

IgE Binding Properties of the Recombinant Ovomucoid Third Domain Expressed in *Escherichia coli*

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Ovomucoid, a major allergen in hen's egg white, consists of three tandem domains. The third domain (DIII) cDNA was subcloned into pGEMT-vector and the resultant plasmid (pGEMDIII) was inserted into a pGEM-4T-2 glutathione-S-transferase (GST) fusion vector. The GST-DIII fusion protein was expressed in *Escherichia coli*. The 56-residue fragment corresponding to DIII (Leu¹³¹-Cys¹⁸⁶) was liberated using cyanogen bromide to cleave off the GST that had been hydrolyzed with thrombin, which left an additional peptide at the terminus of the recombinant protein. Measurement of circular dichroism spectra indicated that the recombinant third domain (DIII*) had a structure that was slightly less compact than that of the native form. Immunoblot analysis showed that the human IgE binding activity of DIII* was identical to that of native DIII, while its activity was significantly increased to IgE antibodies from egg-allergic patients when tested with an enzyme-linked immunosorbent assay. These results indicate that recombinant DIII* has similar sequential epitopes, but may have more predominant conformational epitopes than native analogues. This might have important implications in egg-allergic reactions. © 2001 Academic Press

Food allergy is a major health concern in both children and adults. Food-hypersensitivity reactions affect about 8% of children and 2% of adults (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-thirds of children diagnosed with food allergies are reactive to egg white (4). Egg-white protein contains 24 different glycoproteins and ovomucoid, which comprises 10% of the egg white and is well known as the dominant allergen in egg white (5, 6). Ovomucoid has a molecular weight of 28 kDa and contains 20–25% carbohydrate. It is composed of 186 amino acids that are arranged in three

tandem domains, each 60 amino acids in length, it is highly homologous and is a pancreatic trypsin inhibitor (7). Each domain is cross-linked by three intradomain disulfide bonds; however, they lack any interdomain disulfide bonds (7). This unique structural characteristic (compared with other egg-white proteins) might explain the higher stability of ovomucoid against digestion with proteinase (8) and heat (9), as well as its strong allergenicity (10, 11). Our previous work has shown that there is significantly more human IgG and IgE binding activity to the third domain (DIII) than to the first (DI) and second (DII) domains of ovomucoid in sera derived from egg-allergic patients (12). This work also showed that the carbohydrate moieties in DIII did not affect its IgG and IgE binding activity. Furthermore, it has been suggested that hydrophilic residues appear to be more important for the human IgG binding site, whereas hydrophobic residues might be more important for the IgE binding site by means of chemical modification of DIII (13).

Recombinant DNA technology is becoming more commonly used for producing allergenic proteins *in vitro*. During the last ten years, in addition to molecular approaches to the immunological and structural studies of allergenic proteins, many recombinant allergens have been produced that offer important new tools for the diagnosis and therapy of allergies. Recombinant proteins can be produced in large quantities and with great purity, making it easier to study the molecular design of the protein when attempting to reduce the allergenic structure by genetic engineering. To date, more than 40 food allergens from fruits, vegetables, nuts, milk, and seafood have been expressed as recombinant proteins (14). To study the structure-function relationship of DIII allergenicity, the recombinant ovomucoid third domain (DIII*) was produced using an *Escherichia coli* expression system. In the present study, we constructed a secretory expression system for the DIII gene, and investigated the structural and immunological characterization of the recombinant DIII*.

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MATERIALS AND METHODS

Reverse transcriptase-polymerase chain reaction. Poly (A⁺) RNA was isolated from chicken oviducts using a Poly AT tract mRNA isolation kit (Promega, Madison, WI) and following the protocol provided in the instruction manual. The first-strand cDNA was synthesized from 1 µg of the poly (A⁺) RNA using random primers and AMV reverse transcriptase. Following the reverse transcription, the ovomucoid gene cDNA was amplified by polymerase chain reaction (PCR) using one-seventh of the first-strand cDNA as the template and oligonucleotide primers 5'-AGACGCAGACCATTACCTTG-3' and 5'-CTCAGCCAGCATCAGCAGTT-3'. The PCR mixture contained (per 50 µl) 1 × PCR buffer (10 mM Tris-HCl, pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTP, 0.5 µM of each primer, and 2.5 units of *Taq* polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR parameters were 40 cycles of denaturing, each for 1 min at 94°C, 1 min annealing at 57°C, and 1 min elongation at 72°C. The amplified cDNA was subcloned into a pGEM T-easy vector and sequenced. The resultant plasmid was named pGEMOvo. To obtain the ovomucoid gene cDNA that encodes the DIII region, PCR was performed using 100 ng of the pGEMOvo as the template and oligonucleotide primer ovoDIII 5'-GTAGGAAGGAATGCTTGCTGCTGTG-3' and oligonucleotide primer ovo3L 5'-CTCAGCCAGCATCAGCAGTT-3'. The protocol for making the PCR mixture was the same as above, and the PCR parameters were 30 cycles of denaturing, each for 1 min at 94°C, 1 min annealing at 59°C, and 1 min elongation at 72°C. The amplified DNA fragment was subcloned into a pGEM T-easy vector and sequenced.

Construction of the expression vector. The pGEMDIII plasmid was digested with *Eco*RI (Amersham Pharmacia Biotech), and then a 3-kbp fragment was agarose-gel purified and self-ligated to make a plasmid pGEME. To insert the Sal I site before the ATG codon, PCR was performed using 100 ng of the pGEMDIII as a template and oligonucleotide primer DIII_{Sal} 5'-CTAGTGATTGTCGACAGG-AATGCTTG-3' and oligonucleotide primer ovo3L 5'-CTCAGCCAGCATCAGCAGTT-3'. The PCR fragment was digested with *Eco*RI and Sal I, and the digested DNA was inserted into the *Eco*RI and Sal I site of the pGEME. The resultant plasmid pGEMDIII_{Sal} was digested with Sal I and Not I, and a fragment of approximately 200 bp was purified from an agarose gel. The purified fragment was inserted into the Sal I and Not I sites of pGEX4T-2 (Amersham Pharmacia Biotech) expression vector to make a glutathione S-transferase (GST)-ovomucoid DIII fused protein expression plasmid pGEXDIII.

Expression of the fusion protein. The GST and ovomucoid DIII fused protein was expressed by transforming the plasmid into competent *E. coli* JM109 (Life Technologies, Gaithersburg, MD). 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-thiogalactopyranoside (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 7000g for 5 min. The cell pellet was re-suspended in 10 ml of lysis buffer (40 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid, 0.5% Triton-X 100) and sonicated three times for 30 s using a sonicator (Model 250 Sonifier, Branson, Danbury, CT) at 40 W. The cell suspension was centrifuged at 15,000g for 10 min to obtain the supernatant containing solubilized fusion protein.

Purification of the recombinant ovomucoid third domain (DIII*). 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 transferred to a disposable column (Bio-Rad Laboratories, Hercules, CA) 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 of 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 Co., St. Louis, MO) in the presence of 70% of formic acid, as described previously (12). 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 flow rate 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), after which the protein was transferred to a polyvinylidene fluoride transfer membrane (ProSorb PE, Applied Biosystems) and subjected to N-terminal sequence analysis.

Preparation of the native ovomucoid third domain. The native ovomucoid third domain (DIII) without carbohydrate was prepared by hydrolysis of ovomucoid with *Straphylococcus aureus* V₈ (Spase V₈) protease (Sigma), as described in a previous paper (12).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (15) in a Mini-Protean II Electrophoresis Cell (Bio-Rad). Samples were run on 10% 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), and loaded onto the gel at a concentration of 15 µg/well. Gels were run at a constant current (18 mA/slab gel), after which they were stained with either 0.025% Coomassie Blue R-250 (Sigma) in 10% acetic acid, or subjected to electroblotting.

Tricine SDS-PAGE. Tris-Tricine-ready gels containing 16.5% acrylamide (Bio-Rad), based on the formulation by Schagger and von Jagow (16), were used to analyse DIII*. Samples were diluted in sample buffer containing 20% 1.0 M Tris-HCl, pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Blue R-250 (Sigma), and 2% 2-mercaptoethanol (Fisher Scientific). Electrophoresis was carried out at 100 V, for approximately 110 min at 4–C in running buffer composed of 0.1 M Tris-HCl, 0.1 M Tricine, and 0.1% SDS. Gels were fixed in a 40% methanol, 10% acetic acid solution, followed by staining with 0.025% Coomassie Blue R-250 (Sigma) in 10% acetic acid, and destaining in 10% acetic acid.

Western immunoblot. Immediately following electrophoresis the proteins were transferred electrophoretically onto a nitrocellulose membrane (Micron Separations Inc., Westborough, MA) using a modification of the method of Towbin *et al.* (17). 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 rabbit anti-ovomucoid IgG (×500 dilution with Tris-buffered saline containing 1% BSA) for 2 h, or human serum from egg-allergic patients (×25 dilution with Tris-buffer saline containing 1% BSA) for 12 h at 25°C. The nitrocellulose membrane was incubated with alkaline phosphatase-conjugated goat-anti-rabbit IgG (×5000 dilution with Tris-buffered saline containing 1% BSA) for 1 h, or alkaline phosphatase-conjugated goat-anti-human IgE (×1000 dilution with Tris-buffered saline containing 1% BSA) for 12 h at 25°C, and stained with a nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Life Technologies). Color development was terminated by washing the membranes with deionized water.

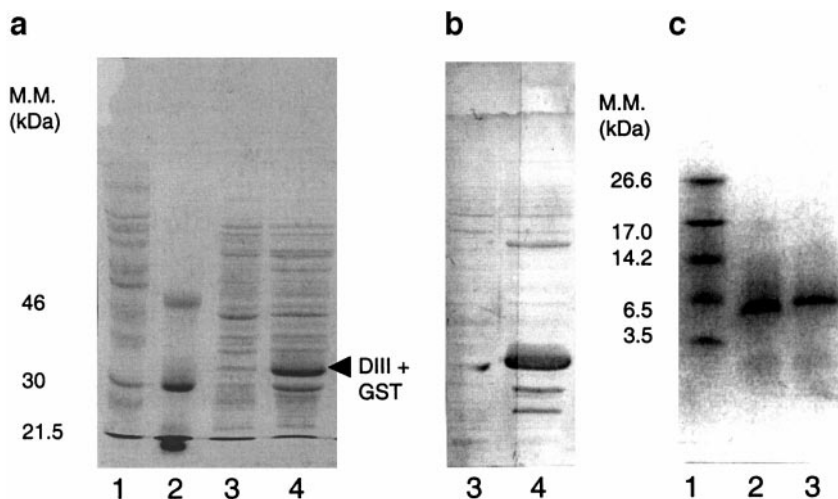


FIG. 1. Expression of recombinant ovomucoid third domain (DIII*). SDS-PAGE of the expression of DIII*+GST fusion protein (a). Lanes 1 and 2, molecular weight markers; lane 3, control (no IPTG); lane 4, IPTG-induced *E. coli* cell lysate and corresponding rabbit anti-ovomucoid IgG immunoblot (b) of the control (lane 3) and IPTG-induced *E. coli* cell lysate (lane 4). Tricine SDS-PAGE (c) of the purified DIII*. Lane 1, molecular weight marker; lane 2, native DIII; lane 3, recombinant DIII*.

Circular dichroism. The far-UV circular dichroism (CD) spectra of DIII* and native DIII were determined at room temperature in 20 mM phosphate buffer, pH 7.5, using a Jasco J600 spectropolarimeter (Easton, MD). Each sample was scanned four times from $\lambda = 200$ –250 nm in a quartz cuvette (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a 0.1-cm path length. A buffer baseline was subtracted 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.* (18).

Enzyme-linked immunosorbent assay. The allergenicity of DIII and DIII* were determined by an indirect enzyme-linked immunosorbent assay (ELISA), according to a modification of the method of Engvall and Perlmann (19). Medical-grade, high-binding polystyrene 96-well microplates (Corning, Cambridge, MA) were precoated with DIII or DIII* (1 μ g/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 IgE serum diluted to 1:25 in PBS containing 1% BSA. The plate was again washed and incubated overnight at 25°C with alkaline phosphatase-conjugated anti-human IgE diluted to 1:1000 in PBS. The plate was washed and developed with *p*-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 μ l 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 eight patients 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.

Production of anti-ovomucoid polyclonal antibodies. Two rabbits (Central Animal Facility, University of Guelph) were injected intramuscularly with 0.2 mg of native ovomucoid that had been resuspended in 0.9% sterile saline and emulsified with an equal amount of Freund's Complete Adjuvant (FCA; Sigma). Two weeks later, the rabbits were given a second intramuscular injection, again with ovomucoid in FCA. Two more injections were administered in

the subsequent two weeks. Antibody titers were monitored by ELISA. The collected antisera were precipitated with saturated ammonium sulfate to a final saturation of 35%. The precipitates were resolved in PBS, dialyzed against PBS for 48 h, and then stored at -80°C until use.

RESULTS AND DISCUSSION

The chicken ovomucoid gene was cloned from the oviduct. The determined nucleotide sequence was essentially the same as that reported previously (20). The cDNA fragment encoding the OVM DIII region (corresponding to Leu¹³¹-Cys¹⁸⁶) was inserted into pGEX-T-2 downstream of the *ptac* promoter, as described under Materials and Methods. The GST-DIII fusion protein (31 kDa) was expressed in *E. coli*, being transformed with the pGEM DIII plasmid in a soluble form, and induction was carried out with 1 mM IPTG, as shown in Fig. 1. The result of the SDS-PAGE suggests that the recombinant fusion protein constitutes a high percentage of the total cell protein, and was identified as GST-DIII fusion protein using an immunoblotting analysis (Fig. 1b). The fusion protein comprised 20% of the total protein in whole-cell lysates (Fig. 1a). A time course showed that the level of expression was maximal 3 h after induction with IPTG (data not shown). The recombinant fusion protein was then purified simply by a GST4B affinity column and cleaved of GST by thrombin, following further purification with HPLC. Approximately 2–3 mg of purified DIII* was obtained from 1 l of media. The DIII* has a molecular mass of 6 kDa, which is identical to the molecular mass of native DIII without carbohydrate (Fig. 1c). The analysis of the amino acid sequence analysis indicates that the purified DIII* starts with Leu¹³¹, as expected. The tertiary

TABLE 1

Secondary Structure Fraction of Native and Recombinant Ovomuroid Third Domains

Forms	Secondary structure (%)		
	α -helix	β -structure	Random coil
Native DIII	17.1	22.7	60.2
Native DIII ^a	19.6	14.3	66.1
Recombinant DIII*	15.6	21.3	63.1

Note. The proportion was estimated from the far-UV CD spectra using a method of Yaung (18).

^a Adapted from Walkenhorst *et al.* (21).

structure of DIII in solution was identified by nuclear magnetic resonance (NMR) as comprising of 19.6% α -helix and 14.3% β -structure (21). The secondary structure based on CD analysis of DIII and DIII* is shown in Table 1. Comparison of CD data with that of NMR analysis reveals that the α -helix of native DIII was slightly lower than that of the NMR, while the β -structure from CD tends to be higher than the NMR analysis. The α -helix content of recombinant DIII* was slightly lower than that of the native DIII, whereas the β -structure content was almost the same. Thus, the DIII* obtained by the present method appeared to have a structure that was slightly less compact than that of native DIII.

E. coli is the most widely used cell type for the expression of food allergens. The recombinant proteins can have structures that vary from those of the native analogues, as they are produced from eukaryotic plant or animal cells. IgE antibodies bind to linear stretches of amino acids (sequential epitopes) on an allergenic protein, or to discontinuous (conformational) epitopes formed by the secondary or tertiary structure of the proteins (22). The IgE binding activity of ovomucoid is distributed over all three domains (23). We found that IgE binds predominantly to the DIII; however, the combination of all three domains may be essential for retaining the full IgE binding activity, resulting in the presence of both sequential and conformational epitopes in ovomucoid (12). Cook and Sampson (24) used synthetic dodeca peptides with overlapping sequences of 10 amino acids, and identified a sequence of 175–186 in DIII as the IgE binding site. The IgE binding fragment of 134–139 (PE-3) has been identified by peptic digestion of ovomucoid (25). Our group has identified seven IgE binding epitopes, two epitopes in DI and DII, and three epitopes in DIII (161–174 and 179–186), using synthetic peptides prepared on cellulose membranes of overlapping peptides (26). It has been reported that no significant changes in IgE binding occurred after oxidation of ovomucoid, while IgE binding of reduced ovomucoid was markedly reduced in some patients, suggesting that conformational epitopes play a significant role in some patients (24). Djurtoft *et al.* (27)

and Zhang and Mine (12) reported the loss of IgE binding to reduced ovomucoid in some patients, while in others reduced ovomucoid still retained its allergenicity. In still other patients, increases in IgE binding of DIII occurred after ovomucoid was reduced. The conformational epitopes in ovomucoid may reflect differences in the hypersensitivity state of individual patients (24). Therefore, minor structural changes of DIII* may alter IgE binding properties and accordingly may modify the allergenic potential of the proteins.

Figure 2 shows Western blotting of native DIII and recombinant DIII* with human IgE antibody derived from egg-allergic patients. The results indicate that immunoreactivity (IgE binding activity) of DIII* was similar to that of the native DIII, suggesting that DIII* has sequential epitopes similar to those in native DIII that are recognizable by human IgE antibodies under Western blotting condition, even after heat treatment in the presence of SDS and 2-mercaptoethanol (the reduced condition) when some loss of conformational epitopes may occur. Thus both proteins could have a similar sequential epitope structure. Figure 3 shows the IgE binding activity of native DIII and recombinant DIII* with the sera from eight different patients. Interestingly, the IgE binding activity of DIII* with some sera (from patients 1, 2, 5, and 7) was significantly higher ($P < 0.05$) than that of native DIII, in spite of similar activity under Western blotting between DIII and DIII* with the same sera. This may be due to the differences of recognition of tertiary structure of DIII and DIII* under ELISA, since both type of specific IgE antibodies (against sequential and tertiary epitopes) can be recognized by ELISA. As pointed by Lorenz and Vieths (14), recombinant food allergens are, in general, not identical copies of the natural analogue, since changes to the IgE binding properties of the recombinant protein may occur. The allergenic reactivity may be altered or even be eliminated through

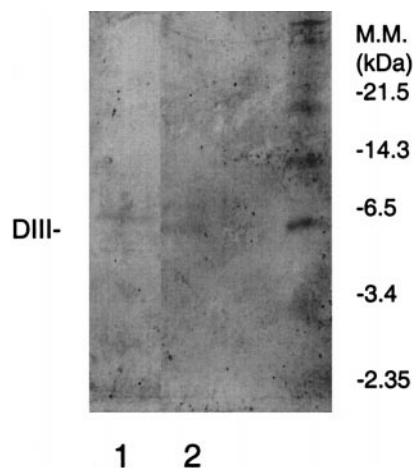


FIG. 2. Human IgE immunoblot analysis of recombinant DIII* (lane 1) and native DIII (lane 2) under reduced conditions.

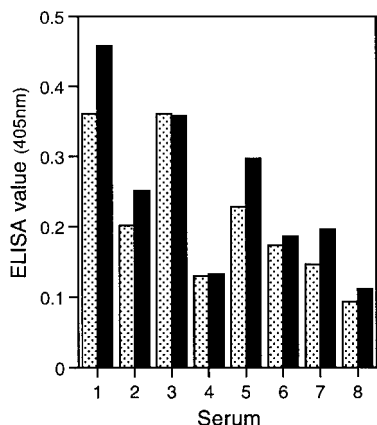


FIG. 3. Comparison of the binding activity of human specific IgE with native DIII and recombinant DIII*. (▨), native DIII; (■), recombinant DIII*.

the loss of linear or conformational IgE binding epitopes. Thus, it is important that the IgE binding properties of a recombinant allergen are well characterized before it is used in research, diagnostic, or therapeutic applications.

In the present study, the IgE binding activity increased with the sera from some patients when recombinant DIII* was used. In our previous work on the immunological and structural characteristics of egg-white proteins (28) and Buckwheat-allergenic proteins (29), we found that IgE binding activity increased when the proteins in the sera from some patients were partially denatured with urea. Conversely, when the protein was fully denatured through chemical modification, the IgE binding activity decreased. These results indicate that food allergens might be sensitized by structural changes, such as partial denaturation, that occur during heat processing, digestion by proteolytic enzymes, or when passing through epithelial cells. An individual patient could be sensitized by such partially denatured allergenic proteins or fragments if they still contain allergenic epitopes. In fact, we have already reported that DIII can retained its allergenicity after heat and peptic digestion *in vitro* (11). Once an individual has been sensitized, a wide variety of IgE-reactive epitopes might repetitively elicit allergenic reactions.

In conclusion, with recombinant DIII* it is possible to characterize the location of both sequential and conformational epitopes. Furthermore, site-directed mutagenesis of DIII* could reveal the structural characteristics of epitopes that are relevant to IgE binding capacity, which would enable us to reduce the allergenic properties by altering the molecular structure using genetic engineering.

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