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Reduction of antigenicity and allergenicity of genetically modified egg white allergen, ovomucoid third domain

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Abstract

Ovomucoid (Gal d1) is a major allergen in hen egg white, consisting of three tandem domains. In this study, five genetically modified third domain (DIII) mutants, which were substituted single or double amino acids within its IgE and IgG epitopes were compared with those prepared and their antigenicity and allergenicity with native analogue using Western immunoblot and enzyme-linked immunosorbent assay. The replacement of phenylalanine at 37 (F37) position with methionine caused drastical loss of IgG and IgE binding activities of human sera derived from egg allergic patients as well as disruption of the α-helix structure which comprises a part of the IgG and IgE epitopes. Substituting glycine at 32 position in conjunction with F37 showed a synergistic effect of decreasing antigenicity. The present study indicated that glycine 32 and phenylalanine 37 have an important role on its antigenicity and allergenicity as well as structural integrity of ovomucoid DIII.

Keywords: Food allergy; Ovomucoid third domain; Antigenicity; Allergenicity; Site-directed mutagenesis; Mutant; Epitope

Approximately 2–3% of adults and 5–7% of children are plagued by food allergy [1]. Hen egg white is one of the most common and serious of immediate food allergic reactions in infants and young children [2,3]. Approximately, two-third of children diagnosed with food allergies is reactive to egg white [4]. Ovomucoid (Gal d1) has been identified as the dominant allergen found in egg white and is the cause of most allergic reactions in children [5–9]. Ovomucoid is a highly glycosylated protein containing 20–25% carbohydrate, with a molecular weight of 28 kDa and an isoelectric point of 4.1 [10]. It is composed of 186 amino acids that are arranged in three tandem domains, each of 60 amino acids in length. It exhibits varying degrees of inhibitory activity towards a number of serine proteases. Each domain is cross-linked by three intradomain disulfide bonds; however, they lack any interdomain disulfide bonds [11]. This unique structural characteristic might be because of its higher stability against proteolysis [12], and heat [13], as well as its strong allergenicity [13–16].

Our previous study has shown that there are significantly more human IgG and IgE binding activities to the third domain (DIII) than to the first and second domains of ovomucoid in sera derived from egg allergic patients [17]. We have already reported on cloning and expression of the genes of the third domain of ovomucoid in *Escherichia coli* as a fusion protein with glutathione *S*-transferase and its characterization with regard to the IgG and IgE binding activity to egg allergic patients' sera [18]. More recently, we have determined the detailed sequential IgG and IgE epitope mapping in the whole ovomucoid molecules. We have also identified the amino acids within each of the IgG and IgE binding epitopes that are critical for immunoglobulin binding [19]. We have identified two IgE binding sites (K29-S44 and T49-C56) and three IgG binding sites (T30-F37, C35-S44, and T47-F53). Using synthetic peptides, K34-F37, F37C38, E43S44, and T47-H52 in IgG epitopes and Y31-N33, F37C38, and K55C56 in IgE epitopes were determined as critical amino acids, respectively [19]. These information will enable us to alter ovomucoid genes by site-directed mutagenesis to reduce the allergenicity of ovomucoid.

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It has been reported earlier that differences in the amino acid sequence can alter the IgE-binding pattern of allergens. Mutation studies have earlier revealed that the substitution of one single amino acid drastically reduced the ability to bind IgE [20,21].

In view of these facts, we investigated the effect of genetic modifications of ovomucoid DIII with respect to protein structural change and its IgG and IgE binding activities with sera from egg allergic patients. In this study, we designed four single site mutations at G32, F37, T49, and K55) and a double site mutation at G32 and F37 based on our previous work. The genes were constructed with the substitution of a single or double amino acid and their effects on the binding activity were analyzed by Western immunoblot and enzyme-linked immunosorbent assays.

Materials and methods

Reverse transcriptase-polymerase chain reaction. Poly(A)$^+$ RNA was isolated from chicken oviduct using the Poly AT tract mRNA isolation kit from Promega (Promega, Madison, WI, USA) following the instruction manual. The first strand cDNA was synthesized from 1 μg of the poly(A)$^+$ RNA using random primers and reverse transcriptase. The cDNA gene was amplified using one-seventh of the first strand cDNA as the template and oligonucleotide primers 5′-AGAC GCAGACCATTACCTTG-3′ and 5′-CTCAGCCAGCATCAGCAG TT-3′. PCR was performed using 50 μl PCR mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 9.0), 0.2 mM dNTP, 0.5 μM of each primer, and 2.5 units *Taq* Polymerase enzyme (Amersham Pharmacia Biotech, Uppsala, Sweden). The parameters used for PCR were 40 cycles of 1 min denaturing at 94 °C, 1 min annealing at 57 °C, and 1 min elongation at 72 °C. The amplified cDNA product was sub-cloned in pGEM-T easy vector system (Promega) and subjected to sequencing analysis. The resultant plasmid was used as a template to amplify the DIII gene using oligonucleotides 5′-GTAGGAAGGAATGCTTGCTGCTGTG-3′ and 5′-CTCAGCC AGCATCAGCAGTT-3′. The resulting amplified product was subcloned into pGEM-T easy vector system and sequenced for its authenticity. The resultant plasmid was used as a template for amplification of the mutant genes. The DIII gene cloned into the pGEM-T easy vector system was further amplified using primers 5′-CTAGTG ATTGTCGACAGGAATGCTTG-3′ and 5′-CTCAGCCAGCATCA GCAGTT-3′ and cloned into pGEX-4T-2 expression vector (Pharmacia) to make a fusion with glutathione *S*-transferase (GST).

Construction of mutant expression vectors. PCR-based mutagenesis was performed for the construction of mutant domain III genes. The recombinant ovomucoid DIII gene was used as a template, oligos were designed to substitute a single amino acid, and five different mutant constructs (G32M, F37A, T49A, K55A, and GMFA) were cloned into pGEM-T easy vector, and thereafter cloned into the pGEX 4T-2 vector and expressed in *E. coli*. The constructs were analyzed for sequence and the substitution of the appropriate nucleotides was confirmed.

The sequences of mutagenesis primer were as follows:

G32M-sense 5′-CAAAACATATATGAACAAGTGCAAC-3′
G32M-reverse 5′-GTTGCACTTGTTCATATATGTTTTG-3′
F37A-sense 5′-CAAGTGCAACGCCTGCAATGCAGT-3′
F37A-revers 5′-GACTGCATTGCAGGCGTTGCACTTG-3′
T49A-sense 5′-CGGGACTCTCGCTTTAAGCC-3′
T49A-reverse 5′-GGCTTAAAGCGAGAGTCCCG-3′
K55A-sense 5′-GCCATTTTGGAGCATGCTGAATAT-3′
K55A-reverse 5′-ATATTCAGCATGCTCCAAAATGGC-3′

The separate amplified 5′ and 3′ fragments were purified by agarose gel and then combined by PCR without template. The PCR parameter was 5 cycles of 1 min denaturing at 94 °C, 1 min annealing at 42 °C, and 1.5 min elongation at 72 °C and then 15 cycles of 1 min denaturing at 94 °C, 1 min annealing at 55 °C, and elongation at 72 °C. The combined fragments were re-amplified with Ovo DIIIsal and Ovo3L primers. The amplified PCR products were ligated into the pGEM T-easy vector (Promega, Madison, WI, USA). Obtained clones were analyzed their sequencing and the substitution of appropriate nucleotides was confirmed.

Expression and purification of recombinant domain III. The GST and ovomucoid DIII fused protein was expressed by transforming the plasmid into competent *E. coli* JM109 (Life Technologies, Gaithersburg, MD, USA). The transformed cells were incubated overnight in a 5-ml culture of 2XTY medium at 37 °C. The cultured medium was inoculated into 500 ml of fresh 2XTY medium and grown to log phase. This preparation was cultured for 3 h. In order to induce ovomucoid gene expression, 1 mM isopropyl-β-D-thiogalactophyranoside (IPTG; Amersham Pharmacia Biotech) was then added to a final concentration of 1 mM. After induction of the fusion protein, cells were harvested by centrifugation at 7000*g* for 5 min. The cell pellet was re-suspended in 10 ml of lysis buffer (40 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid, and 0.5% Triton X-100) and sonicated three times for 30 s using a sonicator (Model 250 Sonifier, Branson, Danbury, CT, USA) at 40 W. The cell suspension was centrifuged at 15,000*g* for 10 min to obtain the supernatant containing solubilized fusion protein.

Glutathione–Sepharose 4B (GS4B; Amersham Pharmacia Biotech) was prepared according to the manufacturer's instructions. The GS4B was combined with an equal volume of cold phosphate-buffered saline (PBS) to produce a 50% slurry and 1 ml of the slurry was added to the cell extract. The mixture was incubated overnight at 4 °C with gentle shaking, then transferring to a disposable column (Bio-Rad Laboratories, Hercules, CA, USA), and washed thoroughly with cold PBS to remove unbound material. Site-specific cleavage of the fusion protein was carried out by incubating the bound GS4B-GST-DIII with 1 U/μl thrombin protease (Amersham Pharmacia Biotech) for 6 h at room temperature with gentle shaking. The DIII was removed from the column by washing with cold PBS. The purified DIII was then dialyzed against water and lyophilized. The extra N-terminal sequence (Gly–Ser–Pro–Gly–Ile–Pro–Gly–Ser–Thr–Gly–Met) raised from the hydrolysis of GST-DIII with thrombin was cleaved with cyanogen bromide (CNBr; Sigma Chemical, St. Louis, USA) in the presence of 70% of formic acid, as described previously [17] except a mutation of G32M. Because the mutation of G32M contains Met residue and is susceptible to CNBr treatment, the incubation time was changed to 2 h instead of 18 h to obtain a selective cleavage of the targeted Met site at the N-terminal sequence. The DIII was further purified using high-performance liquid chromatography (HPLC) ion-exchange chromatography. An aliquot (5 mg) of each sample was dissolved in 0.5 ml of 20 mM Tris–HCl buffer, pH 7.0, and then applied to a Bio-Scale Q5 column (Bio-Rad) equilibrated with the same buffer. The column was eluted with a linear gradient of 0–1.0 M NaCl in 20 mM Tris–HCl buffer, pH 7.0, at a flowrate of 1.0 ml/min using a Bio-Rad Biologic HPLC system. The N-terminal sequence of DIII was analyzed with a 491 protein sequencer (PE Applied Biosystems, Foster City, CA, USA), after which the protein was transferred from SDS–PAGE gel to a polyvinylidene fluoride transfer membrane membrane (ProSorb PE, Applied Biosystems) and subjected to N-terminal sequence analysis.

Preparation of the native ovomucoid third domain. The native ovomucoid DIII without carbohydrate was prepared by hydrolysis of ovomucoid with *Staphylococcus aureus* V_8 (Spase V_8) protease (Sigma), as described in a previous paper [17].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli [22] in a Mini-Protean II Electrophoresis Cell (Bio-Rad). Samples were run on 15% homogeneous gels. Proteins were dissolved in sample buffer in the presence of 5% (w/v) β-mercaptoethanol, heated for 5 min at 95 °C in an Eppendorf Thermomixer 5436 (Fisher Scientific, Unionville, CA, USA), and loaded onto the gel at a concentration of 15 μg/well. Gels were run at a constant current (18 mA/slab gel).

Western immunoblot. Immediately following electrophoresis, the proteins were transferred electrophoretically onto a nitrocellulose membrane (Micron Separations, Westborough, MA, USA) using a modification of the method of Towbin et al. [23]. Electroblotting was carried out at 100 V, for 120 min at 4 °C, on a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After electroblotting, the nitrocellulose membrane was blocked with 2.0% bovine serum albumin (BSA) in 20 mM Tris-buffered saline (pH 7.5) and probed with pooled human sera from egg-allergic patients (25× dilution with Tris-buffer saline containing 1% BSA for IgE and 100× dilution for IgG) for 12 h at 25 °C. The nitrocellulose membrane was incubated with alkaline phosphatase-conjugated goat-anti-human IgG (10,000× dilution with Tris-buffered saline containing 1% BSA) or monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma; 1000× dilution) for 12 h at 25 °C, and stained with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Life Technologies). Color development was terminated by washing the membranes with deionized water.

Circular dichroism. The far-UV circular dichroism (CD) spectra of recombinant DIII and native DIII were determined at room temperature in 20 mM phosphate buffer, pH 7.5, using a Jasco J600 spectropolarimeter (Easton, MD, USA). Each sample was scanned four times from $\lambda = 190$ to 250 nm in a quartz cuvette (Japan Spectroscopic, Tokyo, Japan) with a 0.1-cm path length. A buffer baseline was substracted from the spectra of each sample. The percentages of the different secondary structures (α-helix, β-structure, and random coil) were estimated using a Jasco protein secondary structure estimation program, based on the method of Yang et al. [24].

Enzyme-linked immunosorbent assay. The antigenicity and allergenicity of recombinant DIII and its mutants were determined by an indirect enzyme-linked immunosorbent assay (ELISA), according to a modification of the method of Engvall and Perlmann [25]. Medical-grade, high-binding polystyrene 96-well microplates (Corning, Cambridge, MA, USA) were pre-coated with DIII or its mutants (1 μg/well in 100 mM carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBST), pH 7.2, using a Bio-Rad Immunowash Microplate Washer. The plate was blocked with 2.5% BSA in PBS for 2 h at 37 °C, washed with PBST, and incubated overnight at 25 °C with human serum (diluted to 1:25 for IgE and 1:100 for IgG assay) in PBS containing 1% BSA. The plate was again washed and incubated overnight at 25 °C with rabbit alkaline phosphatase-conjugated anti-human IgE diluted to 1:1000 or goat anti-human IgG diluted in 1:10,000 in PBS. The plate was washed and developed with ρ-nitrophenol phosphate (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at room temperature. The reaction was terminated by the addition of 25 μl of 3 N NaOH and the plate was read at $\lambda = 405$ nm using a Bio-Rad Microplate Reader.

Human serum. Human serum was collected from four patients (3–5 years old) exhibiting class 2–4 radioallergosorbent test towards egg. All of the patients were allergic to egg white. All serum samples were kindly provided by Dr. A. Urisu (Department of Pediatrics, Fujita Health University, Japan) and Dr. Morikawa (Department of Pediatrics, Gunma University, Japan) and stored at −80 °C until use.

Statistical analysis. Data were analyzed by using analysis of variance (ANOVA-SPSS version 7.5 for windows: SPSS Chicago, IL, USA) and the level of significance between antibody activity was defined at $p < 0.05$.

Results and discussion

The cDNA containing the DIII region of ovomucoid was cloned into pGEX-4T-2 vector downstream of the ptac promoter as published earlier [18]. Mutants with the substitution of single or double amino acids were made and the genes were cloned into the PGX-4T-2 vector and expressed in *E. coli*. Upon induction with IPTG, the proteins were expressed in a soluble form and followed the confirmation by SDS–PAGE and immunoblot as well as previous work of expressing a wild-type [18] (data are not shown). All the mutant proteins were expressed in a soluble form using GST fusion protein. The recombinant proteins were purified on glutathione–Sepharose 4B and the following HPLC. The reactivity of human pooled sera (IgE) from egg allergic patients with each mutant was investigated using Western immunoblot after transferred the protein onto the nitrocellulose membrane from a SDS–PAGE gel (15%) and proved with pooled human patients' sera as shown in Fig. 1. It was observed that the mutants cloned were of the same molecular size with the recombinant wild-type and native DIII. The mutants substituted of a single amino acid of phenylalanine with alanine (F37A) and two point mutations of glycine (G32) and phenylalanine (F37) with methionine and alanine (GMFA) did not exhibit any bands corresponding to DIII, resulting in complete loss of IgE binding capacity. We also examined the IgG binding in this manner and found that only two mutants of F37A and GMFA completely lost

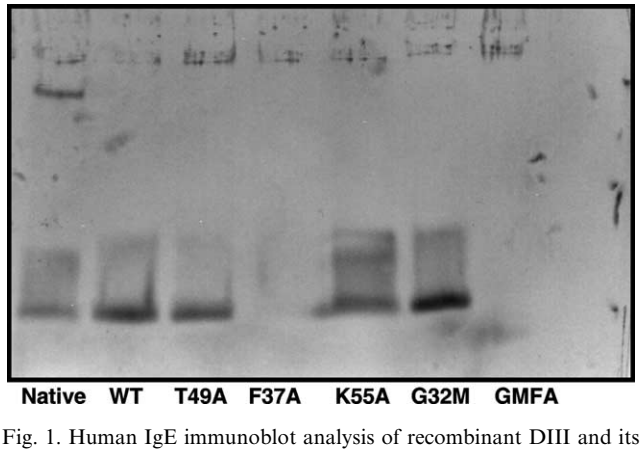

Fig. 1. Human IgE immunoblot analysis of recombinant DIII and its various mutants under reduced conditions. The letters on the left of the number correspond to the one-letter code for the residue normally occurring at that position and the letters on the right represent those substituted with alanine or methionine residue at each position. WT indicates the wild-type DIII with no amino acid substitutions.

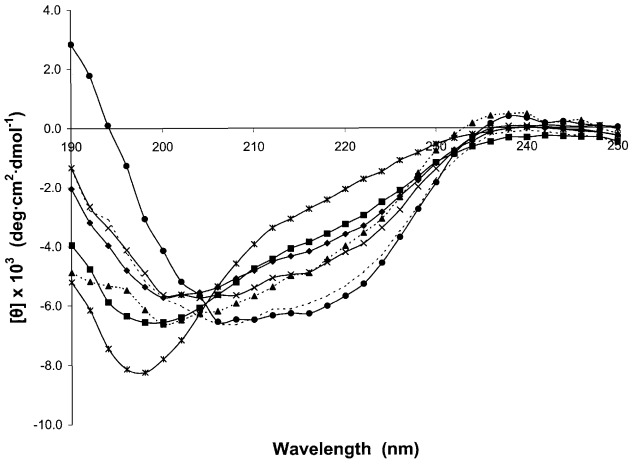

Fig. 2. Far-UV spectra of native and various mutants of DIII. A 0.15 mg/ml solution of each sample was prepared in 10 mM phosphate buffer, pH 7.0. The spectrum was obtained by scanning the sample four times from 190–250 nm. ––: Native, -●-: WT, -1-: GMFA, -X-, T49A; -▲-, G32M; -■-, F37A; -◆-, K55A.

Table 1
Secondary structure fraction of native and recombinant mutants of ovomucoid third domains

Forms	Secondary structure (%)			
	α-helix	β-sheet	β-turn	Random coil
K55A	13.6	21.3	19.6	45.5
F37A	14.6	15.5	22.4	47.4
Native	20.8	7.0	22.5	49.7
G32M	16.8	11.5	24.4	47.3
T49A	15.5	18.3	19.9	46.2
GMFA	10.6	21.3	21.8	46.4
WT	17.1	22.7	16.3	43.9

Note. The proportion was estimated from the far-UV CD spectra using a method of Yang et al. [24].

their IgG binding activity as well as IgE binding activity (data are not shown). These results suggested that sequential epitopes which might be important for binding human IgG and IgE derived from egg allergic patients were destroyed by substituting at phenylalanine 37, while their binding activity was still retained by mutating other single amino acids of threonine 49, lysine 55, and glycine 32. From the immunoblot analysis, it was not clear about the difference of IgG and IgE binding between F37A and GMFA with respect to structural and the following changes of antibody binding.

The secondary structures of the native, recombinant wild-type, and various mutants DIII were analyzed by CD (Fig. 2) and the percentage of secondary structure is summarized in Table 1. The CD spectra of native and WT DIII were almost identical. The secondary structure of recombinant DIII (WT) was comprised of 17.1% α-helix, 39.0% β-structure, and 43.9% random coil, a slightly lower α-helix content compared to that of native analogue. This is in good agreement with our previous work [18]. All DIII mutants had different CD profiles to that of native or WT form, resulting in the fact that π–π^* transition at 209 nm was shifted to a higher energy, decreasing the α-helix structure. Substitution of lysine at 55 position with alanine decreased the α-helix content, but did not affect human IgG and IgE binding activities. Mutating two amino acids of glycine at 32 and phenylalanine at 37 positions dramatically decreased α-helix structure and caused complete loss of its antibody binding capacity. Previously, we mapped IgG and IgE epitopes in ovomucoid DIII, which are comprised of T30F37, C35S44, and T47F53 for IgG epitopes, and K29S44 and T49C56 for IgE ones, respectively. Ovomucoid

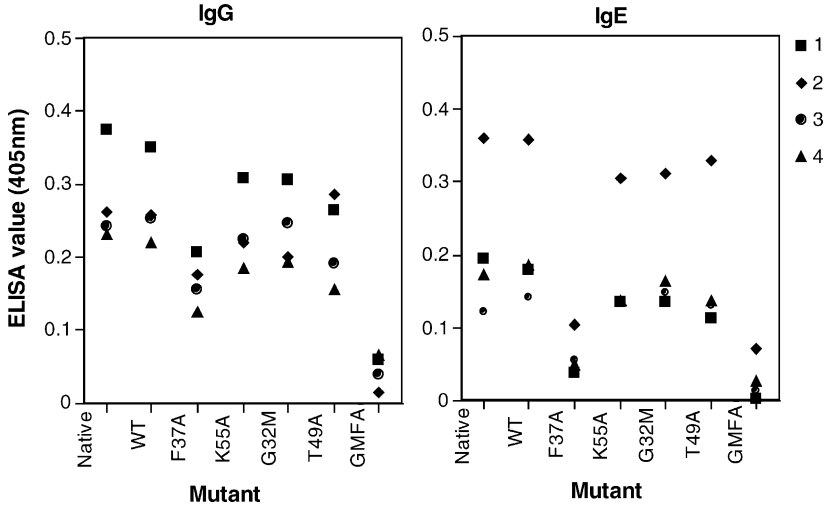

Fig. 3. Comparison of antigenicity and allergenicity of recombinant DIII and its various mutants. Four individual serums from egg allergic patient were used, showing four different symbols. The binding activities of human specific IgG and IgE with the proteins were analyzed by ELISA.

DIII is consisting of one α-helix (residues 34K-43E) and three β-sheet (23V-25G, 30T-31Y, and 52H-53F) structures [26]. The epitopes of T30F37, C35S44 (IgG), and K29S44 (IgE) comprise of β-sheet (30T-31Y) and α-helix (34K-43E). Substitution of G32, which is adjacent to the α-helix and is comprising of the β-bend, did not affect its significant change of α-helix compared to that of WT. Displacing a single amino acid of F37 with alanine did not affect the drastic decrease of α-helix structure, regardless it being in the center of the α-helix of DIII. However, two site mutations of G32 and F37 caused the disruption of the α-helix coil of DIII, indicating that α-helix (K34-E43) within IgG and IgE epitopes might have important implication in egg allergic reactions.

Fig. 3 shows the IgG and IgE binding activities of native DIII and various recombinant DIII mutants with individual serum from four egg allergy patients. The binding activities of IgG and IgE of WT antigen were almost the same as that of native form when tested with an ELISA ($p > 0.05$), indicating that WT DIII was identical in its antigenicity and allergenicity against human sera used in this study to the native form. Three mutants of K55A, G32M, and T49A did not exhibit any significant changes of IgG and IgE binding activities, regardless of these amino acids being identified as a critical one [19]. Interestingly, substituting phenylalanine with alanine (F37A) significantly decreased their binding activities of IgG and IgE from egg allergic patients, while G32M mutant retained the activities. Both antigenicity and allergenicity of GMFA DIII was almost completely lost. The loss of activities in the GMFA seems to have been caused largely by the replacement at residue F37 and substituting G32 as well as F37 contributing to the complete loss of IgG activity.

In conclusion, it is suggested that the F37, a core of α-helix structure, has an important role on antigenicity and allergenicity as well as a key element of structural integrity of ovomucoid DIII. G32 which is located in β-bend could have synergistic effect on the antigenicity of DIII. The present study provides useful information on the molecular approaches to alter ovomucoid DIII gene to reduce its antigenicity and allergenicity as well as a better understanding of structure–functional relationships of allergenic epitopes in food allergens.

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