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Genetic attachment of undecane peptides to ovomucoid third domain can suppress the production of specific IgG and IgE antibodies

Yoshinori Mine* and Prithy Rupa

Department of Food Science, University of Guelph, Guelph, Ont., Canada N1G 2W1

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Abstract

An undecane peptide (Gly–Ser–Pro–Gly–Ile–Pro–Gly–Ser–Thr–Gly–Met) was genetically attached to the N-terminus of ovomucoid third domain (DIII) to investigate structural characteristics of linear IgE and IgG (B cell) epitopes in DIII with respect to modulation of the immune response towards antigenicity and allergenicity. Balb/c mice were sensitized with native DIII, wild type recombinant DIII, and recombinant modified DIII containing the extra amino acid stretch. The immune responses to the antigens were compared using enzyme-linked immunosorbent assay. Interestingly, specific IgE and IgG levels were suppressed when the modified DIII was used as antigen. This was further confirmed by synthesizing immunodominant IgE and IgG epitopes of DIII on cellulose acetate membrane (SPOTs) and probing them with antibodies raised against DIII antigens. Anti-recombinant wild type DIII anti-serum showed strong binding activities to immunodominant IgE and IgG epitopes, while anti-modified DIII serum did not show any significant binding to the IgE and IgG epitopes. Thus, it is clearly demonstrated that the amino acid stretch in DIII is masking the immune reactive epitope. Genetical attachment of peptides into DIII was found to be effective in reducing the production of specific IgE and IgG antibodies in mice.

Keywords: Food allergy; Egg white; Ovomucoid third domain; IgE and IgG epitope; Balb/c mice; SPOTs assay

Allergic response involving the combination of antigen and IgE antibodies of a sensitive individual can result in anaphylaxis. Cross-linking of mast cell-bound IgEs by allergen leads to the release of inflammatory mediators responsible for the immediate allergic reaction [1]. Several approaches have been used to suppress IgE response to allergens. The progress of recombinant techniques for producing allergens and allergen derivatives has led to a dramatic improvement in the ability of developing novel vaccines for the treatment of allergy. Genetic modifications of allergens [2,3], chemical modifications of allergens [4–6], oral tolerance of the allergens [7,8], mucosal tolerance [9], and different immune regulatory processes [10] have been defined, to suppress or inhibit IgE response. Modifications of recombinant allergens with a reduced capacity to bind cell-bound IgE would ensure a lower risk of IgE-mediated side effects and the retention of their T-cell epitopes within the allergen, would allow for a modulation of the immune response [11]. Ovomucoid is one of the best characterized egg white allergens, which is composed of 186 amino acids that are arranged in three tandem domains [12]. Our observations show that IgG and IgE binding activities to the third domain (DIII) are significantly more when compared to the first (DI) and the second (DII) domains [13]. We had also cloned and characterized the third domain of ovomucoid in *Escherichia coli* to study the allergenic properties of the third domain in detail [14]. Recently we have reported cloning and immunological characterization of the whole ovomucoid molecule [15]. Detailed epitope mapping has been reported and amino acids that are critical for IgG and IgE immunoglobulin binding have been identified [16].

Induction of murine IgE responses requires both a genetically based IgE high-responder phenotype and defined experimental condition [17]. Protein structure is believed to play a key role in determining the IgE epitopes that are important to immunoglobulin binding [18]. It has been demonstrated earlier that a change in

* Corresponding author. Fax: +1-519-824-6631.
E-mail address: ymine@uoguelph.ca (Y. Mine).

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the amino acid sequence can alter the IgE binding pattern of allergens [2,11,19]. So it is important to assess the protein-specific IgE response, as well as the IgG1 response in mice, with regard to the immune response of allergens in an in vivo model system. In the present study, we have evaluated the effect of IgE and IgG immune responses that is caused in mice due to an addition of extra stretch of amino acid in the recombinant DIII of ovomucoid. The native ovomucoid third domain (DIII), the wild type recombinant DIII, and the modified recombinant DIII with undecane peptide at N-terminus were purified separately on high-performance liquid chromatography (HPLC) ion exchange chromatography. Balb/c mice were sensitized and challenged with the purified antigens and total and specific serum IgE and IgG after challenge were compared using enzyme liked immunosorbent assay. The immune reactive response was also checked against the immunodominant epitope of DIII synthesized on cellulose acetate membrane using SPOTs analysis. In this study, we found that genetically attached undecane peptide into DIII led to a significant inhibition of IgE and IgE production in mice. This allergy-related antibody response in the mice model could lead us to a new strategy to reduce allergy in humans.

Materials and methods

Animals and reagents. Female Balb/c mice were maintained at central animal facility, University of Guelph. Six to eight week mice were obtained and bred in specific pathogen free environment. SPOTs membrane was purchased from Genosys Biotechnologies (Woodlands, Texas). Cyanogen bromide and dimethylformamide were obtained from Sigma (St. Louis, USA). All animal studies were conducted under guidelines and permission from University of Guelph, Animal Utilization Committee.

Preparation of the purified antigens of 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 earlier [13]. The protein was further purified on HPLC using ion exchange Bio-scale Q 5 column (Bio-Rad Laboratories, Hercules, CA, USA). The recombinant ovomucoid DIII was cloned and expressed as a fusion protein with glutathione *S*-transferase in *E. coli* from our laboratory which was published earlier [14]. The recombinant DIII was purified using glutathione–Sepharose 4B (Amersham–Pharmacia Biotech, Uppsala, Sweden) and followed by hydrolysis of the fusion protein with thrombin according to the manufacturer's instructions. The DIII was further purified using Q5 column on Bio-Rad Biologic HPLC system. The extra N-terminal sequence consisting of the 11 amino acids (Gly–Ser–Pro–Gly–Ile–Pro–Gly–Ser–Thr–Gly–Met) which was genetically inserted into DIII was cleaved with cyanogen bromide (CNBr) in the presence of 70% formic acid, as described previously [14]. After the cleavage of the peptide, the DIII was dialyzed against 20 mM Tris–HCl buffer, pH 7.0, further purified on HPLC ion exchange chromatography using Mono Q column (Pharmacia) equilibrated with the same buffer, and eluted with a linear gradient of 0–1.0 M NaCl in 20 mM Tris–HCl buffer, pH 7.0, at a flow rate of 1.0 ml/min.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed according to a method of Laemmli [20]. Fifteen percent homogeneous gels were run in a Mini-Protean II Electrophoresis Cell (Bio-Rad). The purified proteins were dissolved in the sample buffer in the presence of 5% (w/v) β-mercaptoethanol and run under denaturing conditions at a constant current (18 mA/slab gel).

Immunizations in mice. Six to eight week old female Balb/c mice were immunized subcutaneously with 50 μg of the purified proteins with Complete Freund's adjuvant (Sigma). Pre-immune sera were collected before sensitization and non-immunized mice were maintained as controls. Two weeks after the first injection, the animals were given a booster injection of the same doses of antigens with Incomplete Freund's adjuvant (Sigma). After seven days the blood was collected and checked for titer levels using immunoassays. Four mice were used for sensitization per antigen.

Enzyme-linked immunosorbent assay. Serum titers of ovomucoid-specific and total IgE and IgG were determined according to a previously reported method, with some modifications [21]. Enzyme-linked immunosorbent assay was performed to determine the production of total IgG and IgE levels in the anti-sera raised in mice against the native and recombinant ovomucoid DIII antigens. One-hundred microliters of goat anti-mouse IgG (Cedarlane, Hornby, ON, Canada) and anti-mouse IgE monoclonal antibodies (BD Bio Science, San Diego, CA) (1 μg/well) in 50 mM sodium carbonate buffer (pH 8.5) was added to each well of a 96-well ELISA high-binding polystyrene microtiter plate (Corning, Cambridge, MA). The plate was kept at 4 °C overnight. After the plate was washed three times with Tris-buffered saline (TBS) containing 0.05% Tween (TBST) using a Bio-Rad Immunowash Microplate Washer, incubation was followed with 150 μl of 2% BSA in TBS for 2 h at 37 °C. The plate was washed with TBST three times and incubated for 2 h at 37 °C with 100 μl of different dilutions of the serum obtained from mice. Standard pure mouse IgG and IgE antibodies (BD Bio Science) were used as controls. After the plates were washed with TBST four times, 100 μl of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) (1:2000 dilution), for measuring IgG levels, and monoclonal anti-mouse IgE conjugated to biotin (1:1000 dilution) (Sigma), for determining IgE levels, was added to each well and incubated for 2 h at 37 °C. For IgE assay, the plate was further incubated with avidin conjugated alkaline phosphatase (1:3000 dilution) (Sigma) for 1 h at 37 °C. The plate was washed six times and developed with 100 μl of *p*-nitrophenol phosphate (1 mg/ml) (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at 37 °C. The reaction was terminated by adding 25 μl of 3 N sodium hydroxide. Absorbance at 405 nm was read by microplate reader (Model 550, Bio-Rad Laboratories) For specific IgG and IgE determination, recombinant and native DIII was coated (1 μg/well) instead of anti-mouse IgG and IgE antibody coating and followed the same procedures.

Peptide synthesis (SPOTs assay) and probing of membrane. An immunodominant region (amino acids 29–44 and 47–56) of DIII recognizing IgE and IgG epitopes by human patient's sera reported earlier [16] was synthesized on a derivatized cellulose membrane using Fmoc amino acid active esters (SPOTs synthesis) according to manufacturer's instructions (Genosys). Briefly, synthesis of each peptide began by esterifying a Fmoc amino acid to the cellulose membrane. Coupling reactions were followed by acetylation with acetic anhydride in *N*,*N*-dimethylformamide to render peptides unreactive during the reactions. Fmoc protective groups were removed by addition of piperidine to render the nascent peptides reactive. All the remaining amino acids were added by the same process of coupling, blocking, and deprotection until the desired peptide was generated. After addition of the last amino acid, the side chains of the peptide were deprotected with a 1:1:0.05 mixture of dichloromethane:trifluoroacetic acid:tributylsilane and washed with methanol. Membrane containing the synthetic peptide was blocked with the blocking buffer (Genosys Biotechnologies) at room temperature overnight, washed with TBST, and probed with mice sera raised against recombinant wild type DIII and modified DIII for 2 h at room temperature. The membrane was further washed with

TBST and for detection of IgE, biotinylated anti-mouse IgE (1:1000) (Sigma) was added and for IgG epitopes, alkaline phosphatase conjugated with rabbit anti-mouse IgG (1:10,000) (Sigma) was added and incubated for 2 h at room temperature. The membrane was further washed with PBST and developed with chemiluminescent substrate CDP-Star (Boehringer Ingelheim, Mannheim, Germany) and enhancer Nitro-Block II (Tropix, Bedford, MA) both diluted to 1:100 with 0.1 M Tris–HCl, 0.1 M sodium chloride, pH 9.5. The membrane was visualized under a light imager (EG & G Berthold, Bad Wildbad, Germany). The image processing was done using Win Light software (EG & G Berthold).

Statistical analysis. Differences between immune response were analyzed by using analysis of variance (ANOVA-SPSS version 7.5 for windows: SPSS Chicago, IL, USA). Duncan's post hoc test was performed and the level of significance between antibody activity was defined at an alpha error level of 0.05%.

Results

Purification of ovomucoid DIII antigens

The native and recombinant ovomucoid DIII proteins were purified and analyzed for their purity on SDS–PAGE gel which is shown in Fig. 1. All the three proteins, the native DIII, recombinant wild type DIII, and modified DIII, were purified to homogeneity. No impure bands were detected. There is a slight difference in the migration position between the native and the recombinant DIII due to the genetic modifications. Silver staining of the proteins was done to further confirm that the antigens did not contain any impurities (data not shown).

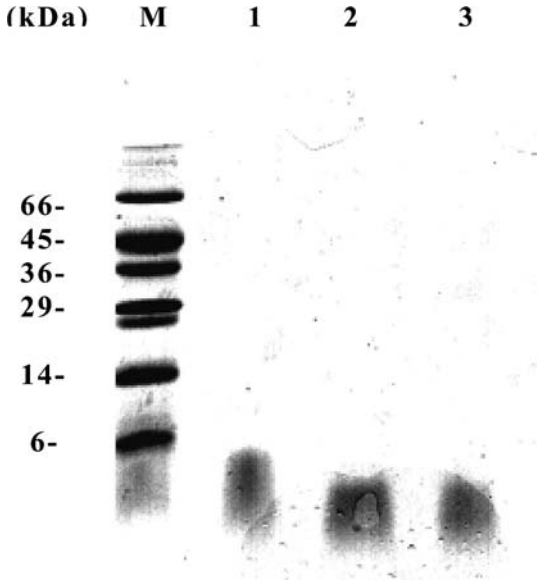

Fig. 1. Sodium-dodecyl sulfate–polyacrylamide gel electrophoresis. A 15% SDS–PAGE gel was run to analyze the different ovomucoid DIII samples. M denotes the low range molecular markers from Sigma. Lane 1 represents the native DIII, lane 2 represents the purified recombinant wild type DIII, and lane 3 represents the modified DIII which was genetically attached undecane peptide.

Effect of sensitization in mice (serum total and specific IgE and IgG levels)

Total and specific IgG and IgE levels were determined using an enzyme-linked immunosorbent assay which is demonstrated in Fig. 2. As shown in the figure, the levels of total IgG antibodies among three antigens were not significantly different and showed high response, while total IgE level of recombinant modified DIII was slightly lower than others. Interestingly, the levels of specific IgG and IgE were significantly reduced in the recombinant modified DIII and the suppression appeared to be associated with the undecane peptide attached to DIII. This was compared to the immune response of native DIII and to the recombinant DIII devoid of the extra amino acid sequence which showed high levels of response and indicated that the extra stretch of amino acids plays a key role in masking the antigenicity and allergenicity in mice. As much as 80.9% of antigenicity (IgG) and 84.8% of allergenicity (IgE) of DIII were suppressed by genetical attachment of undecane peptide into DIII, respectively.

Reactivity of the immunodominant peptide of DIII (SPOTs assay)

Fig. 3 represents the reactivity of the immunodominant epitope (amino acid residues 29–44 and 47–56) to the IgE and IgG antibodies to recombinant wild type DIII (Fig. 3A) and the recombinant modified DIII (Fig. 3B). Fig. 3A showed clear recognition of the IgE epitope against wild type DIII (spots 2 and 3). The IgG against wild type DIII showed weak positive reaction to spot numbers 2, 3, and 11. Mice recognized the amino acid residues 29–37 in IgE response, while amino acid residues 29–37 and 47–55 were recognized by IgG antibody. Alternatively in Fig. 3B, no signal was detected and it was significant that there was an immunosuppression of activity of anti-recombinant modified DIII sera to the IgE and IgG epitopes.

Discussion

Hypersensitivity reactions to foods affect up to 6% of children in the first few years of life [22]. Egg allergy is present in about two-thirds of food-allergic children with atopic dermatitis [23,24]. Egg white proteins more frequently cause allergic reactions than egg yolk [25]. Ovomucoid has been shown to be the dominant allergen [26,27]. This may be due to the unique characteristics of ovomucoid such as relative stability against digestion with proteases [28], heat [29], and its strong allergenicity [30] compared with other egg white components. Several methods to reduce the allergenicity of protein allergens have been conducted in the past [2–10]. However, the

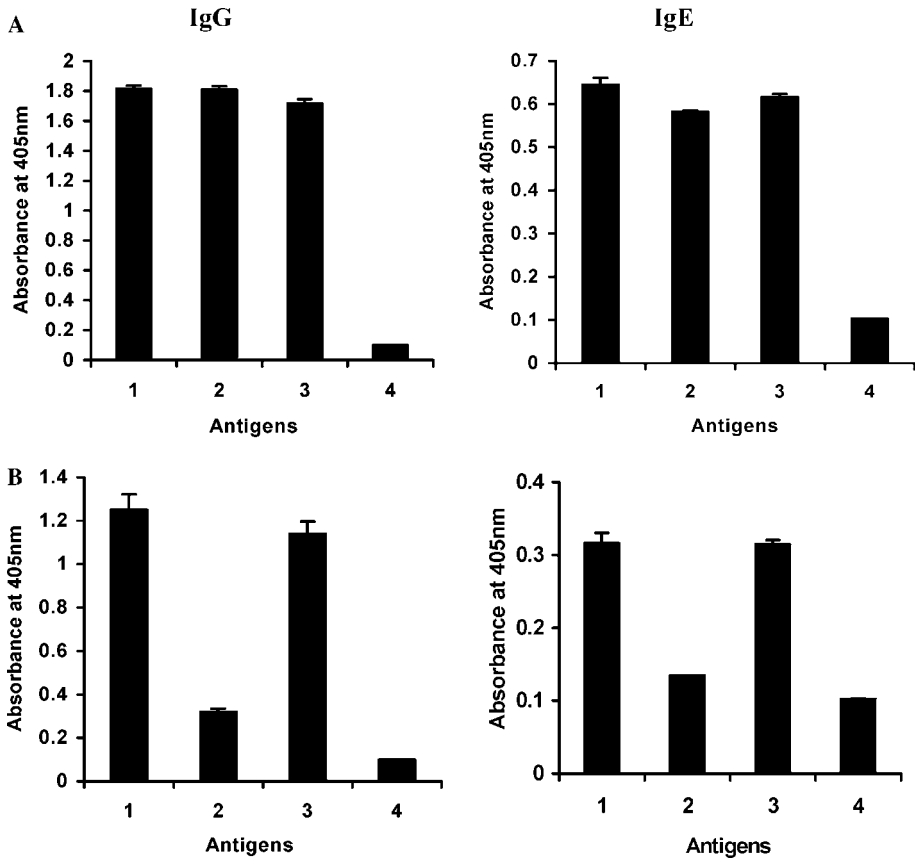

Fig. 2. Enzyme-linked immunosorbent assay. (A) Total IgG and (B) specific IgG and IgE levels in mice. (1) native DIII, (2) recombinant modified DIII, (3) recombinant wild type DIII, and (4) control (without antigen).

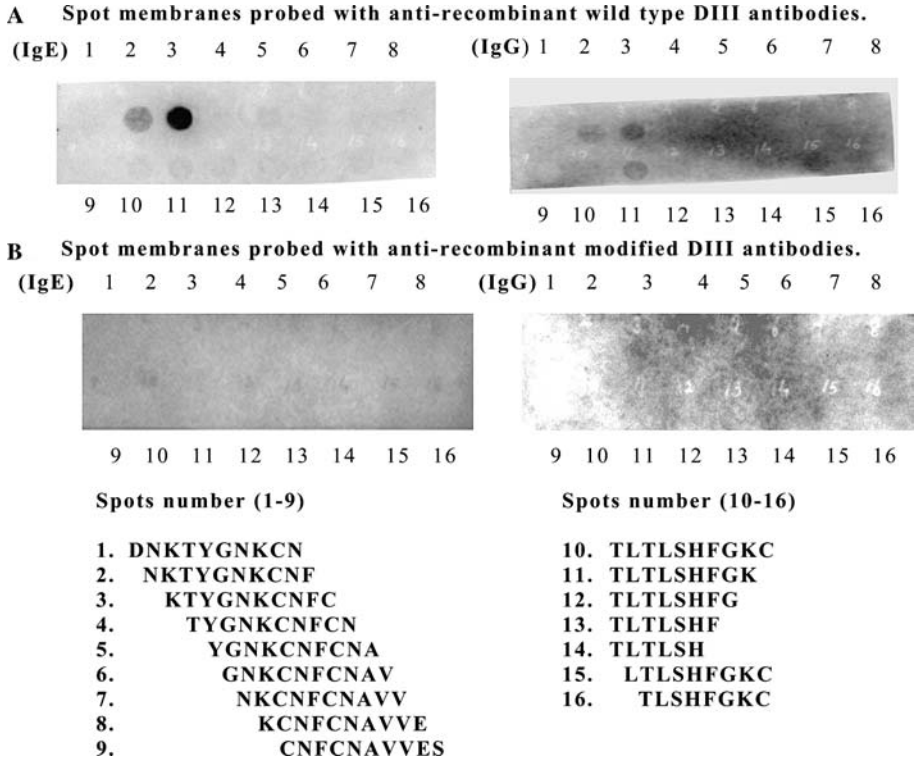

Fig. 3. SPOTs assay (peptide synthesis assay). (A,B) The membrane probed with antibodies against the recombinant wild type DIII and recombinant modified DIII, respectively. The amino acid sequence of each spot was shown.

mechanism for the reduced antigenicity and allergenicity of proteins is still unclear. It has been proposed that masking of the allergenic structure or modulation of the T-cell response to allergens could be effective in decreasing the allergenicity of the protein [31,32]. Genetic attachment of polysaccharide to lysozyme or conjugation it to native lysozyme and soybean allergen, P34 protein, using the Maillard reaction was found to be effective in reducing the production of IgE and IgG antibodies in mice [31].

Previously, we mapped IgG and IgE epitopes in ovomucoid DIII, which are composed of T30F37, C35S44, and T47F53 for IgG epitopes, and K29S44 and T49C56 for IgE ones using patients' sera from egg allergy [16]. Ovomucoid DIII consists of one α-helix (residues 34K-43E) and three β-sheet (23V-25G, 30T-31Y, and 52H-53F) structures. The epitopes of T30F37, C35S44 (IgG), and K29S44 (IgE) comprise of β-sheet (30T-31Y) and α-helix (34K-43E) [2]. The present results clearly demonstrate that genetic attachment of undecane peptide into ovomucoid DIII caused dramatic decrease of both specific IgE and IgG antibody production. It is assumed that it is because of suppression of immunological recognition of dominant IgG and IgE epitopes in DIII due to masking or structural hindrance caused by the peptides. To demonstrate the hypothesis that the genetically attached peptide is masking the antigenic and allergenic regions of ovomucoid DIII, we synthesized peptides that represented an immunoreactive IgE and IgG region against mice sera. The recognition of IgE and IgG epitopes by mouse was slightly different from those by egg-allergic patients. Mouse IgE recognized a sequence of K29F37 more strongly, which comprises of β-sheet and α-helix. Mouse IgG antibody recognized the amino acid residues of 29–37 and 47–55. The synthetic peptide corresponding to the immunoreactive region in mice had no IgE and IgG binding ability against anti-recombinant modified DIII sera, while the synthetic peptide displayed the strongest IgG and IgE binding regions against anti-recombinant wild type DIII sera (Fig. 3). These results are consistent with those observed on the immunoassay on ELISA in Fig. 2. Ribbon diagram representing the corresponding amino acids at the amino terminal end which is shown in Fig. 4 clearly indicates that the undecane peptide seems to be masking the immunodominant epitope (residues 29–37) in the DIII. It has been shown that the F37, a core of α-helix structure, has an important role in antigenicity and allergenicity as well as a key element of structural integrity of ovomucoid DIII. The G32, which is located in β-bend, has a synergistic effect on the antigenicity of DIII [2]. It could be of great interest to further investigate how an addition of extra stretch of amino acid in the recombinant DIII of ovomucoid can interact with particular amino acids in the epitope and cause changes in the immunological responses.

It could be concluded that the masking immunodominant epitopes are responsible for loss of activity of recognition of the IgG and IgE binding regions. We conclude that the epitopes could be masked by modifications in the gene of the respective allergen by genetic engineering. To date, there is little known about the

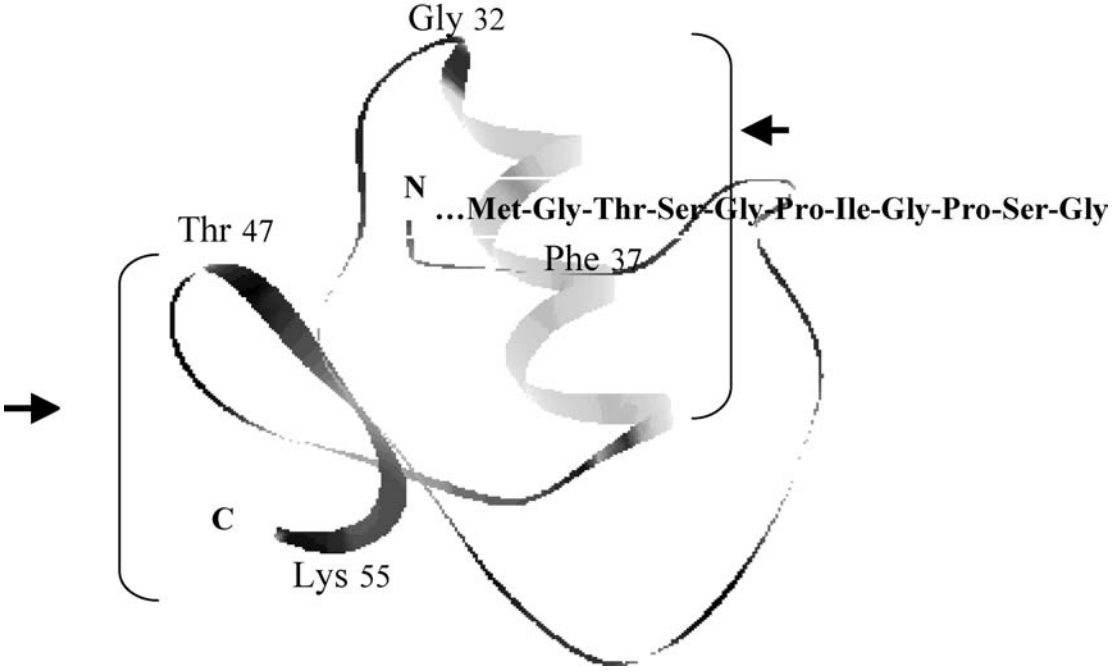

Fig. 4. Molecular structure of ovomucoid third domain. The genetically attached undecane peptide is shown in the molecular structure of the DIII. N represents the amino terminal end and C represents the carboxyl terminal end of DIII. Arrows indicate the immunodominant allergenic and antigenic epitopes (amino acid residues 29–37 and 47–55) in mice.

factors that determine the allergenicity of food proteins. Structural characteristics of food allergens appear to play an important role in the capacity of a protein to modulate the immune response towards allergic reactions. Increasing knowledge on structure–function of IgE binding epitopes may offer a new insight in developing hypoallergenic foods.

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