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# Characterization of IgE and IgG Epitopes on Ovomucoid Using Egg-White-Allergic Patients' Sera

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**To investigate the importance of linear and conformational structure and carbohydrate chains in hen ovomucoid epitopes, the IgG and IgE binding activities of three native and reduced carboxymethylated (RCM) domains (DI, DII, and DIII) were compared using human sera from egg-white-allergic patients. There was significantly more IgG and IgE binding activity to DIII than to DI and DII. The IgG binding activity to RCM domains was similar to the native form, while RCM-DIII had significantly greater binding activity to IgE antibody ($p < 0.05$). It indicated that the main IgE and IgG epitopes on each domain were of linear structure. However, the total reactivity of the three domains was estimated to be about 50–60% (IgG binding) and 55–75% (IgE binding) compared with total reactivity in ovomucoid, resulting in some ovomucoid epitopes consisting of conformational epitopes on domain I~II or II~III. The carbohydrate moieties in DIII had a rather inhibiting effect on its IgG and IgE binding activities.** 

Hen's egg is one of the more frequent causes of food hypersensitivity in infants and young children (1, 2). Ovomucoid is the immunodominant protein fraction in egg white (3) even though it comprises only 10% of total egg white protein. Ovomucoid has a $M_r$ of 28,000 and contains about 20–25% carbohydrate. It is comprised of three well-separated domains (the first domain (DI), the second domain (DII), and the third domain (DIII), each about 60 amino acids in length. There are two kinds of DIII, either with or without a carbohydrate chain; about half the ovomucoid molecules have the carbohydrate-containing domain and the remainder the carbohydrate-free ones (4, 5). Each domain is cross-linked by three intradomain disulfide bonds, but there are no interdomain disulfide bridges (5).

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It has been reported that domain I~II and domain II~III retained allergenic reactivity as much as intact ovomucoid and DI and DIII, however, had no detectable reactivity to each domain (6). More recently, Cooke and Sampson reported that no significant difference in the amount of IgE binding to DI and DIII and there was significantly more IgE activity to DII, while quantities of patient IgG antibody were no different for all three domains (7). Matsuda *et al.* (8) found the carbohydrate chain attached to DIII played an important role in antigenic determinants of the carbohydrate-containing third domain (DIII-CHO+) against the human IgE antibody, whereas Besler *et al.* (9) has reported only epitopes on the ovomucoid backbone are responsible for IgE binding sites while carbohydrate residues do not participate in allergenic structures of ovomucoid. Hence the issue of the allergenic and antigenic epitope structures of ovomucoid and the role of the carbohydrate moiety on its immunogenic activity are still controversial.

In the present study, enzyme-linked immunosorbent assay (ELISA) was carried out to characterize the allergenic and antigenic epitopes of ovomucoid using human sera from egg-white allergic patients. The importance of linear and conformational IgG and IgE epitopes on ovomucoid three domains were investigated by determining ovomucoid-specific IgG and IgE antibody binding to the native domains and the reduced-carboxymethylated domains. The role of the carbohydrate chain was also determined by comparing the ovomucoid-specific IgG and IgE antibody binding to the DIII-CHO+ with the carbohydrate-free third domain (DIII-CHO−).

## MATERIALS AND METHODS

*Isolation of ovomucoid and its three domains.* Crude ovomucoid was isolated from egg white according to the procedure of Fredericq and Deutsch (10). The further purification of ovomucoid was carried out by HPLC (Bio-Rad Laboratories, Hercules, CA) using a Bio-Scale Q5 column. The first domain (DI) was prepared by cleaving ovomucoid with cyanogen bromide (CNBr) (Sigma Chemical Co., St. Louis, MO) in the presence of 70% formic acid and the second (DII) and the third domains (DIII-CHO+, DIII-CHO−) were prepared from ovo-

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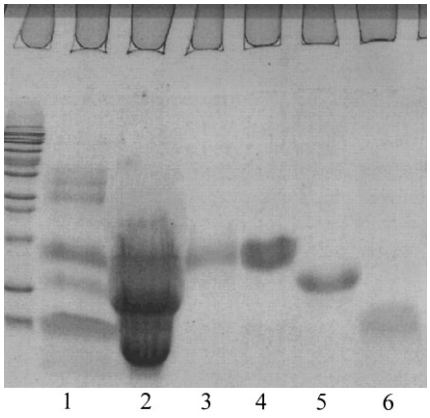


**FIG. 1.** SDS–PAGE analysis of ovomucoid domains: lane 1, ovomucoid hydrolyzed by Spase V8; lane 2, ovomucoid cleaved by CNBr; lane 3, DI; lane 4, DII; lane 5, DIII-CHO+; lane 6, DIII-CHO−.

mucoid by hydrolysis with *Straphylococcus aureus* $V_8$ (Spase $V_8$) protease (Sigma), as described by Kato *et al.* (11). All three domains were chromatographically purified using Bio-Scale S5 and Bio-Scale Q5 columns (Bio-Rad Laboratories, Hercules, CA).

*Patient sera.* Human sera from children (0.5–5 years) with egg-white allergy were kindly provided by Dr. Urisu (Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan) and Dr. 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.

*Preparation of reduced carboxymethylated ovomucoid (RCM-ovomucoid) and domains (RCM-DI, RCM-DII, RCM-DIII-CHO+, RCM-DIII-CHO−).* RCM-ovomucoid and RCM-domains were prepared according to the method of Aitken and Learmonth (12). Ovomucoid and its domains (30 mg) were dissolved in 3 ml of 6 M guanidium hydrochloride in Tris–HCl, pH 8.6, respectively. Five microliter of 4 M dithiothreitol (DTT) (Sigma) was added and incubated under $N_2$ for 3 h at room temperature. Following this incubation, 0.3 ml of colorless 500 mM iodoacetate solution (Sigma) was added and incubated in the dark for 30 min at 37°C. All the samples were dialyzed overnight at 4°C against 20 mM phosphate buffer, pH 7.2.

*Enzyme-linked immunosorbent assay (ELISA).* Antigenic and allergenic properties of ovomucoid and its domains were determined by an indirect ELISA. Ovomucoid 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 $\mu$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 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature. The plate was washed with PBST, and incubated with 100 $\mu$l of sera diluted 1/25 for IgE detection and 1/500 for IgG detection in PBS containing 1% BSA (Sigma). The plate was washed and 100 $\mu$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 $\mu$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$\mu$l of 3 N sodium hydroxide. Absorbance at 405 nm was read by the microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). The IgE or IgG domain-specific binding activity was expressed as a percentage of IgE or IgG binding to intact ovomucoid.

*Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).* The SDS–PAGE (15% acrylamide) was performed according to the method of Laemmi (13). Gel sheets were stained with Coomassie brilliant blue R-250 and destained with methanol/acetic acid/water (40:7:53, v/v).

*Statistics.* Student's *t* test was used to determine significant differences between antibody activity. The level of statistical significance was 0.05.

## RESULTS

The purity of ovomucoid and each domain was evaluated by amino acid sequence analysis and SDS–PAGE. The amino acid sequences of DI, DII and DIII preparations were identified to exactly with those expected for residues 1–68, 65–130, and 131–186 (data are not shown). The purity of domains was also determined by SDS–PAGE as shown in Fig. 1. Serum samples from 9 patients with high titer of IgE or IgG antibody to ovomucoid were used in ELISA to determine the ovomucoid-specific IgE and IgG reactivities with each of the native and RCM domains. Figure 2 showed the binding activity of ovomucoid-specific IgG (Fig. 2A) and IgE (Fig. 2B) to each domain, respectively. The median percentage of ovomucoid-specific IgG and IgE antibody binding activity to each domain was shown in Table 1. The median percentage of ovomucoid specific-IgG antibody to DIII-CHO+ was 28.2% (range 3.4–44.2%) compared with 8.9% (range 5.0–16.4%) for DI and 12.8% (range 3.4–26.3%) for DII. The percentage of IgG antibody to DIII was significantly greater ($p < 0.05$) than that of other two domains. Ovomucoid-specific IgG and IgE antibodies from differ-

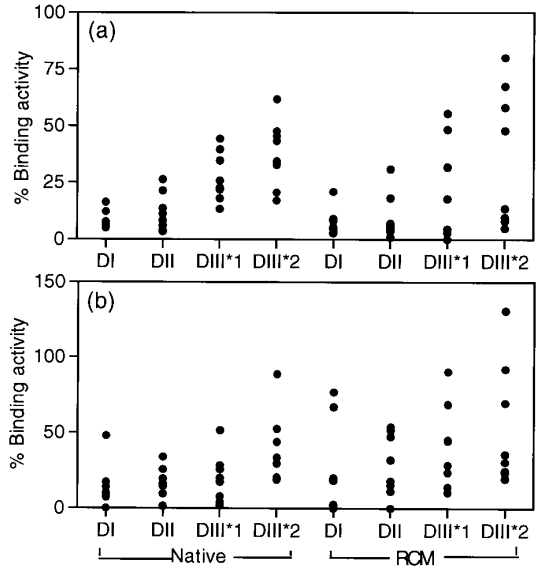


**FIG. 2.** Binding activity of human ovomucoid-specific IgG and IgE antibodies to native and RCM ovomucoid domains. A, IgE antibody; B, IgG antibody. DI, domain I, DII, domain II, DIII*1, domain III with carbohydrate, DIII*2, domain III without carbohydrate.

**TABLE 1**

The Medium Percentage Binding Activities of Human IgG and IgE Antibodies Specific to Ovomucoid Domains from Egg-Allergic Patients

| | Native | | | | RCM− | | | |
|---|---|---|---|---|---|---|---|---|
| Antibody | DI | DII | DIII-CHO+[a] | DIII-CHO−[b] | DI | DII | DIII-CHO+ | DIII-CHO− |
| IgG | 8.9 | 12.8 | 28.2 | 39.1 | 13.6 | 8.6 | 26.1 | 39.5 |
| IgE | 11.5 | 16.4 | 27.4 | 46.9 | 24.9 | 27.4 | 40.1 | 52.2 |

[a] The ovomucoid third domain with carbohydrate.
[b] The ovomucoid third domain without carbohydrate.

ent patients varied in their ability to recognize each domains and the RCM− domains. There was no significant difference in the amount of ovomucoid-specific IgG antibody binding to domains between in native form and RCM− domains ($p < 0.05$). Interestingly, the DIII-CHO− had higher IgG binding activity (39.1%) than DIII-CHO+ (28.2%), and there was no significantly differences between native and RCM forms. The median percentage of ovomucoid-specific IgE antibody directed to each domains showed similar results with IgG binding activity. The median percentage of IgE binding activity directed to DIII-CHO+ was 27.4% (range 1.8–51.8%) compared with 11.5% (range 0–48.0%) for DI and 16.4% (range 1.5–34.1%) for DII. The percentage of IgE antibody to DIII was significantly greater than DI. The RCM− domains were similar to native domains in their ability to bind to ovomucoid-specific IgG antibody, while the RCM-DIII had significantly more IgE binding activity than native form ($p < 0.05$). The median percentage of IgE antibody binding to DIII-CHO− was significantly greater than to DIII-CHO+ in either native form or RCM− form ($p < 0.05$).

## DISCUSSION

It has been believed that IgE is responsible for food-induced allergic reactions of the immediate hypersensitivity type (Type I), while it is becoming increasingly recognized that IgG-mediated hypersensitivity reaction (Type III) may contribute to some allergic conditions, particularly food allergies (14). Epitopes are either depend on the tertiary structure of the protein (conformational epitope) or several amino acid sequence on the protein surface (linear epitope). Ovomucoid has been shown to be the dominant allergen in hen's egg (3). To characterize IgG and IgE epitopes on ovomucoid domains, reduced carboxymethylation was employed to break disulfide bonds in the domains. There was no significant difference in the amount of ovomucoid-specific IgG antibody binding to the native and RCM− domains, while the RCM-DIII significantly had more IgE binding activity than native form ($p < 0.05$). It indicated the main IgG and IgE epitopes on each domain were linear structure. However, the combination of all three domains may be essential for full showing of the immunoreactivity *in ovo*mucoid, because the total reactivity of three domains was estimated about 50–60% (IgG binding) and 55–75% (IgE binding) compared with total reactivity *in ovo*mucoid. This suggests that some epitopes which are the paring of structurally adjacent amino acids (conformation epitopes) in DI∼II or DII∼III may present in native ovomucoid. In fact, it was reported that DI∼II and DII∼III have full allergenic reactivity, but 60% loss in the reactivity of DII using specific IgE antibody from mouse (6, 15). These conformational epitopes may be disrupted during the process of the preparation of each domain. Although, it was reported the more human serum IgE activity to DII but not significantly difference in the IgG binding to three domains (7), the present study strongly indicate that major linear IgG and IgE epitopes are located in DIII. As depicted in Fig. 2A, IgG antibodies from some antibodies recognized more native DIII than RCM-DIII, suggesting the importance of conformational epitopes on DIII in some patients.

Matsuda *et al.* have reported that carbohydrate moiety in DIII contributes to the allergenic structure of ovomucoid (8). As represented in Table 1, ovomucoid-specific IgG and IgE antibodies binding activity to DIII-CHO− was significantly greater than to DIII-CHO+ ($p < 0.05$). It indicated the carbohydrate moiety on DIII had rather inhibition effect on ovomucoid-specific IgG and IgE binding to native DIII. The ovomucoid is heavily glycosylated (20–25%) protein and DIII-CHO+ has a carbohydrate chain with a $M_r$ of about 3000 (16). This may interfere IgG binding to DIII backbone, because of its high content and spatial distribution along the polypeptide chain in DIII. Interestingly, when DIII-CHO+ was denatured by RCM, ovomucoid-specific IgE binding activity to DIII-CHO+ was significantly enhanced from 27.4 to 40.1%, suggesting that IgE antibody may have some difficulties approaching the native DIII-CHO+ compared with denatured forms.

In conclusion, the major epitopes on each domain were linear structure, but there was more IgG and IgE binding activities to DIII. The carbohydrate moieties of DIII had rather inhibition effect on its IgG and IgE binding activities. However, the full allergenicity and antigenicity of ovomucoid needed the combination of all ovomucoid three domains, suggesting the presence of some conformational epitopes which are the paring of structurally adjacent amino acid in DI~II or DII~III.

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