

Identification and Fine Mapping of IgG and IgE Epitopes in Ovomucoid

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Ovomucoid is a major allergen in hen egg white which causes a serious IgE-mediated food allergy reaction. This study determined eight IgG epitopes, 5–11 amino acids in length, and nine IgE epitopes, 5–16 amino acids in length, within the primary sequence in ovomucoid using arrays of overlapping peptides synthesized on cellulose membranes. Pooled sera from eight egg-allergic patients were used to probe the membrane. We also analyzed the amino acids that are critical for antibody binding by substituting a single amino acid within each epitope. Mutational analysis of the epitopes indicated that charged amino acids (aspartic acid, glutamic acid, and lysine) and some hydrophobic (leucine, phenylalanine, and glycine) and polar (serine, threonine, tyrosine, and cysteine) amino acids were important for antibody binding. These results provide useful information for the molecular design necessary to reduce the allergenicity of ovomucoid, and a better understanding of structure–function relationships of allergic epitopes in food proteins. © 2002

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Food allergy is a major health problem in both children and adults, with food-hypersensitivity reactions affecting about 8% of children and 2% of adults (1). Ovomucoid is the dominant allergen found in egg white and is the cause of most allergic reactions in children (2–6). Ovomucoid is a glycoprotein with a molecular weight of 28 kDa (7), and constitutes 11% of the total protein content of egg white. It has a *pI* of 4.0 and is a trypsin inhibitor. Being a dominant allergen it has prominent carbohydrate domains (8), but it has been demonstrated that the carbohydrate moieties have no impact on the IgE binding of ovomucoid (9, 10). On the other hand, the importance of the carbohydrate epitope in N-glycosylated domain III (N175)

to allergic reactions to egg white has been documented (11), while it has been shown that the carbohydrate region in domain III inhibits the effect of IgE binding activities (12).

Epitopes are dependent on either the tertiary structure of the protein (conformational epitope) or several amino acid sequences on the protein surface (linear epitope). Previous studies have suggested that the allergenicity and antigenicity of ovomucoid are quite resistant to heat and denaturation (13, 14), suggesting that antibody binding is directed toward a linear epitope. We have shown that the major epitopes on each domain primarily had a linear structure, and that the IgG and IgE binding activities were more dominant to the third domain (12). Each domain has common epitope(s) (homology) which are recognized by peripheral blood T cells from allergic patients (3). The importance of linear and conformational IgE and IgG epitopes of ovomucoid has been studied previously (12), and a few epitopes recognizing the IgE and IgG binding regions have been identified by several groups (15–17) using synthetic peptides and enzyme digested fragments and its recognition to patients' sera.

The purpose of this study was to identify the major IgG and IgE epitopes in the entire ovomucoid protein backbone, investigate the essential amino acid residues in IgG and IgE epitopes, and structurally analyse their allergenic properties. In this communication we have determined the detailed epitope mapping and binding regions in the whole ovomucoid molecule, and identified the major IgG and IgE binding epitopes. We have also identified the amino acids within each of the IgG and IgE binding epitopes that are critical for immunoglobulin binding. These results will enable us to further investigate how to reduce the risk of this egg-white allergen by lowering the IgG and IgE binding activities.

MATERIALS AND METHODS

Peptide synthesis and SPOTs assay for determining the overlapping peptides. Epitopes were identified using the arrays of synthetic peptides prepared on cellulose membrane (SPOTs synthesis)

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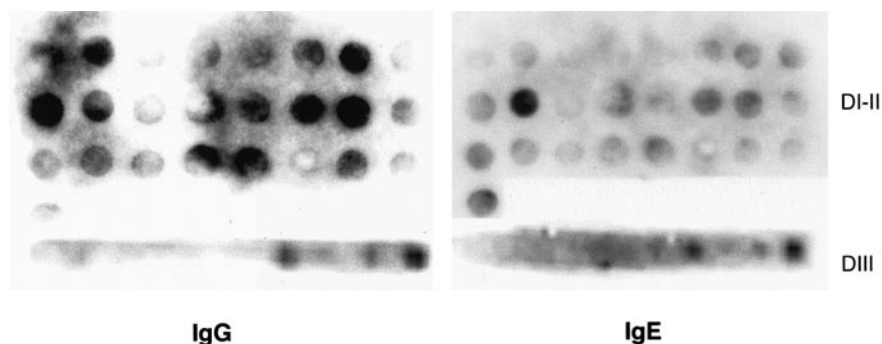


FIG. 1. SPOTs membranes with overlapping ovomucoid peptides probed with pooled sera from egg allergic patients.

(18). Based on the information derived from the amino acid sequence of ovomucoid (7), the synthesis of peptides using the SPOTs technique allows synthesis of desired peptides on a membrane. In this study the individual peptides were synthesized on a derivatized cellulose membrane using *N*-(9-fluorenyl) methoxycarbonyl (Fmoc) amino-acid-active esters according to the manufacturer's instruction (Genosys Biotechnologies, The Woodlands, TX) (19). Peptide synthesis reactions were monitored at certain stages using the color development of bromophenol blue. The SPOTs membrane and Fmoc amino acids were purchased from Genosys Biotechnologies. In short, the primary sequence was synthesized so that it contained synthetic peptides that were ten amino acids long and offset by five amino acids, by coupling Fmoc amino acids as per the manufacturer's instruction. The synthesis begins by esterifying a Fmoc amino acid to the cellulose membrane derivatized with a dimmer of β -alanine-NH₂ groups. Following the coupling of each amino acid, the peptides were rendered unreactive by acetylation with acetic anhydride (4% v/v) in *N,N*-dimethylformamide, following which the Fmoc groups were removed by the addition of piperidine to render the nascent peptides reactive. Once the desired peptides were synthesized, the side chains were deprotected with a solution containing dichloromethane, trifluoroacetic acid, and triisobutylsilane in the ratio 1:1:0.05 (v/v/v). The immobilized synthetic peptides on the membranes were either probed directly or stored in a freezer (-80°C) until further use.

Probing the SPOTs membrane. After washing with methanol, the SPOTs membrane was blocked overnight with blocking buffer (Genosys Biotechnologies) diluted 10 $\times$ in TBST (50 mM Tris, pH 8.0, 136 mM NaCl, 2.7 mM KCl and 0.05% Tween 20) with 5% (w/v) sucrose. The membrane was washed with TBST for 10 min. The membrane was then incubated for 4 h at 25°C at a 1:10 dilution with pooled sera obtained from eight patients with egg-white allergy (provided by Dr. Urisu, Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan, and by Dr. Moridawa, Faculty of Medicine, Gunma University, Gunma, Japan). The membrane was further washed twice with TBST and incubated with monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma, St. Louis, MO; 1:1000 dilution) at room temperature for 2 h. After incubation the membrane was washed four times with TBST. The bound antibodies were detected using the chemiluminescent substrate CDP-Star (Boehringer Ingelheim, Mannheim, Germany) and enhancer Nitro-Block II (Tropix, Bedford, MA) both diluted 100 $\times$ in 0.1 M Tris-HCl, 0.1 M sodium chloride, pH 9.5. The membrane was visualized with a molecular light imager (EG & G Berthold, Bad Wildbad, Germany). The image processing and evaluation was performed using WinLight software (EG & G Berthold). For the detection of IgG epitopes, the membrane was incubated in a 1:100 dilution of pooled sera from eight egg-allergic patients, then incubated in goat anti-human IgG alkaline phosphatase conjugate (Sigma; 1:10000) and developed using the chemiluminescent substrate CDP-Star and enhancer Nitro-Block II.

To determine the exact amino acid sequence of the IgG and IgE binding regions in ovomucoid, detailed epitope mapping was per-

formed by synthesizing overlapping peptides on a SPOTs membrane. The peptides were numbered, and each peptide was overlapped with its adjacent neighbour by one residue. The overlapping peptides (10–15 amino acid residues in length), which were stepwise different from the previous peptide by one residue, were prepared and probed with pooled sera from eight allergic patients. The membrane was probed using the technique described above.

Analysis of the critical amino acids. The amino acids required in ovomucoid IgG and IgE epitopes were identified by synthesizing multiple peptides with single amino acid changes at each position. Peptides were synthesized on a SPOTs membrane with each amino acid sequentially replaced with either alanine or methionine, and probed as described in the previous section.

RESULTS AND DISCUSSION

Pooled sera from egg-allergic patients were used to map the linear IgE and IgG binding region in ovomucoid using the overlapping decapeptides on the SPOTs membrane. Thirty-five overlapping peptides were synthesized to determine which regions of the ovomucoid primary sequence were recognized by human sera. Although there were many common positive spot IgG and IgE binding sites, there also appeared to be specific IgG or IgE binding regions. We have previously reported IgE and IgG binding activities to all three ovomucoid domains, especially to the third domain (12). The allergenicity (IgE) and antigenicity (IgG) might have different epitopes, although some regions shared a common sequence. The results obtained by cleaving (chemically and enzymatically) were equivalent to epitope mapping using synthetic peptides for some regions showing homology with the results published previously (16). To determine the exact amino acid sequence of the IgG and IgE binding regions, detailed epitope mapping was performed using synthetic overlapping peptides comprising 10–15 amino acids (offset by one amino acid) with respect to IgG and IgE binding regions derived from the results of Fig. 1 for the patients' sera. Figure 2 shows a representative spot of the immunoblot showing the binding and amino acid binding sequence of the individual overlapping peptide to IgG binding of domain III (amino acids 157–172). The immunoblot shows positive binding regions to the IgG antibody of egg-allergic patients' sera, and the residues

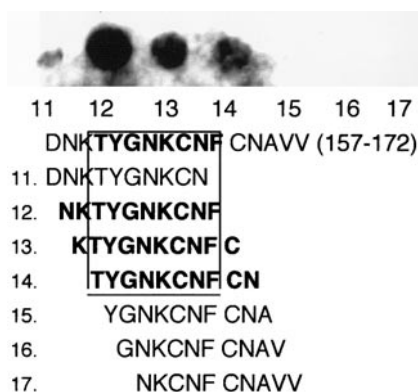


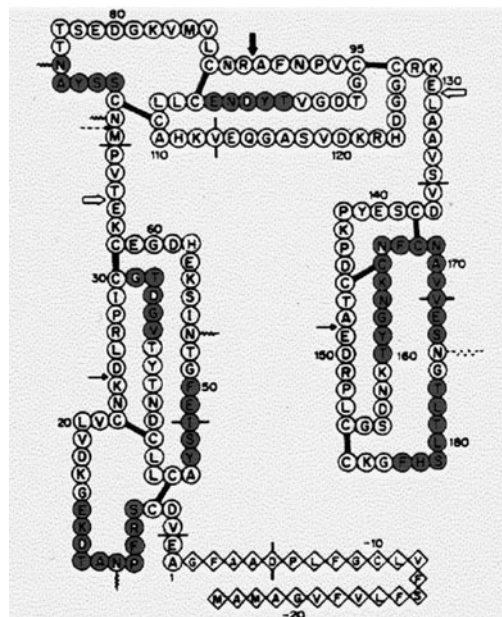
FIG. 2. Identification of a core IgG-binding epitope on ovomucoid. The data shown represent amino acids 157–172. Residues in bold letters corresponding to common IgG-binding amino acids of the spots are shown in the box.

in boldface correspond to the amino acids in the IgG binding epitope. The figure shows that each peptide was ten amino acids in length and offset from the previous peptide by one residue, and the IgG binding region is highlighted. This approach enabled us to map exactly the entire IgG and IgE linear epitopes.

Figure 3 provides a schematic representation of the entire epitope sequence binding to specific IgG and IgE patients' sera in the whole ovomucoid molecule comprising all the three domains. Pooled patients' sera identified three main IgE binding epitopes in domain I (amino acids 32–42, 40–50 and 56–66), five IgE binding epitopes (amino acids 71–75, 80–90, 101–105, and 121–130) in domain II and two major regions (amino acids 159–174 and 179–186) in domain III. The figure also shows the IgG binding domains in the whole ovomucoid molecule that were similarly recognized. Pooled patients' sera recognized three IgG epitopes on domain I (amino acids 6–15, 31–35, and 46–50), two on domain II (amino acids 71–75 and 101–105) and three on domain III (amino acids 160–167, 165–174 and 177–183). Among these epitopes, three IgE epitopes (amino acids 56–66, 80–90, and 121–130) and one IgG epitope (6–15) were recognized by only either the IgE or the IgG antibody. Cooke and Sampson (15) identified five IgE epitopes (amino acids 1–20, 49–56, 85–96, 115–122 and 175–186) and seven IgG epitopes (amino acids 3–11, 23–56, 69–74, 87–104, 119–124, 138–149, and 175–186) using the SPOTs membrane to probe the sera of seven pooled egg-allergic patients. Two distinct IgE epitopes (amino acids 90–121 and 134–186) were determined by trypsin cleavage of ovomucoid and subsequent immunoblot probing with egg-allergic patients' sera (16). More recently, Holen *et al.* (17) reported one novel IgE epitope (amino acid 61–74) and another common IgE and IgG epitope (amino acid 101–114) using synthetic overlapping peptides. In addition to these two epitopes, four epitopes (amino acids 41–56, 71–84, 131–144, and 171–186) were identified as ex-

clusively T-cell epitopes with no affinity to specific antibodies (17). Comparing the present work with earlier published data, five of nine IgE epitopes (amino acids 71–75, 80–90, 101–105, 159–174 and 179–186) overlap, while the other four epitopes (amino acids 32–42, 40–50, 56–66, and 121–130) were first identified in

IgG



IgE

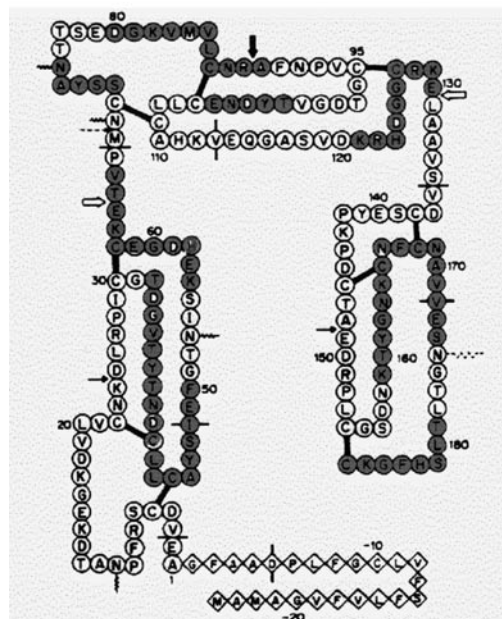


FIG. 3. Schematic representation of IgG and IgE epitopes in ovomucoid. The shaded amino acid sequences are shown each epitope.

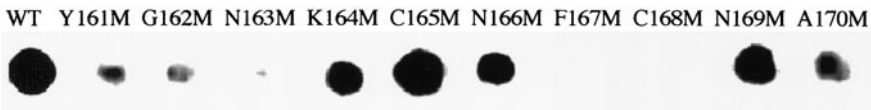


FIG. 4. Critical amino acid analysis of ovomucoid epitopes. The data shown represent IgE epitope, amino acids 161–170. The epitope was characterized by synthesizing each peptide with each amino acid sequentially substituted with methionine (M). The letters to the left of each lane indicate the one-letter amino acid code for the residues normally at that position and right one for the amino acid substituted for that residue. The numbers indicate the position of each residue in the ovomucoid.

this study. In contrast, there was more homology of our IgG epitopes with other published data: five of eight IgG epitopes (amino acids 6–15, 31–35, 71–75, 101–105 and 177–183) fully or partially overlap with previous work (15), but three IgG epitopes (amino acids 46–50, 160–167, and 165–174) were first identified in the present work. With regards to IgE binding epitopes, domains II and III were homologous to some extent with work published previously, but domain I was entirely different in our data.

The amino acids that were critical for IgE and IgG binding to ovomucoid were determined by synthesizing multiple peptides with a single amino acid mutation at each IgE and IgG epitope. Epitopes were synthesized with an alanine or methionine residue substituted for one of the amino acids to determine whether the substitution affects IgG or IgE binding activity. Figure 4 shows an immunoblot strip containing the wild-type and mutant peptides for the IgE epitope comprising amino acids 159–174. The pooled sera either did not recognize or decrease in binding was observed when methionine was substituted at Y161, G162, N163, F167 and C168 in the IgE epitope, indicating that

these amino acids were critical for IgE binding activity. Amino acids within each of the IgG and IgE epitopes of ovomucoid that are critical to allergic reactions were identified in this manner and are summarized in Table 1. The substitution of one amino acid in some peptide positions produced an interestingly loss or reduction of activity against IgG and IgE binding, which indicates that those amino acids are critical for binding. For example, the S174 is essential for IgG (but not IgE) binding to domain III epitope and the carbohydrate moiety that is connected to domain III at N175, which may result in the spatial hindrance of IgE antibody binding. These statement is in agreement with our previous report that the specific binding activity of human IgE against N-glycosylated domain III (N175) was lower than against nonglycosylated domain III in egg-allergic patients (12). This was confirmed by another group that observed no difference between IgE binding of glycosylated and deglycosylated ovomucoid (9). The carbohydrate moiety at N175 may act to stimulate the antigenicity but suppress the allergenicity of ovomucoid. Although the IgG and IgE binding epitopes overlapped, the critical amino acid residues were different. There was no obvious consensus in the type of mutated amino acid that led to complete loss or a decrease of IgE and IgG binding. However, it is worth noting that we classified the amino acid in each of the IgG and IgE epitopes based on hydrophobic, polar or charged residues (Fig. 5). There were a total 55 amino

TABLE 1
Amino Acids Critical to IgG and IgE Binding

	Amino acid sequence	Position
IgG epitope		
1	SR F PNATD K E	6–15
2	G T DG V	31–35
3	Y S I E F	46–50
4	SS Y AN	71–75
5	T YD N E	101–105
6	TYGN K C N F	160–167
7	CN F CNAV V ES	165–174
8	T L T LS H F	177–183
IgE epitope		
1	T DG V TYTND C L	32–42
2	D CLLCAYS I E F	40–50
3	KEHDGE C K E TV	56–66
4	SS Y AN	71–75
5	D G K V MVLCN R A	80–90
6	T YD N E	101–105
7	K R H D GGCR K E	121–130
8	KTYGN K CN F CNAV V ES	159–174
9	TL S H F G K C	179–186

Note. The amino acids that, when altered, led a decrease in IgG or IgE binding are shown as the bold, underlined residues.

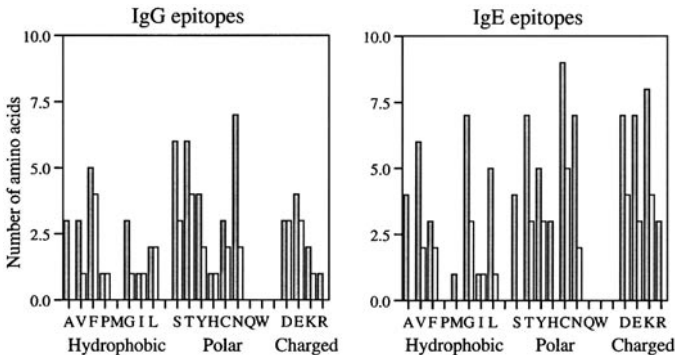


FIG. 5. Classification of amino acids in IgG and IgE epitopes in ovomucoid. The closed boxes represent the total number of a particular type of amino acid residues identified in all of the ovomucoid IgG and IgE epitopes, whereas the open boxes represent the total number of critical amino acids which are important for antibody binding.

acids present in the eight IgG epitopes of ovomucoid identified in this study. Polar residues were most frequent (27/55), followed by hydrophobic residues (18/55) and charged amino acid (10/55): aspartic acid and glutamic acid (charged amino acid residues) were critical in the IgG epitopes, and phenylalanine and leucine (hydrophobic residues) and serine and threonine (polar residues) were also important.

There were a total of 87 amino acids present in the nine IgE epitopes of ovomucoid. The IgE epitopes consisted of a higher proportion of charged (25/87) and polar (35/87) amino acids than the IgG epitopes. Aspartic acid, glutamic acid and lysine were critical (charged) amino acids in IgE epitopes, since the mutation of these residues within IgE epitopes produced the most dramatic decreases in antibody binding. Valine, threonine, tyrosine, phenylalanine and cysteine were also critical amino acids.

In summary, the elucidation of the IgG and IgE binding epitopes in ovomucoid and the determination of which amino acids within these epitopes are critical provide useful information for the molecular design necessary to alter ovomucoid genes by site-directed mutagenesis to reduce the allergenicity of ovomucoid. The present work also provides useful information on the structure–function relationships of allergenic epitopes in food proteins.

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