

Characterization of Residues in Human IgE and IgG Binding Site by Chemical Modification of Ovomucoid Third Domain

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Chemical modification of ovomucoid third domain (DIII) has been conducted to characterize the binding site residues that determine antigenicity and allergenicity of DIII. Nitration of Tyr, ethoxyformylation of His and succinylation of Lys residues led to a decrease of α -helix content of DIII. Modification of His, Tyr, Glu, Asp and Lys residues on DIII resulted in a reduction of human IgG binding activity, but little effect on IgE binding activity. These results suggest that hydrophilic residues appear to be more critical for human IgG binding site, whereas hydrophobic residues may be more important for IgE binding site. © 1999 Academic Press

Ovomucoid, an abundant glycoprotein, is well known as the immunodominant protein fraction in avian egg white (1). Ovomucoid has a Mw of 28,000 and their polypeptide chain consists of three tandem homologous domains (2). They can be cleaved into their three components domains without any intein domain nicking (2). Ovomucoid is also well known as a major allergenic component of egg white which frequently causes atopic allergic reactions (1, 3). Our previous work has revealed that there was significantly more IgG and IgE binding activity to the third domain (DIII) than to the first (DI) and the second domain (DII) using egg allergic patients' sera. The main IgE and IgG epitopes of each domain were linear (4).

Chemical modification of the side-chain of residues in protein antigen is powerful method to investigate epitopes (5, 6). Alteration of the structure of key residue in an epitope by a chemical modification reagent will greatly change its reactivity with an antibody to that epitope. Chemical modification has the advantage that it can be applied to discontinuous as well as continuous epitopes (6). In this study, we prepared four derivatives of DIII specifically modified with chemical

reagents, and the IgG and IgE binding activity of each derivative with antiserum from egg allergic patients was conducted to identify residue type as being a key epitope constituent.

MATERIALS AND METHODS

Isolation of ovomucoid and its third domains. Crude ovomucoid was isolated from egg white according to the procedure of Fredericq and Deutsch (6). The further purification of ovomucoid was carried out by HPLC (Bio-Rad Laboratories, Hercules, CA) using a Bio-Scale Q5 column. The third domains containing carbohydrate chain (DIII) was prepared by hydrolysis with *Staphylococcus aureus* V₈ (Spase V₈) protease (Sigma, St Louis, MO), as described by Kato *et al.* (2). DIII was chromatographically purified using Bio-Scale Q5.

Patient sera. Human sera from children (0.5–5 years) with egg-white allergy were kindly provided by Dr. A. Urisu (Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan) and Dr. H. Morikawa (Faculty of Medicine, Gunma University, Gunma, Japan). The sera with high titer of ovomucoid-specific IgG and IgE antibodies were selected by ELISA using purified ovomucoid as a coating antigen.

Chemical modification of ovomucoid DIII. The imidazole group of histidine was modified with diethylpyrocarbonate (DEP) (Sigma) by the modified procedure of Melchior & Fahrney (8). Three milligrams of DIII was dissolved in 5 mL of 0.1 M acetate buffer, pH 5.0, and 100 μ L of 5% DEP solution in absolute ethanol was added to the solution over a period of 10 min at room temperature. The reaction mixture was dialyzed against 0.02 M phosphate buffer for 10 h at 4°C. The number of modified histidine residues was measured by spectrophotometric assay at 240 nm using an extinction coefficient of 5200 M⁻¹ cm⁻¹ (8). Phenolic hydroxyl groups of tyrosine residues were nitrated with tetranitromethane (TNM) (Sigma) by the modified method of Riordan *et al.* (9). Three milligrams of DIII was dissolved in 5 mL of 0.05 M Tris-HCl buffer, pH 8.0 and 50 μ L of 0.5 M TNM in 95% ethanol was added to the solution. The mixture was incubated with occasional stirring for 1.5 h at 4°C and then at room temperature for 30 min with shaking. The reaction was quenched by adding 2 μ L of 2-mercaptoethanol and the mixture was incubated for 15 min at room temperature with shaking. The mixture was diluted with 0.02 M phosphate buffer and dialyzed against with same buffer for 8 h at 4°C and stored at 4°C. The number of 3-nitrotyrosines was determined by measuring the O.D. at 428 nm using ϵ 4100 M⁻¹ cm⁻¹ (9). The ϵ -amino groups of lysine were modified by succinylation (10). Three milligrams of DIII was dissolved in 5 mL of 0.2 M sodium borate buffer, pH 8.0, and 20 mg of solid succinic anhydride (Sigma) was added over a period of 30 min. The pH was kept at 8.0 by 1 N

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TABLE 1
Degree of Modification of DIII

Derivatives	Modified residue type	Degree of modification* (%)
DEP-DIII**	Histidine	46
Nitrated-DIII	Tyrosine	28
Amide-DIII	Aspartate, Glutamate	83
Succinylated	Lysine, $-\text{NH}_2$	72

* Number of the modified residues/total of the objective residues.

** DEP: diethylpyrocarbonate.

NaOH during incubation. The reaction mixture was dialyzed against 0.2 M phosphate buffer for overnight at 4°C and stored at 4°C. The number of unmodified lysine residues was measured using a trinitrobenzenesulfonic acid (TNBS) (Sigma). The optical density of the protein preparation at 345 nm and an extinction coefficient of 14,000 $\text{M}^{-1} \text{cm}^{-1}$ (11). The carboxylic side chains of aspartic and glutamic acid and a C-terminal carboxyl group were modified by amidation (12). The carboxylic side chains were coupled with glycine amide in

the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma). Three milligrams of DIII was dissolved in 10 mL of 0.1 M MES buffer, pH 5.5, mixed slowly with 69.8 mg glycine ethyl ester (Sigma) to give a final concentration of 0.05 M and 19 mg of EDC to a final concentration of 0.01 M. The pH of the mixture was kept at 5.5 with 1 N HCl at 4°C during the incubation period of 6 h. The reaction was quenched by adding 10 mL of acetate buffer, pH 5.0. The mixture dialyzed against 0.02 M phosphate buffer for 8 h at 4°C and stored at 4°C. The fraction of modified carboxyl groups was measured by counting the increased number of glycine residues with an amino acid analyzer using a Beckman system gold amino acid analyzer equipped with a post-column ninhydrin detection.

Enzyme-linked immunosorbent assay (ELISA). Antigenic and allergenic properties of DIII and its chemical derivatives were determined by an indirect ELISA. Ovomucoid DIII and its derivatives were coated to a 96-well microtiter plate (Corning Costar Corporation, Cambridge, MA) in 0.1 M sodium carbonate buffer (pH 9.6) at a concentration of 10 $\mu\text{g/mL}$ and incubated overnight at 4°C. The plate was washed four times with phosphate buffer saline containing 0.05% Tween-20 (PBST). The plate was blocked with 2.5% bovine serum albumin (BSA) in PBS for 2 h at room temperature. The plate was washed with PBST, and incubated with 100 μL of sera diluted 1/25 for IgE detection and 1/500 for IgG detection in PBS containing

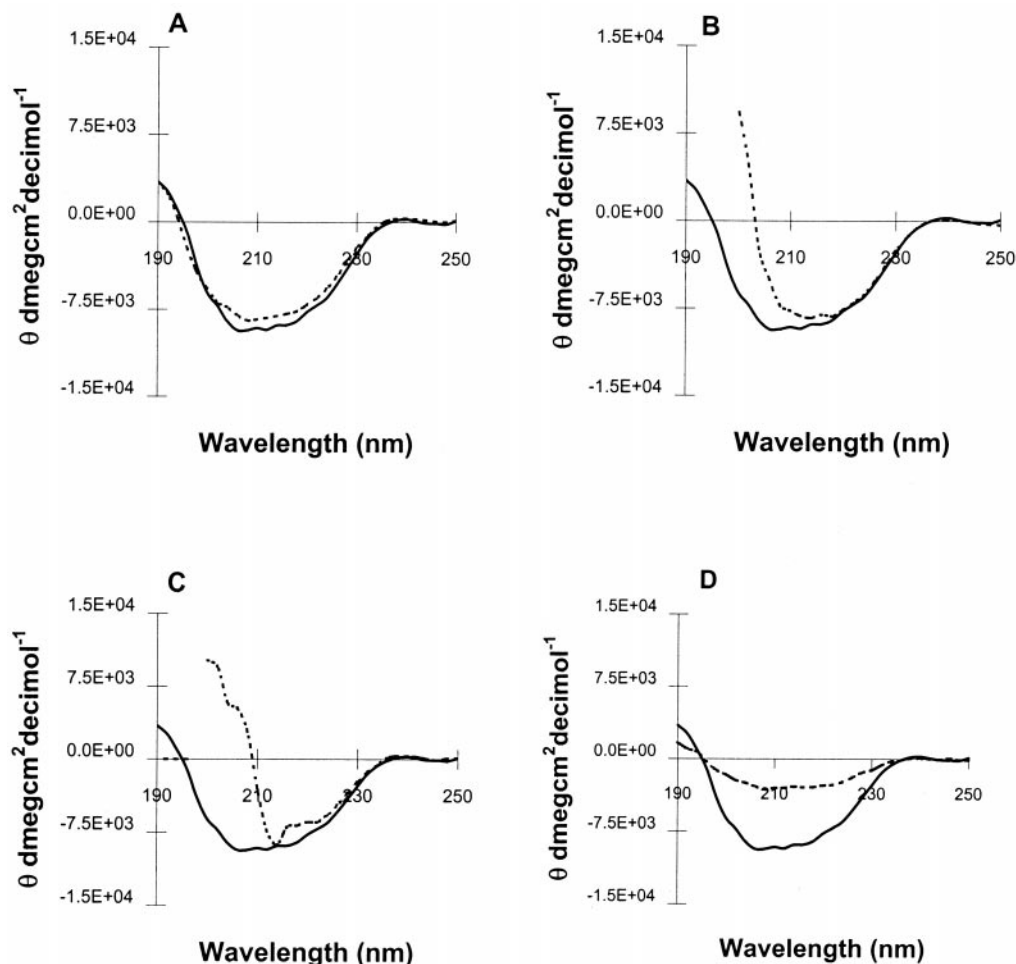


FIG. 1. Far-UV CD spectra of the chemically modified DIII. CD measurements were carried out in 0.02 M phosphate buffer, pH 7.2, in the protein concentration of 0.007–0.015% (w/v). The solid line represents native DIII and the dotted curve derivatives. (A) amide, (B) succinylated, (C) DEP, and (D) nitrated DIII.

1% BSA (Sigma). The plate was washed and 100 μ L of monoclonal anti-human IgE conjugated alkaline phosphatase (1/1000) (Sigma) for IgE detection and goat anti-human IgG conjugated alkaline phosphatase (1/20000) (Sigma) for IgG detection were added. The plate was washed and developed with 100 μ L of *p*-nitrophenol phosphate (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at room temperature. The reaction was terminated by 25 μ L of 3 N sodium hydroxide. Absorbance at 405 nm was read by the microplate reader (Model 550, Bio-Rad Laboratories). The IgE or IgG of chemical modified DIII-specific binding activity was expressed as a percentage of IgE or IgG binding to intact DIII.

Circular dichroism (CD). The far-UV CD spectra of DIII, and chemically modified derivatives were determined at room temperature in 0.02 M phosphate buffer (PB), pH 7.5, using a Jasco-J600 spectropolarimeter (Easton, MD). Each sample was scanned four times from 200–250 nm in a quartz cuvette (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a 0.1 cm pathlength. A buffer baseline was determined using 0.02 M PB, and was subtracted from each sample spectra. The data were expressed as mean residue ellipticities in $\text{deg}/\text{cm}^2 \cdot \text{decimol}$, taking a mean residue weight of 110 established from amino acid composition. Sample protein concentration was determined by MicroBCA assay (13). The percentage of the different secondary structure (α -helix, β -sheet, β -turn and random coil) were estimated using a Jasco Protein Secondary Structure Estimation Program (Easton, MD) which was based on the method of Yang *et al.* (14).

Statistics. The data were analyzed using analysis of variance (ANOVA). The significance of difference was established at $p < 0.05$.

RESULTS AND DISCUSSION

TNM reacts with phenolic side chain of tyrosine under relatively mild conditions to give 3-nitrotyrosine. This induces the decrease of pK_a from 10 to 7. DEP reacts with various nucleophiles. At low pH, the reaction is quite selective for histidine. Several reactions have been described for the modification of the carboxylic side chains of aspartic and glutamic acid, using an amine (methyl esters of glycine) and a water soluble, coupling carbodiimide reagent, is most often employed. The modified product is stable. Protein-COOH is converted into protein $-\text{CO}-\text{NHCH}_2\text{COOH}$. Hence, the mild modification that affects only the size, but not the charge of the aspartyl or glutaryl side chains of the protein was achieved. Acylation or alkylation of ϵ -amino group of lysine alter both the size and the charge of the amino group (15). The percentage of modified residues are listed in Table 1. More than 80% of the carboxyl group in Glu and Asp was modified with glycine amide in the presence of EDC. In succinylation of DIII, 72% of the objective residue was modified, while DIII modified with DEP and nitrated one having lower level of modification were obtained. The reactivity of a certain type side chain in a protein varies by several orders of magnitude, such as the interactions with neighboring groups that affect the accessibility and reactivity. Chemical modification can have indirect effects on the display of an epitope, notably by disrupting the protein's structure. Loss of activity on treatment with a reagent might be the result of conformational changes or other changes that occur far

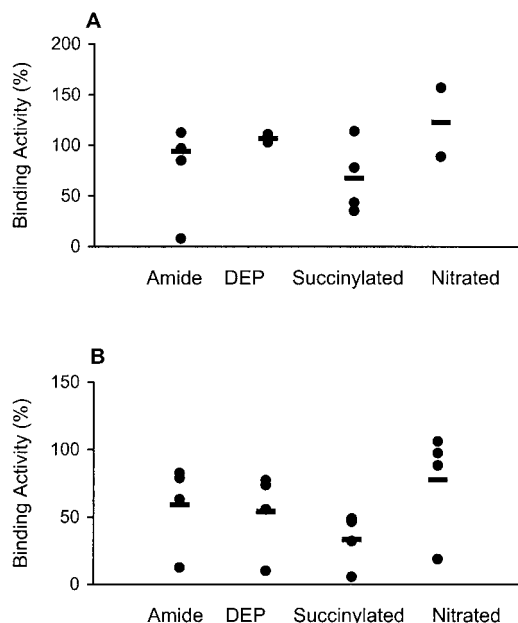


FIG. 2. Binding activity of human IgE and IgG antibodies from egg allergic patients' sera to chemically modified DIII. (A) IgE antibody, (B) IgG antibody. Binding activity was expressed as a percentage of IgE and IgG binding activity to native DIII.

from the active site (5, 6). With chemical modification studies, it is essential to make some independent assessment of the structural integrity of the protein after modification.

The CD spectra of chemically modified DIII are shown in Fig. 1. The CD spectrum of DIII in the far ultraviolet region had one negative maximum at about 208 nm and two shoulders at about 218 and 222 nm. The CD spectra curve of amide DIII was similar to that of native one (Fig. 1A). The major change in the CD spectra occurred in succinylation of ϵ -amino group of Lys was that the ellipticity between 190 and 220 nm decreased. The α -helix and β -turn contents decreased noticeably and the β -sheet increased (Table 1). The maximum ellipticity of DEP-DIII was slightly shifted to a longer wavelength than that of initial DIII. The α -helix and random coil contents decrease, whereas β -turn increased noticeably. The pattern of CD spectrum of nitrated DIII showed a typical curve of decreasing α -, resulting in 84% loss of α -helix (Table 1).

Figure 2 shows the binding activity of ovomucoid-specific IgE (Fig. 2A) and IgG (Fig. 2B) to each modified DIII. Interestingly, the mean percentage of ovomucoid-specific IgE antibody binding to nitrated DIII and DEP-DIII was 107% and 123%, respectively, compared to 94% for amide DIII and 67% for succinylated DIII. Ovomucoid-specific IgG antibody from 4 patients varied in their ability to recognize succinylated DIII. There were no significant differences of IgE binding activity between native DIII and its chemical deriva-

TABLE 2
Secondary Structure Fraction of Native DIII and
Its Chemically Modified Derivatives

Derivatives	Secondary structure (%)			
	α -helix	β -sheet	β -turn	random coil
Native DIII	25.0	21.3	16.4	37.3
DEP-DIII	0.5	17.6	52.0	29.9
Nitrated-DIII	4.0	50.4	10.4	35.1
Amide-DIII	25.0	15.0	16.7	42.7
Succinylated-DIII	5.7	47.6	7.8	38.9

Note. The proportion was estimated from the far-UV CD spectra shown in Fig. 1 using a method of Yaung (11).

tives ($P > 0.05$). Since the main IgE epitope on DIII are linear structure and the Asp, Glu, Lys and His in DIII which were modified in this study are all hydrophilic amino acid residues. This result suggests hydrophilic amino acid group may not be critical for IgE binding site in DIII. When four patients were tested for ovomucoid specific IgG antibody binding to the chemically modified DIII derivatives, the mean percentage were all less than 100% and significantly low binding activity ($P < 0.05$). As shown in Fig. 2B, a 67% loss in the binding activity of succinylated DIII, a 46% loss of DEP-DIII, a 46% loss of amide DIII and 23% loss of nitrated DIII were caused by chemical modification. Although there were some changes on secondary structure of these derivatives, it is possible to evaluate the changes within the antigenic sites since the main epitopes on DIII were linear structure (4). Hence, Asp, Glu, Lys, His and Tyr may play a significant role in the antigenicity of DIII, suggesting the hydrophilic amino acids are more critical to IgG binding site than hydrophobic and polar amino acids.

Chemical modification techniques have been widely used to determine the types of residues that are critical in an enzyme's active site. Atassi *et al.* investigated antigenic determinants on the structure of lysozyme and myoglobin by chemical modification (5, 6). Their work showed that Lys and Glu were most critical amino acids in epitopes. Furthermore, some of eptopes in food allergens have been identified last ten years. For example, Ara h1, an abundant peanut protein, contains 24 linear IgE binding epitopes (16). A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE binding epitope (17). However, any

differences on biochemical and structural analysis of IgE and IgG binding sites have been reported. The present work suggests that structure of key residues in IgG and IgE epitopes may be different. Further studies on complete IgG and IgE epitope mapping via the synthesis of overlapping peptides of DIII is currently being explored in our laboratories.

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