 0.1% trifluoroacetic acid was used as the eluting solvent, and 50 mg/mL synthetic peptides were loaded.

IgE-binding assay

An IgE ELISA was performed as described by Cocco and others (2003). Before immunological detection, the purified peptides were coupled with BSA. Then, the peptides reacted with BSA were added to 96-well plates. Peptides were incubated with individual serum for IgE-binding epitopes or the serum pool for identifying the critical aa. These sera were from patients with CMA diluted (1:10) in a solution containing Tris-NaCl (10 mM Tris-HCl and 500 mM NaCl, pH 7.5) and 1% BSA for 2 h at 37 °C. The primary antibody was detected using monoclonal mouse-anti-human IgE (1:2000; Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphthol staining, and then expressed as the optical density at a wavelength of

450 nm. Between each step, plates were washed 5 times with Tris-buffered saline containing 0.1% Tween 20. Nonspecific binding was studied using serum from nonallergic individuals and allergic individuals (but not cow milk allergic). An epitope can be considered immunodominant if it is recognized by above 65% serum IgE from the majority of patients with cow milk hypersensitivity. Critical aa were defined as those that resulted in loss of IgE binding when substituted by alanine residue.

IgG-binding assay

A modified IgG ELISA was performed as described previously (Järvinen and others 2001). Peptides were incubated with individual serum sample for identifying IgG-binding epitopes or the serum pool for identifying the critical aa from patients with cow milk hypersensitivity diluted (1:20) in a solution containing Tris-NaCl (10 mM Tris/HCl and 500 mM NaCl; pH 7.5) and 1% BSA for 2 h at 37 °C. The primary antibody was detected using monoclonal goat-anti-human IgG (1:3000; Pharmacia & Upjohn Diagnostics) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphthol staining, and then expressed as the optical density at a wavelength of 450 nm. Between each step, plates were washed 5 times with Tris-buffered saline containing 0.1% Tween 20. Nonspecific binding was studied using serum from nonallergic individuals and allergic individuals (but not cow milk allergic). An epitope can be considered immunodominant if it is recognized by above 65% serum IgG from the majority of patients with cow milk hypersensitivity. Critical aa were defined as those that resulted in loss of IgG binding when substituted by alanine residue.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010. All determinations were carried out in triplicate. Significant difference between data was assessed after ANOVA using the Kruskal-Wallis test at a confidence level of 95%.

Results

Peptide synthesis

Based on a lot of synthesizing peptides, it was possible to identify individual IgE- and IgG binding epitopes within the larger regions of the α -lactalbumin molecule. As shown in Table 1, 23 peptides were synthesized, and the purity of these peptides was above 85%.

Table 2–The IgE-binding epitopes of α-lactalbumin.

seru m	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23
No.1																							
No.2																							
No.3																							
No.4																							
No.5																							
No.6																							
No.7																							
No.8																							

A total of 23 peptides were recognized by 8 sera. P1 to P23 represents the code of 23 peptides; No.1 to No.8 represents the code of 8 sera.  strong binding (OD 0.50 to 0.70)  moderate binding (OD 0.25 to 0.49)  weak binding (OD 0.1 to 0.24)  No/weak binding (OD < 0.10).

Identification of IgE-binding epitopes

As shown in Table 2, IgE antibodies from 8 patients showed binding to at least 1 linear peptide when 23 peptides were recognized. Peptides coded as P1 at aa1–15 and P21 at aa101–115 were identified by IgE from 8 sera. The sequences at aa6–20 and aa46–60 were identified by 7 of 8 sera. The region at aa71–85 was identified by 6 of 8 sera. The peptides coded as P11, P14, P20, and P22 were recognized by 3 of 8 sera. Other peptides coded as P3, P4, P6, P7, P8, P9, P12, P13, P16, P17, P18, P19, and P23 were also recognized by individual serum samples. Therefore,

5 IgE-binding epitopes were identified at aa1–15, aa6–20, aa46–60, aa71–85, and aa101–115, depicted in red on the 3D structure of α-lactalbumin in Figure 1. Sera from non–milk-allergic children showed weak reaction with synthesized peptides (data not shown).

Identification of IgG-binding epitopes

The intensity of IgG binding to each peptide was determined as a function of that patient’s specific IgG binding to these 23 peptides (Table 3). Individual sera showed different IgG-binding ability to these 23 peptides. The sequence of aa6–20 (coded as P2)

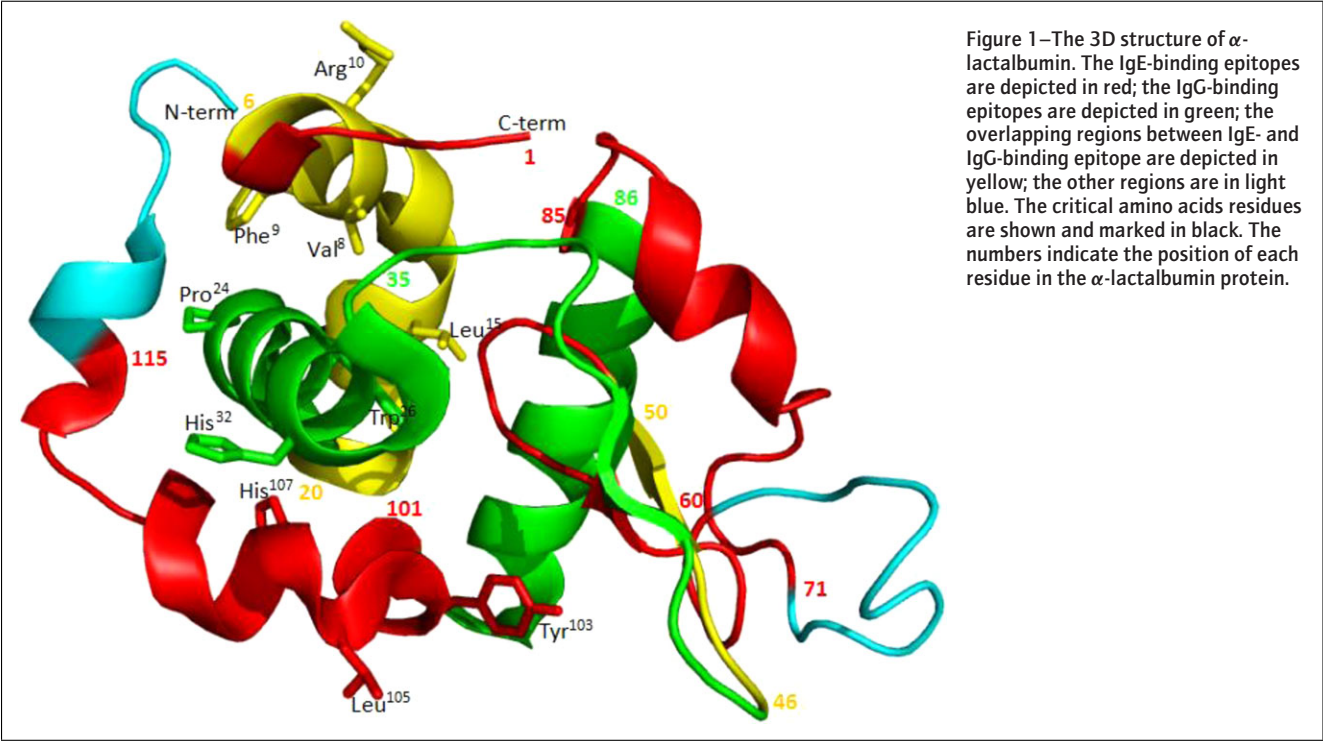
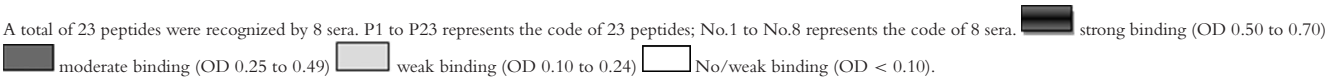


Figure 1–The 3D structure of α-lactalbumin. The IgE-binding epitopes are depicted in red; the IgG-binding epitopes are depicted in green; the overlapping regions between IgE- and IgG-binding epitope are depicted in yellow; the other regions are in light blue. The critical amino acids residues are shown and marked in black. The numbers indicate the position of each residue in the α-lactalbumin protein.

Table 3–The IgG-binding epitopes of α-lactalbumin.

Seru m	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23
No.1																							
No.2																							
No.3																							
No.4																							
No.5																							
No.6																							
No.7																							
No.8																							

A total of 23 peptides were recognized by 8 sera. P1 to P23 represents the code of 23 peptides; No.1 to No.8 represents the code of 8 sera.  strong binding (OD 0.50 to 0.70) moderate binding (OD 0.25 to 0.49) weak binding (OD 0.10 to 0.24) No/weak binding (OD < 0.10).

and aa21–35 (coded as P5), considered as IgG-binding epitopes, were recognized by 8 of 8 patients sera. The peptide at aa36–50 (coded as P8) and aa86–100 (coded as P18), also considered as IgG-binding epitope, were identified by 6 of 8 sera. The peptides coded as P4, P9, P11, and P13 were recognized by 3 of 8 sera. P1, P3, P6, P7, P10, P12, P14, P15, P17, P19, P20, P22, and P23 were all identified by individual serum samples. Therefore, 4 IgE-binding epitopes were identified at aa6–20, aa21–35, aa36–50, and aa86–100, depicted in green on the 3D structure of α-lactalbumin in Figure 1. Sera from non-milk-allergic children showed weak reaction with synthesized peptides (data not shown).

Characterization of the critical aa of the IgE-Binding epitopes at aa1-15 and aa101-115

The aa essential to IgE binding in the epitope at aa1–15 were determined by synthesizing duplicate peptides with single aa changes at each position. These peptides were then probed with pooled serum IgE from 8 patients with cow milk hypersensitivity to determine if the change affected cow milk-specific IgE binding (results are shown in Figure 2). Peptides at aa1–15 with alanine substituted for Val⁸, for Phe⁹, and for Arg¹⁰ were not recognized by pooled serum IgE. Compared with the control group (no aa substitutions), the intensity of other peptides with alanine substituted for one of the aa residues showed no significant difference.

The critical aa for IgE-binding epitope at aa101–115 were also probed with pooled serum IgE from 8 patients, data shown in Figure 3. Peptides with alanine substituted for Tyr¹⁰³, for Leu¹⁰⁵, and for His¹⁰⁷ were not identified by pooled serum IgE. The OD value of other peptides with alanine substituted for one of the aa residues showed no significant difference compared with the control group.

Based on above experiments, Val⁸, Phe⁹, and Arg¹⁰ are the aa essential to IgE binding epitope at aa1–15. Tyr¹⁰³, Leu¹⁰⁵, and His¹⁰⁷ are the critical aa for the epitope at aa101–115. These critical aa residues were depicted in Figure 1.

Characterization of the critical aa of the IgG-binding epitope at aa6-20 and aa21-35

To identify the aa critical for IgG binding in the sequence of aa6–20, a series of related peptides were synthesized with alanine substitutions at each position and their IgG-binding abilities were

evaluated (Figure 4). A significant loss of IgG binding was observed for 2 aa, one with alanine substituted for Phe⁹ and the other with alanine substituted for Leu¹⁵. Substitution of other position on this peptide did not cause a consistent decrease in IgG binding.

A significant loss of IgG binding was observed for 3 aa in the sequence of aa21–35, shown in Figure 5. Peptides with alanine substituted for Pro²⁴, for Trp²⁶, and for His³² were not identified by pooled serum IgE. Compared with the control group, the OD value of the peptide with alanine substituted for Thr²⁹ significantly decreased, the other peptide showed no significant difference.

Therefore, Phe⁹ and Leu¹⁵ are the aa essential to IgE binding epitope at aa6–20. Pro²⁴, Trp²⁶, and His³² are the critical aa for the epitope at aa21–35. These critical aa residues were depicted in Figure 1.

Discussion

CMA is an IgE-mediated type I immunological reaction, causing the systemic clinical symptoms of urticarial, bronchial constriction, angioedema and anaphylactic reactions following contact

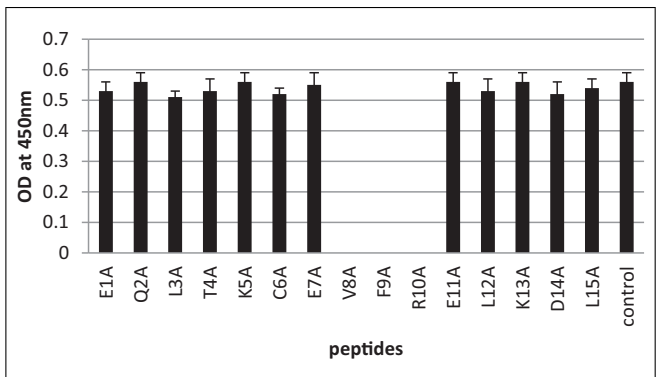


Figure 2–Single aa changes to peptide at aa 1–15 result in loss of IgE binding to this epitope.

The letters across the bottom of each panel indicate the 1-letter aa code for the residue normally at that position and the aa that was substituted for this residue. The numbers indicate the position of each residue in α-lactalbumin protein. The control group indicates the native epitope (no aa substitutions). OD, optical density.

with cow's milk. However, further delayed or late immunological reactions mediated through cytokines and immunoregulatory cells are included in the clinical spectrum of CMA, for example, atopic dermatitis (Savilahti 1981). In this study, all 8 patients presented atopic dermatitis as clinical symptom. We were interested in recognition patterns of sequential epitopes of ALA compared to the previous similar work which would have used sera from patients with immediate reactions in western countries. Different recognition patterns of sequential epitopes of ALA might reflect the clinical reactivity of ALA-sensitized individuals. Identification of the conformational epitopes of ALA will be our future work.

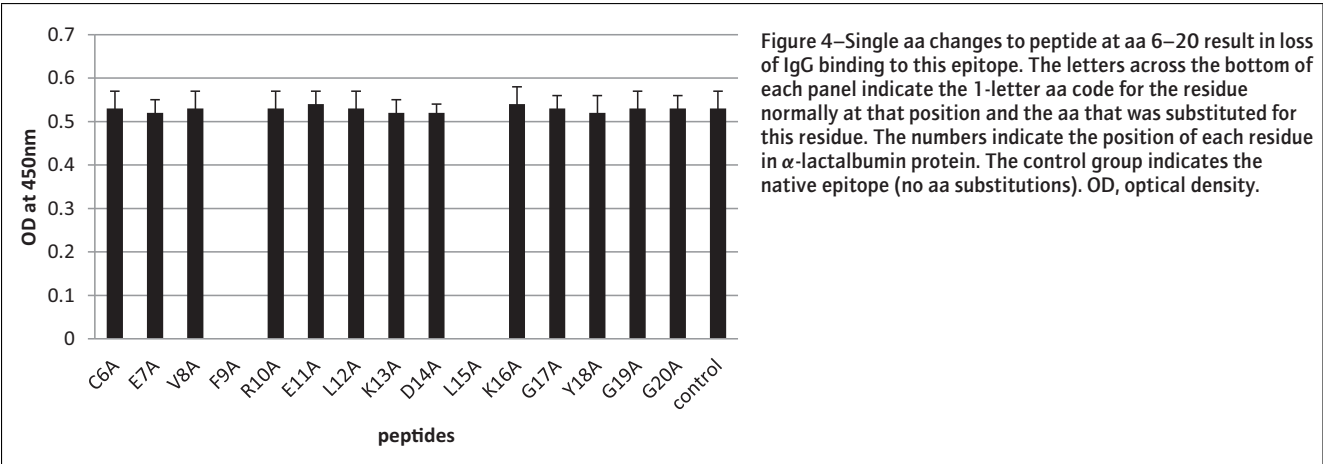
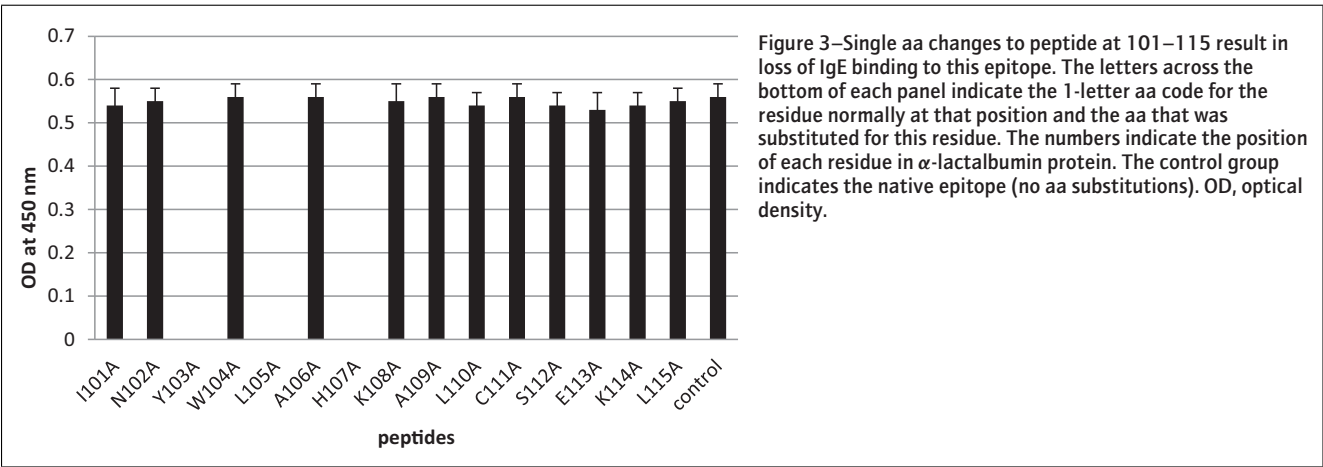
The IgE binding epitopes at aa1-15 observed coincide with allergenic epitopes reported by Adams and others (1991) although exact comparisons are difficult to perform because the tryptic cleavage of ALA yields considerably longer residues or the different synthetic design based on the sequence of ALA. Our study found the other IgE-binding epitopes at aa101-115, aa6-20, aa46-60, and aa71-85. Furthermore, some peptides were not recognized by any patient sera because they may be located in hydrophobic region in the inner core of ALA.

We identified 4 IgG binding sequences on ALA which were aa6-20, 21-35, 36-50, and 86-100, in partial agreement with earlier findings using rabbit antisera (Hopp and Woods 1982), and those predicted by the hydrophilicity profile. In addition, the IgG binding regions identified in our study partial localized with the IgE binding epitopes in milk-allergic patients, depicted in yellow in Figure 1. We found the sequence at aa6-20 can be identified by

both IgE and IgG from cow milk patients' sera. In previous study, Jarvinen and others (2001) showed IgE epitope and IgG epitope share overlap district on ALA. This suggests that allergenic sites of bovine ALA do not totally correspond to antigenic determinants, which was also reported by Maynard and others (1997). The epitopes are exposed on the surface of the allergen, making them accessible to immunoglobulin interaction, as has been found with other allergens (Breiteneder and others 1992; van Kampen and others 1994; Burks and others 1997).

In the search for critical aa for IgE binding, alanine was used for aa substitution because they are relatively small neutral aa that do not significantly alter the charge or solubility of peptides. This approach has been used successfully for other allergens (Rabjohn and others 1999; Robotham and others 2002; Elsayed and others 2004). In the study by Adams and others (1991), the fragment corresponding to aa5-18 was shown by RAST inhibition to exhibit IgE binding capacity. In another study using trypsin-digested ALA, a sequence corresponding to aa17-58 and larger peptides sharing this sequence were most strongly and frequently recognized, in addition, the sequence at aa59-94 showed IgE binding ability with cow milk patients sera (Maynard and others 1997) and binding to smaller peptides corresponding to sequences aa6-10:S-S: 115-123 and aa109-123 was also reported. Based on above results, we choose the sequences at aa1-15, aa101-115, aa6-20, and aa21-35 to determine essential aa for IgE and IgG binding, respectively.

Determination of aa critical for IgE and IgG binding could provide crucial information for altering ALA cDNA to encode a



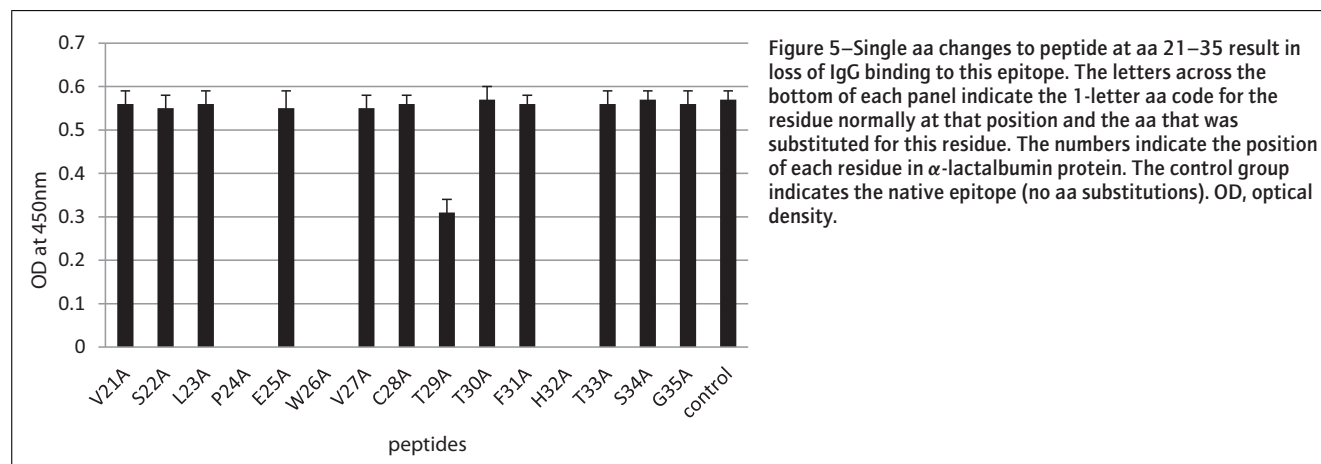


Figure 5—Single aa changes to peptide at aa 21–35 result in loss of IgG binding to this epitope. The letters across the bottom of each panel indicate the 1-letter aa code for the residue normally at that position and the aa that was substituted for this residue. The numbers indicate the position of each residue in α -lactalbumin protein. The control group indicates the native epitope (no aa substitutions). OD, optical density.

protein with reduced IgE and IgG binding capacity. Such mutated proteins or peptides could be used in future immunotherapeutic approaches for this disease. Our data indicated that modification of Val⁸, Phe⁹, and Arg¹⁰ for IgE binding epitope at aa1–15 could decrease the allergenicity of ALA. Likewise, Tyr¹⁰³, Leu¹⁰⁵, and His¹⁰⁷ are the critical aa for the epitope at aa101–115. A significant loss of IgG binding was observed for 2 aa for IgG-binding epitope at aa6–20, one with alanine substituted for Phe⁹ and the other with alanine substituted for Leu¹⁵. Substitution of other position on this peptide did not cause a consistent decrease in IgG binding. A significant loss of IgG binding was observed for 3 aa in the sequence of aa21–35, Pro²⁴, Trp²⁶, and His³². The results could be used in future developing safe immunotherapeutic agents for food allergy, or modified, recombinant proteins need to be engineered that will not bind serum and mast cell IgE, this possibility is actively being pursued in our laboratories. From what we have learned from epitope mapping with linear peptides, it is important to note that IgE epitopes are primarily composed of charge and hydrophobic aa, and many of them contain at least 1 lysine (Colombo and others 1998; Chapman and others 2000; Tsai and others 2000; Mine and others 2002; Cocco and others 2003). The same conclusion was found in this paper.

Our initial study found Phe⁹ is the common critical aa for IgE- and IgG-binding epitopes in ALA, this data shows that it may be possible to mutate ALA to no longer bind IgE or IgG by modification of this aa. This raises the possibility that an altered ALA gene could be used to replace its allergenic homologue in the ALA genome. Moreover, the same aa residues between IgE and IgG binding epitopes may provide important information for elucidation of association between IgG and IgE antibodies generation.

Conclusions

A series of aa was identified by alanine substitution scanning. Interestingly, a heterogeneous pattern of critical aa was found between IgE and IgG binding epitopes. Val⁸, Phe⁹, Arg¹⁰, Tyr¹⁰³, Leu¹⁰⁵, and His¹⁰⁷ are the critical aa to IgE binding epitope. Moreover, Phe⁹, Leu¹⁵, Pro²⁴, Trp²⁶, and His³² were the critical aa of IgG-binding epitopes of ALA. This information is necessary to engineer recombinant proteins with decreased allergenicity for use in immunotherapeutic reagents for cow milk allergy. Furthermore, knowledge of the aa position of those that are not as critical for IgE binding may help in developing strategies to silence epitopes of other allergens. In addition, the low incidence of cow milk allergy

in China may increase the difficulty of collecting of cow milk-allergic patients' sera. However, this study is very important, as it provides basic data and theoretical methods for drawing general conclusions in the future. We will continue to collect cow milk-allergic patients' sera for studying the immunological mechanism of cow milk allergens in the next few years.

Acknowledgments

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References

- Adams SL, Barnett D, Walsh BJ, Pearce RJ, Hill DJ, Howden MEH. 1991. Human IgE-binding synthetic peptides of bovine β -lactoglobulin and α -lactalbumin. In vitro cross-reactivity of the allergens. *Immunol Cell Biol*. 69:191–7.
- Breiteneder H, Ferreira F, Reikerstorfer A, Duchene M, Valenta R, Hoffman-Sommergruber K, Ebner C, Breitenbach M, Kraft D, Scheiner O. 1992. Complementary DNA cloning and expression in *Escherichia coli* of Aln g I, the major allergen in pollen of alder (*Alnus glutinosa*). *J. Allergy Clin. Immunol*. 90:909–17.
- Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA. 1997. Mapping and mutational analysis of the IgE-binding epitopes on Ara h1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem*. 245:334–9.
- Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomés A. 2000. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 106:409–18.
- Cocco RR, Järvinen KM, Sampson HA, Beyer K. 2003. Mutational analysis of major, sequential IgE-binding epitopes in α _{s1}-casein, a major cow's milk allergen. *J Allergy Clin. Immunol*. 112:433–7.
- Colombo P, Kennedy D, Ramsdale T, Costa MA, Duro G, Izzo V, et al. 1998. Identification of an immunodominant IgE epitope of the *Parietaria Judaica* major allergen. *J Immunol* 160:2780–5.
- Cong Y, Hong Y, Yuting Q, Linfeng L. 2013. Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes on α _{s1}-casein by alanine scanning analysis. *J Dairy Sci*. 96:6870–6.
- Cong YJ, Li LF. 2012. Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes in β -lactoglobulin by alanine scanning analysis. *J. Dairy Sci*. 95:6307–12.
- Elsayed S, Hill DJ, Do TV. 2004. Bovine-milk α _{s1}-casein using extensively purified synthetic peptides. *Scandinavian Journal of Immunology* 60:486–93.
- Hopp TP, Woods KR. 1982. Immunochemical studies on α -lactalbumin. *Mol Immunol*. 19:1453–63.
- Host A, Husby S, Gjesing B, Larsen JN, Lowenstein H. 1992. Prospective estimation of IgG, IgG subclasses and IgG antibodies to dietary proteins in infants with cow milk allergy. Levels of antibodies to whole milk protein, BLG and ovalbumin in relation to repeated milk challenge and clinical course of cow milk allergy. *Allergy* 47:218–9.
- James JM, Sampson HA. 1992. Immunologic changes associated with the development of tolerance in children with cow milk allergy. *J Pediatr*. 121:371–7.
- Järvinen KM, Chatchatee P, Bardina L, Beyer K, Sampson HA. 2001. IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol*. 126:111–8.
- Little CH, Georgiou GM, Shelton MJ, Cone RE. 1998. Production of serum immunoglobulins and T cell antigen binding molecules for cow's milk antigens in adults intolerant to cow's milk. *Clin Immunol Immunopathol* 89:160–70.
- Maynard F, Jost R, Wal JM. 1997. Human IgE binding capacity of tryptic peptides from bovine α -lactalbumin. *Int Arch Allergy Appl Immunol*. 113:478–88.
- Mine Y, Zhang JW. 2002. Identification and fine mapping of IgG and IgE epitopes in ovomucoid. *Biochem Biophys Res Commun*. 292:1070–4.

- Osterballe M, Hansen TK, Mortz CG, Host A, Bindslev-Jensen C. 2005. The prevalence of food hypersensitivity in an unselected population of children and adults. *Pediatr Allergy Immunol* 16:567–73.
- Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. 1999. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 103:535–42.
- Robotham JM, Teuber SS, Sathe SK, Roux KH. 2002. Linear IgE binding epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J Allergy Clin Immunol* 109:143–9.
- Ruiter B, Trégoat V, M'Rabet L, Garssen J, Bruijnzeel-Koomen CAFM, Knol EF, van Hoffen E. 2006. Characterization of T cell epitopes in α_{s1} -casein in cow's milk allergic, atopic and non-atopic children. *Clin. Exp. Allergy* 36:303–10.
- Savilahti E. 1981. Cow's milk allergy. *Allergy* 36:73–88.
- Sampson HA. 2003. Food allergy. *J Allergy Clin Immunol* 111:S540–S547.
- Stanley JS, King N, Burks AW, Huang SK, Sampson HA, Cockrell G, et al. 1997. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h2. *Arch Biochem Biophys* 342:244–53.
- Tsai LC, Chao PL, Hung MW, Sun YC, Kuo IC, Chua KY, et al. 2000. Protein sequence analysis and mapping of IgE and IgG epitopes of an allergenic 98-kDa *Dermatophagoides farinae* paramyosin, Der f 11. *Allergy* 55:141–7.
- Van Kampen V, Becker WM, Chen Z, Rihs HP, Mazur G, Raulf M, Liebers V, Isringhausen-Bley S, Baur X. 1994. Analysis of B-cell epitopes in the N-terminal region of Chi t 1 component III using monoclonal antibodies. *Mol. Immunol*, 31:1133–40.
- Wal JM. 1998. Cow's milk allergens. *Allergy* 53:1013–22.